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RESEARCH ARTICLE

Pasteurisation of soil from an extremely phosphorus-impoverished habitat increases phosphorus availability and enhances the growth of *Agonis flexuosa* (Myrtaceae)

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Abstract

Background and aims Soil pasteurisation will unavoidably affect soil abiotic properties. Such changes are often overlooked in extremely phosphorus (P)-impoverished soils, where even slight increases in P availability can influence plant performance. Therefore, we aimed to determine whether pasteurisation of extremely P-impoverished soil affects growth of *Agonis flexuosa* and facilitation by *Banksia attenuata*.

Methods In a controlled experiment, we evaluated plant growth and root traits of target species *A. flexuosa* grown either in monoculture or mixed culture with *B. attenuata*, under pasteurised soil treatment (PST) and inoculated soil treatment (IST).

Results Soil resin P concentration was 20% higher in PST than in IST. In PST compared with IST, *A. flexuosa* had greater leaf P concentration (1.1 vs. 0.3 mg g^{-1}) and total biomass (5.0 vs. 3.0 g), developed thicker roots with lower tissue density, and showed higher rhizosheath carboxylate amounts. Under PST, the total biomass of *A. flexuosa* was greater when grown with *B. attenuata* (7.4 vs. 5.0 g), accompanied by lower rhizosheath acid phosphatase activity than without *B. attenuata*, whereas total biomass was indistinguishable under IST. Moreover, growing with *B. attenuata*, biomass of *A. flexuosa* under IST was 49% of that under PST.

Conclusions Pasteurisation of extremely P-impoverished soil increased P availability, likely through microbial P release and reduced microbial competition, thereby enhancing nutrient-foraging traits of *A. flexuosa*. Pasteurisation may also modify soil redox conditions and promote the release of Mn- and Fe-bound P. Therefore, experiments using soil pasteurisation should assess microbial biomass and changes in soil nutrient pools.

Keywords Soil abiotic properties · Interspecific interactions · Microbial phosphorus · Root traits · Cluster roots

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Introduction

Soil properties, encompassing both abiotic and biotic components, play a vital role in shaping plant

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performance and how they interact with their environment (Bennett and Klironomos 2019; De Long et al. 2023). Abiotic soil conditions, particularly the physicochemical availability of nutrients such as phosphorus (P), determine the resource supply that is freely accessible to plants, while biotic factors, including soil microbial communities, strongly influence nutrient cycling and ecosystem function (De Deyn et al. 2004; Ho et al. 2017). To clearly distinguish abiotic and biotic contributions, some studies rely on soil heat-treatment methods to kill biota (Buchan et al. 2012; Lees et al. 2018; Zuma et al. 2025). However, these heat treatments can introduce unintended side effects, potentially confounding experimental outcomes.

Pasteurisation is a technique used to suppress soil biota and assess the net effect of microbial communities on plant growth, including both beneficial interactions (e.g., mycorrhizal and rhizobial associations) and negative effects (e.g., pathogen infections) (Chauhan et al. 2023; Morgan et al. 2005). However, pasteurisation may significantly alter soil chemistry by releasing nutrients such as P through microbial cell lysis (Anderson and Magdoff 2005; Dietrich et al. 2020; McNamara et al. 2003; Trevors 1996). The inoculum method is commonly used, which involves inoculating pasteurised soil with a small proportion of live soil to reintroduce microbial communities, rather than using entirely untreated soil (Albornoz et al. 2017; Gille et al. 2024). This approach is considered more reliable because it ensures that the abiotic properties of most of the soil used in the experiment are consistent across treatments (Brinkman et al. 2010). Even with this approach, experiments based on soils from extremely P-impoverished systems require particular caution, as plants in these environments are exceptionally sensitive to soil P availability and may rapidly adjust their resource-use strategies in response to even minor changes in nutrient availability (Chen et al. 2025; Lambers 2022; Lynch and Brown 2008). Nevertheless, few studies have investigated whether soil pasteurisation may lead to incorrect interpretation of plant growth responses in experiments focused on extremely P-impoverished soils.

The Warren dune chronosequence in south-western Australia contains some of the oldest, most highly weathered, and most P-impoverished soils in the world (Turner et al. 2018; Zhong et al. 2025). The relatively high rainfall (1185 mm) supports large-stature eucalypt trees (such as *Eucalyptus marginata* and *E. megacarpa*), whose continuous inputs of lignin-rich organic matter help sustain high microbial biomass (Mou et al. 2025; Turner et al. 2018). *Agonis flexuosa* (Myrtaceae), a common species across this region, displays root traits highly responsive to shifts in soil P availability and microbial associations (Guilbeault-Mayers et al. 2026). For example, Huang et al. (2017) reported that, in a glasshouse experiment, leaf P concentration ([P]) of *A. flexuosa* is threefold higher under high P supply (1.2 mg P g^{-1}) compared with low P supply (0.4 mg P g^{-1}). Together, the unique soil conditions of the Warren dune chronosequence and the adaptive traits of *A. flexuosa* provide an ideal context for investigating whether differences in plant performance due to soil pasteurisation are a direct effect of removing microbes or an indirect effect (e.g., nutrient release through cell lysis and reduced immobilisation in microbial biomass).

The Root Economics Space (RES) framework provides a powerful conceptual model for understanding how plants adjust their root traits in response to environmental gradients (Bergmann et al. 2020). Under moderate P limitation, plants tend to produce slower-growing roots with denser tissue structure and rely more heavily on symbiotic partners (Scheifes et al. 2024). However, in extremely P-impoverished soils, both plants and fungi may suffer P limitation, which constrains hyphal development and reduces colonisation success (Parfitt 1979; Treseder and Allen 2002). In such habitats, some species exhibit alternative strategies such as cluster roots or dauciform roots, both of which release carboxylates that mobilise P (Shane and Lambers 2005; Wen et al. 2022; Williams et al. 2022). By analysing differences in root traits in an experimental setting, we can further determine whether plant growth responses post-pasteurisation are driven by direct microbial effects or indirect effects.

Banksia attenuata, a prominent member of the Proteaceae family, is known for its cluster-root strategy that mobilises poorly available soil P via carboxylate exudation (Lambers et al. 2008; Shane and Lambers 2005). Consequently, the localised release of these carboxylates can facilitate P acquisition by neighbouring plant species, which has been demonstrated across multiple studies (Muler et al. 2014; Shen et al. 2024; Staudinger et al. 2024). Leaf manganese concentration ([Mn]) can be used as a proxy

for rhizosphere carboxylate activity (Lambers et al. 2015, 2021), because Mn uptake in plants is generally closely associated with carboxylate-mediated P mobilisation. Given that Huang et al. (2017) found *A. flexuosa* has high leaf [Mn] but does not rely on carboxylate release under extremely P-impoverished conditions, the markedly high leaf [Mn] observed in the field suggests that its P acquisition is facilitated by neighbouring carboxylate-exuding plants. However, it remains to be demonstrated whether *A. flexuosa* can benefit from P facilitation by *B. attenuata*, and how soil pasteurisation through microbial cell lysis and the consequent release of P modulates such interactions under experimental conditions.

In this study, we conducted a multi-factorial glasshouse experiment using pasteurised soil either alone or inoculated with one-third live field soil from the same location to examine whether pasteurisation alters soil P availability and subsequently affects the growth of target species *A. flexuosa* and its interaction with *B. attenuata*. Framed within the RES, we assessed whether *A. flexuosa* adjusts its root traits in response to these abiotic nutrient differences and whether growth with *B. attenuata* further modulates its nutrient-acquisition strategy, including its capacity to establish mycorrhizal symbioses. We hypothesised that in extremely P-impoverished soils, (1) soil pasteurisation would sufficiently increase soil P availability to influence plant growth, (2) *A. flexuosa* would exhibit root traits more towards resource absorption rather than extensive foraging in response to the elevated P availability, and (3) soil pasteurisation would modify the growth response of *A. flexuosa* when grown with *B. attenuata*.

Materials and methods

Soil collection and processing

Soil was collected from the oldest and most severely P-impoverished stage of the Warren dune chronosequence (34.62° S, 115.89° E), located 350 km south of Perth, Australia, in D'Entrecasteaux National Park (Turner et al. 2018). The mean annual rainfall is 1047 mm, and the annual temperature ranges from a minimum of 11.4 °C to a maximum of 20.3 °C, based on climate data from 1991 to 2020 (http://www.bom.gov.au). The soil properties of this region have been well-documented, with P availability known to be extremely low at 2.5 mg P kg^{-1} (Turner et al. 2018). *Agonis flexuosa* (Willd.) Sweet is a widespread woody species native to south-western Australia (https://florabase.dbca.wa.gov.au/). It develops an extensive root system and is dual mycorrhizal (arbuscular mycorrhizal (AM) and ectomycorrhizal) (Howard et al. 2000). Soil was sampled beneath naturally growing *A. flexuosa* plants to capture the soil microbial community typically associated with the target species. Topsoil (0–30 cm depth) was collected from three randomly selected locations and homogenised together to create a representative mixture. A portion of field soil was triple pasteurised by steaming at 80 °C for 2 h on three alternate days over seven days to reduce the native microbial community (Albornoz et al. 2017).

Soil P measurement

Phosphate concentrations in extracts were determined using the malachite green colorimetric method (Turner and Romero 2009). Soil samples were air-dried at 40 °C for 72 h and sieved through a 2 mm mesh to remove roots and debris. Resin-extractable P represents the readily plant-available inorganic P fraction (soil resin P). Soil total [P] was measured by ignition in a muffle furnace at 550 °C for 1 h, followed by extraction of soil resin P with 1 M HCl by shaking for 16 h at ambient temperature (Saunders and Williams 1955). Soil resin P concentration was measured using anion-exchange membrane (AEM) strips (Myers et al. 1999). Briefly, strips were shaken with 3 g soil in 30 mL deionised water for 16 h, washed with deionised water to remove soil particles, and incubated in 10 mL 0.5 M HCl for 1 h to elute adsorbed soil resin P.

Glasshouse experiments

A multi-factorial experimental design was implemented, involving two main treatments: soil treatment (pasteurised soil treatment (PST) vs. inoculated soil treatment (IST)) and growth condition (monoculture vs. mixed culture with *Banksia attenuata* R.Br.). For the PST, each pot contained 3 kg of triple-pasteurised soil; for the IST, each pot contained a mixture of 2 kg of triple-pasteurised soil and 1 kg of non-pasteurised soil that was thoroughly homogenised. Each of the four treatment combinations (PST monoculture, PST

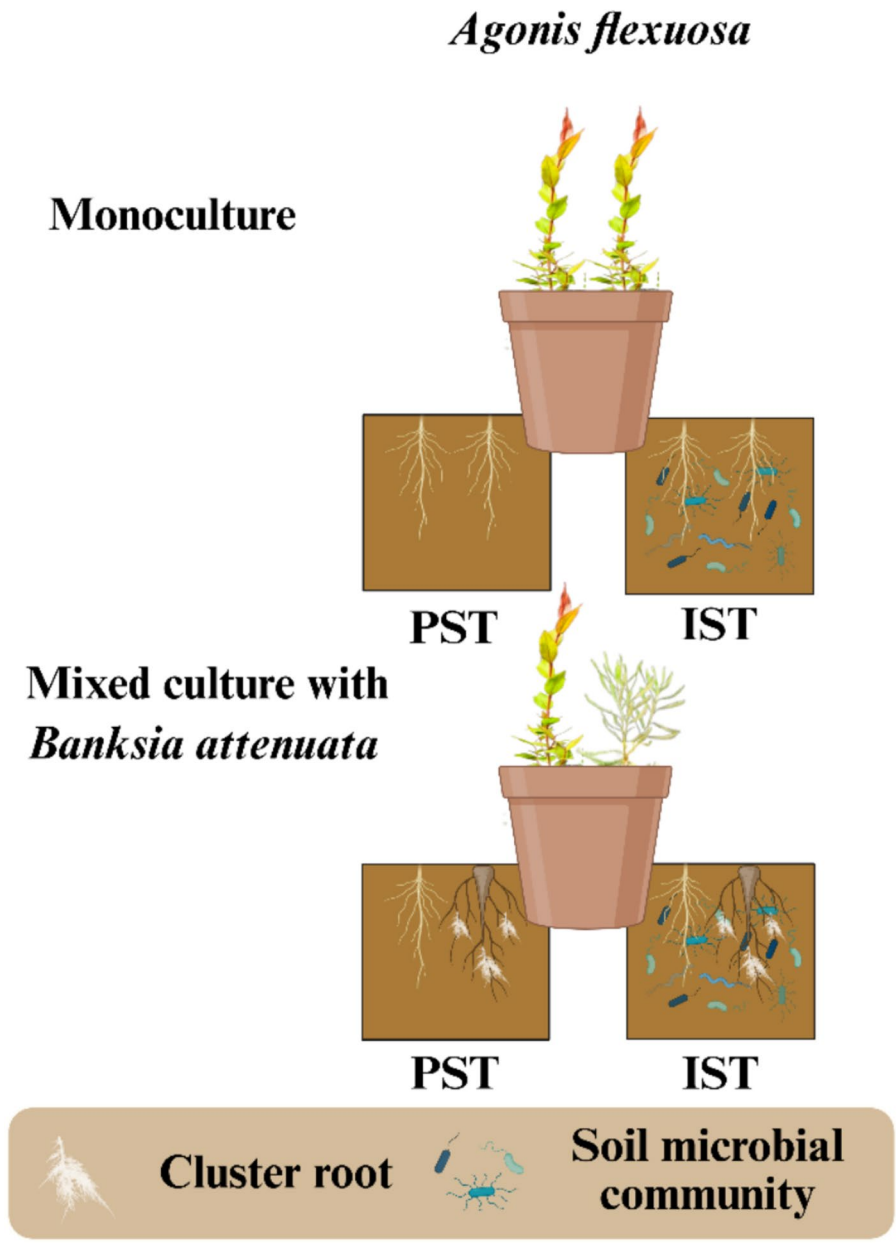


Fig. 1 Schematic diagram of the experimental design, which was carried out in a glasshouse at the University of Western Australia. The target species, *Agonis flexuosa*, was grown in monoculture or mixed culture with *Banksia attenuata*. These two growth conditions were combined with two soil treatments: pasteurised soil treatment (PST) and inoculated soil treatment (IST), resulting in four treatment combinations. There were 10 replicates ($n = 10$) for each treatment

mixed culture, IST monoculture, IST mixed culture) had 10 replicates (Fig. 1).

Seeds of *A. flexuosa* and *B. attenuata* were sourced from Nindethana Seed Service (Albany, WA, Australia). Before sowing, seeds were placed in a tea strainer and immersed in 1% (v/v) sodium hypochlorite for 20 s with continuous shaking, then in 70% (v/v) ethanol for 20 s and rinsed thoroughly with deionised water (Abrahão et al. 2018). Surface-disinfected seeds were sown in seedling trays filled with triple-pasteurised soil and germinated under controlled conditions (15 °C, 12 h photoperiod). After germination, seedlings were transferred to 4.5 L pots (21 cm top diameter, 19 cm height) containing 3 kg of PST or IST soil for 24 weeks. In monoculture, pots contained four seedlings of *A. flexuosa*. In mixed culture, pots initially contained two seedlings each of *A. flexuosa* and *B. attenuata*. Four weeks after transfer, the smallest seedlings were selectively removed, leaving two seedlings per pot (two *A. flexuosa* seedlings

in monocultures; one *A. flexuosa* and one *B. attenuata* seedling in mixed cultures) (Fig. 1).

The experiment was conducted at the University of Western Australia, Perth, Australia (31.57°S, 115.47°E), with average day/night temperatures of 23 °C/13 °C and relative humidity maintained at approximately 65%. Pots were regularly watered to maintain approximately 70% water-holding capacity of the soil. To reduce surface evaporation and minimise cross-contamination of soil microbes between pots, a 10 g layer of sterile polyethene beads (approx. 10 mm) was placed on top of the soil in each pot. Pots were arranged in a random block design to minimise positional effects. The plants were grown for a total of 27 weeks, from germination through transplantation into pots until final harvest.

Plant harvest and measurements

At harvest, the shoot was separated from the root system at the cotyledonary node. Shoots were separated into mature leaves, young leaves, and stems, and their fresh weights were recorded immediately at harvest. Stems were oven-dried at 70 °C for 72 h to constant weight.

Mature and young leaves were freeze-dried for 10 days until constant weight (VirTis BenchTop Pro freeze dryer, SP Scientific, New York, USA). Freeze-dried mature leaves were finely ground (Geno/Grinder 2010 ball-mill, SPEX SamplePrep, Metuchen, NJ, USA) with zirconium beads. Approximately 100 mg of ground leaf powder was acid digested with 4 mL concentrated HNO_3, followed by 1 mL $HClO_4$ (Zarcinas et al. 1987), diluted with deionised water to a final volume of 10 mL, and analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES; PerkinElmer Optima 5300 DV, Shelton, CT, USA) to determine leaf [P] and [Mn] against appropriate standards and blanks.

Root systems were separated from soil by gentle shaking. Soil particles that tightly adhered to the root surface were carefully collected and are hereafter referred to as rhizosheath soil (Pang et al. 2017). Both rhizosheath and bulk soil samples were stored at −20 °C and processed for soil acid phosphatase (APase) assays within 48 h. Soil acid phosphatase activity was assayed using 1 g of fresh soil suspended in a reaction mixture containing 0.2 mL toluene and 4 mL of modified universal buffer (2.4 g L^{-1} TRIS hydroxymethyl aminomethane, 2.3 g L^{-1} maleic acid, 2.8 g L^{-1} citric acid, 1.3 g L^{-1} boric acid, pH 6.5 with 0.1 M NaOH) (Pang et al. 2018). The reaction was initiated by adding 1 mL of 50 mM *para*-nitrophenyl phosphate substrate and incubating at 37 °C for 1 h. Enzyme activity was terminated by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH, and the mixture was subsequently filtered. Filtrates were analysed spectrophotometrically at 420 nm, and APase activity was expressed as μmol *p*-nitrophenol released g^{-1} soil h^{-1}.

For rhizosheath carboxylate analysis, whole root systems with adhering rhizosheath soil were immersed in 40 mL 0.2 mM $CaCl_2$ and gently shaken for 90 s. Subsamples of these solutions were filtered through a 0.2 μm syringe filter, acidified with 20 μL concentrated *ortho*-phosphoric acid, and stored at −20 °C until analysed. Carboxylates were quantified using high-performance liquid chromatography (HPLC; Waters 600E pump, 717 plus autosampler, 996 photodiode array (PDA) detector and Alltima C18 reverse-phase column, Milford, USA) (Cawthray 2003).

After extracting rhizosheath carboxylates, roots were thoroughly washed with deionised water to remove loosely adhering soil particles. Roots were patted dry with paper towels, and the fresh root mass was recorded. Root systems were scanned (800 dpi, Epson Perfection V800 scanner, Seiko Epson, Nagano, Japan), and images analysed (WinRHIZO Pro software v4.1c, Regent Instruments, Québec, Canada) to measure total root length (TRL, cm) and average root diameter (RD, mm). From these data, we calculated specific root length (SRL, cm g^{-1}) and root tissue density (RTD, g cm^{-3}). After scanning, fine root subsamples were stored in 70% (v/v) ethanol at 4 °C for assessment of AM colonisation. The remaining roots were sorted into coarse, fine, and cluster roots (for *B. attenuata*), wrapped in aluminium foil and stored at −80°C for further analyses. Finally, total plant biomass was calculated by summing the dry weights of leaves, stems, and roots.

AM colonisation

Fine lateral roots stored in 70% (v/v) ethanol were cleared by immersing them in 10% (w/v) KOH at

room temperature for seven days, and rinsed in acidified water (pH ~ 2, adjusted with 1% (v/v) HCl) (Phillips and Hayman 1970). Cleared roots were stained with 5% (v/v) ink (Shaeffer black ink)-white vinegar solution for 1 h at room temperature, followed by several rinses in acidified water to remove excess stain (Vierheilig et al. 1998). Stained root segments (~ 1 cm length) were mounted on slides and examined under 100 × magnification (Axioskop optical microscope, El-Einsatz; Carl Zeiss, Oberkochen, Germany) and photographed (Axiocam digital camera, Carl Zeiss Microscopy, GmbH, Oberkochen, Germany). The line-intersect method (Giovannetti and Mosse 1980) was used to calculate the % AM colonisation in roots; at least 100 intersections per sample were scored for the presence of AM hyphae and arbuscules.

Statistical analyses

All statistical analyses and visualisations were conducted using R statistical software (R Development Core Team 2024). In the monoculture treatment, two *A. flexuosa* individuals were grown per pot, resulting in a total of 20 plants from 10 pots. In the mixed-culture treatment, one *A. flexuosa* individual was grown per pot ($n = 10$ plants). Individual plants were used as the observational units in all analyses. Linear mixed-effects models were used to evaluate the impact of experimental treatments on traits of *A. flexuosa*. The models were implemented using the 'lme4' package (Bates et al. 2015), with growth condition and soil treatment defined as fixed effects. Both block and pot were included as random effects to account for spatial variability within the glasshouse and for potential non-independence of plants grown within the same pot. The significance of fixed effects was determined using Satterthwaite's approximation method as implemented in the 'lmerTest' package (Kuznetsova et al. 2017). Model assumptions were verified through Shapiro–Wilk normality tests on residuals. Variables that did not meet normality assumptions were log10 transformed before analysis. We calculated estimated marginal means using the 'emmeans' package (Lenth 2024) and applied Tukey's method for multiple comparisons among treatment combinations. Specifically, for cases with only two treatment levels, Welch's t-test was conducted using base 'stats' functions in R.

To assess broader patterns of trait variation, a principal component analysis (PCA) was carried out using the 'FactoMineR' package (Lê et al. 2008). Before the PCA, the dataset was standardised via z-score transformations, and missing data were imputed using the imputePCA function from the 'missMDA' package (Josse and Husson 2016). PCA results were visualised by plotting the first two principal components (PC1 and PC2) with confidence ellipses (95%) around treatment groups. All plots were generated using the 'ggplot2' package (Wickham 2016). Statistical significance was interpreted at the $P < 0.05$ level unless otherwise specified.

Results

Soil P

At the start of the glasshouse experiment, soil resin [P] in the PST soil (2.0 mg $kg^{-}1$) was about 20% higher than in the IST soil (1.7 mg $kg^{-}1$), whereas total soil [P] was approximately 30 mg $kg^{-}1$ and indistinguishable between PST and IST soils (Fig. 2).

Plant biomass and leaf nutrient concentrations

The linear mixed-effects model revealed that soil treatments significantly influenced total plant biomass, whereas growth conditions had no significant effect (Fig. 3a). Irrespective of the growth conditions, *A. flexuosa* exhibited less total biomass under IST than under PST. The total biomass under IST was 59% and 49% of the biomass under PST in monoculture and mixed culture, respectively (Fig. 3a; Table S1). The presence of *B. attenuata* resulted in greater total biomass for *A. flexuosa* under PST, whereas no significant difference was observed under IST (Fig. 3a). Conversely, the companion species, *B. attenuata*, exhibited greater total biomass under IST than under PST. Neither soil treatment nor growth condition had a significant effect on the root-to-shoot ratio of *A. flexuosa* (Figs. 3b, S1). *Banksia attenuata* also had a similar root-to-shoot ratio under PST and IST (Fig. 3b).

Soil treatment had a significant effect on both leaf [P] and leaf [Mn], whereas growth condition had no significant effect (Fig. 3c, d). Regardless of growth condition, *A. flexuosa* exhibited significantly lower leaf [P] under IST (0.3 mg g^{-1} in Monoculture and 0.5 mg g^{-1} in Mixed culture) than under PST (1.1 mg g^{-1} in

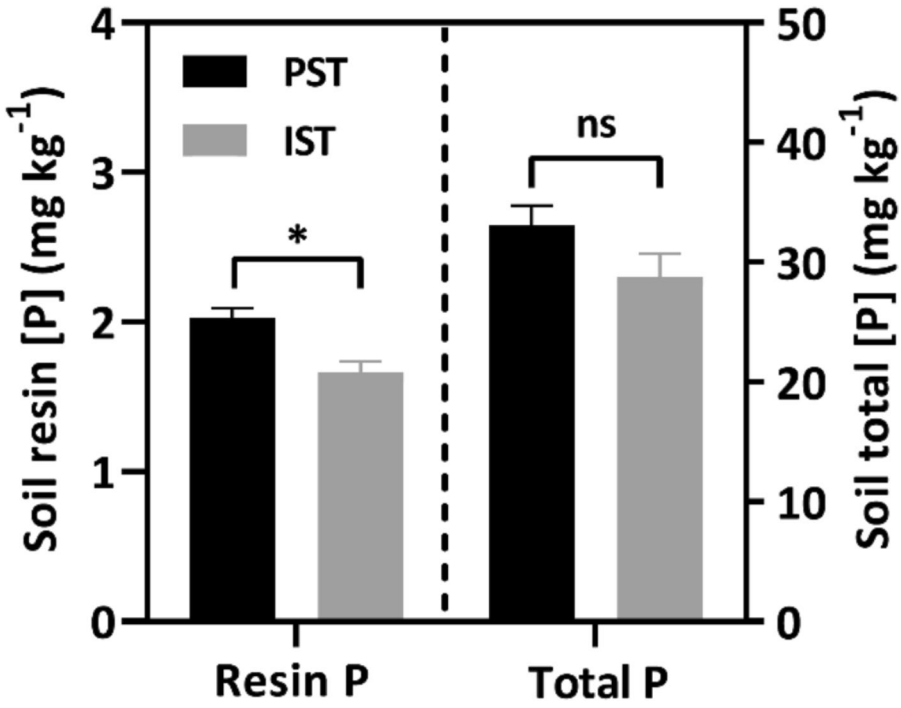


Fig. 2 Concentrations of resin-extractable phosphorus (resin P) and total phosphorus (total P) in the pasteurised soil treatment (PST) and inoculated soil treatment (IST) at the start of the glasshouse experiment. Bars represent means ± SE ($n = 6$). Asterix indicates a significant difference at $P < 0.05$, while ns denotes no significant difference (Welch's t-test)

Monoculture and 0.9 mg g^{-1} in Mixed culture; Table S1; Fig. 3c). Leaf [Mn] of *A. flexuosa* exhibited similar patterns with leaf [P], being significantly lower under IST than under PST, regardless of the growth conditions (Fig. 3d). Within the same soil treatment, the presence of *B. attenuata* did not significantly alter leaf [P] or [Mn] of *A. flexuosa* (Fig. 3c, d). Notably, *B. attenuata* also demonstrated markedly lower leaf [P] and [Mn] under IST than under PST (Fig. 3c, d).

Root morphological traits

Soil treatment had a significant effect on TRL, RD, SRL, and RTD (Fig. 4a-d). Regardless of growth condition, *A. flexuosa* exhibited higher TRL, SRL, and RTD, and lower RD under IST than under PST (Table S1; Fig. 4a-d). Similarly, *B. attenuata* also exhibited higher TRL and RTD, and lower RD under IST than under PST, while SRL was indistinguishable between treatments (Fig. 4a-d). There was a significant interaction between soil treatment and growth condition for both RD and SRL (Fig. 4b, c). Specifically, under PST, *A. flexuosa* exhibited higher RD and lower SRL in mixed culture than in monoculture, while these traits were indistinguishable between monoculture and mixed culture under IST.

Root physiological traits

Soil treatment had a significant effect on rhizosheath carboxylate amount in *A. flexuosa* (Fig. 5a). The concentration of rhizosheath carboxylates for *A. flexuosa* under IST was lower than that under PST, regardless of whether they were grown in monoculture or in mixed culture (Fig. 5a). Within PST, rhizosheath carboxylate amounts were similar between monoculture and mixed culture, with malate, citrate and oxalate being proportionally dominant; however, malate accounted for a relatively greater proportion in mixed culture than in monoculture (54% vs 40%, respectively; Table S1; Fig. 5b). Moreover, under IST, rhizosheath carboxylate amounts of *A. flexuosa* grown in mixed culture were indistinguishable from those in monoculture (Fig. 5b). For *B. attenuata*, rhizosheath carboxylate amounts were indistinguishable from those under PST, with malate (55%) being the dominant component, followed by trans-aconitate (26%) and citrate (18%), whereas under IST, trans-aconitate was dominant at 78% (Figs. S2, S3).

Soil treatment had a significant effect on APase activity (Fig. 6). The APase activity in the rhizosheath of *A. flexuosa* in mixed culture under PST

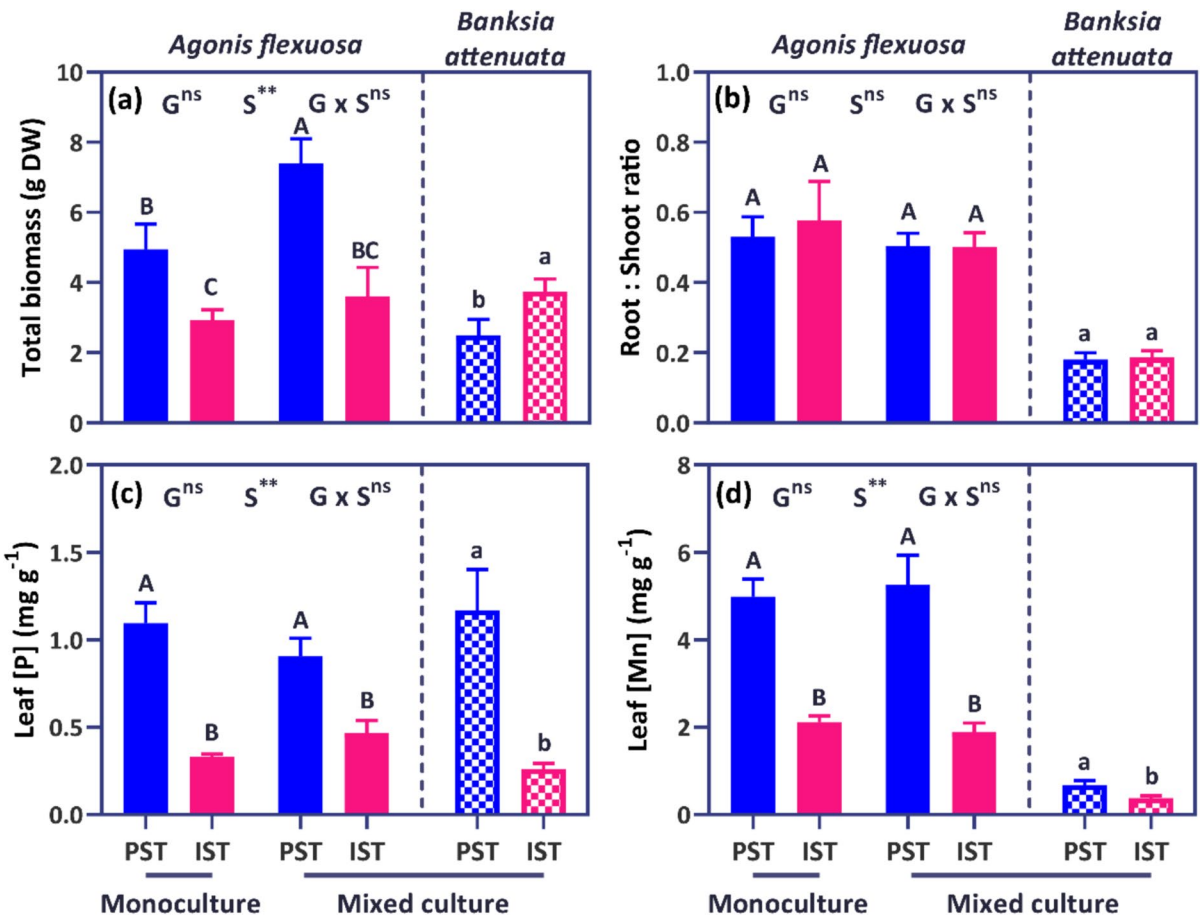


Fig. 3 Effect of growth condition (G) and soil treatment (S) on biomass and leaf nutrient concentrations of *Agonis flexuosa* and *Banksia attenuata*. (**a**) Total biomass, (**b**) root-to-shoot ratio, (**c**) leaf phosphorus concentration ([P]) and (**d**) leaf manganese concentration ([Mn]) of *A. flexuosa* (solid bars) in monoculture or mixed culture with *B. attenuata* (stippled bars), under pasteurised soil treatment (PST, blue) and inoculated soil treatment (IST, red). Bars represent means ± SE (monoculture: $n = 20$; mixed culture: $n = 10$). Uppercase letters indicate significant differences among the four *A. flexuosa* treatments based on estimated marginal means from linear mixed-effects models, followed by Tukey's post hoc tests ($P < 0.05$); lowercase letters denote significant differences ($P < 0.05$) between PST and IST for *B. attenuata* based on Welch's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant

was lower than that in all other treatments and was indistinguishable from the APase activity in the mixed bulk soil (Fig. 6).

AM colonisation

As expected, there was no AM colonisation of *A. flexuosa* roots under PST. Under IST, the AM colonisation rate of *A. flexuosa* roots was greater in monoculture than in mixed culture, as indicated by the greater abundance of both hyphae and arbuscules (Fig. 7). We did not observe evidence of any ectomycorrhizal colonisation in all treatments.

Trait trade-offs and coordination

Principal component analysis (PCA) revealed that *A. flexuosa* occupied distinct regions within the root economics space depending on treatment. The first principal component (PC1) accounted for 41.2% of the variation and was positively associated with SRL, while being negatively associated with RD and RSA (Fig. 8a; Table S2). Individuals of *A.*

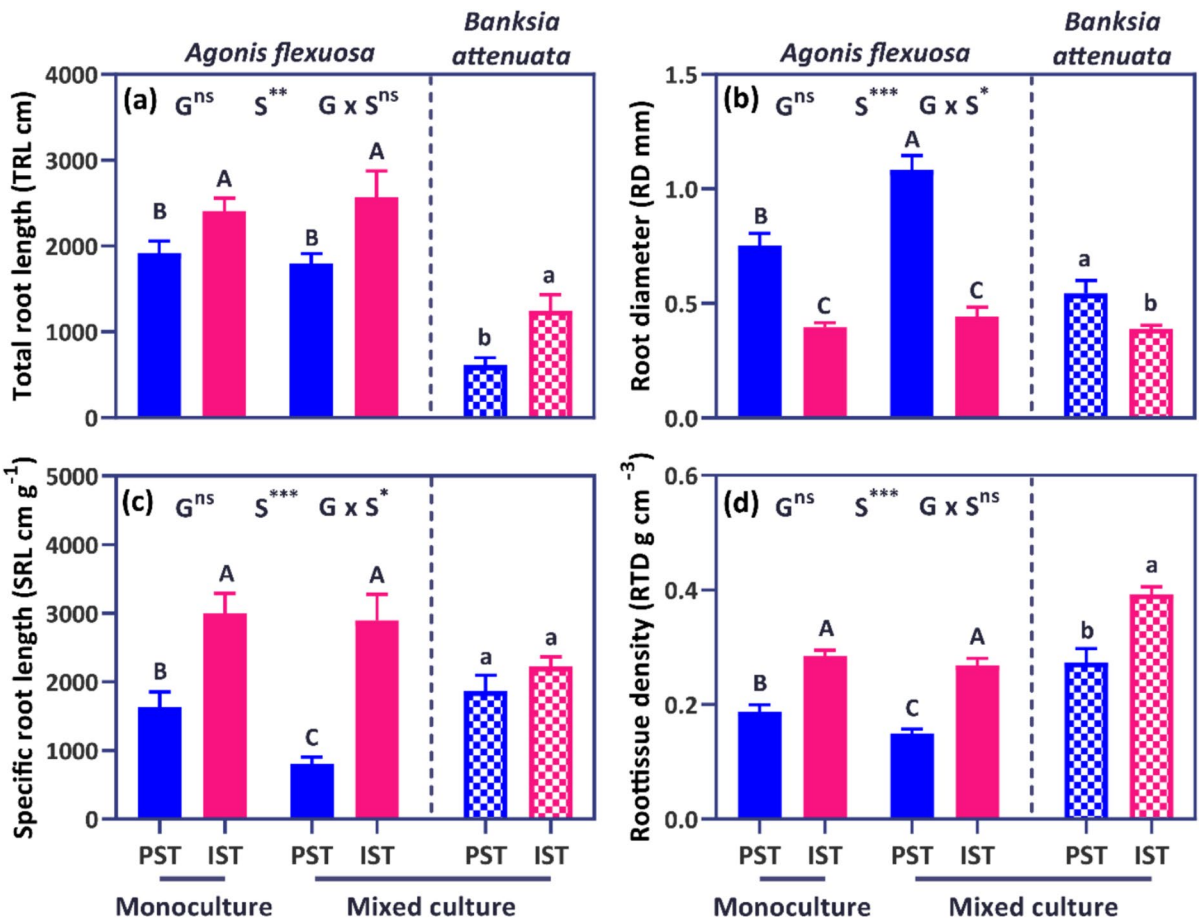


Fig. 4 Effect of growth condition (G) and soil treatment (S) on root traits of *Agonis flexuosa* and *Banksia attenuata*. (**a**) Total root length, (**b**) average root diameter, (**c**) specific root length, and (**d**) root tissue density of *A. flexuosa* (solid bars) in monoculture and mixed culture with *B. attenuata* (stippled bars), under pasteurised soil treatment (PST, blue) and inoculated soil treatment (IST, red). Bars represent means ± SE (monoculture: $n = 20$; mixed culture: $n = 10$). Uppercase letters indicate significant differences among the four *A. flexuosa* treatments based on estimated marginal means from linear mixed-effects models, followed by Tukey's post hoc tests ($P < 0.05$); lowercase letters denote significant differences ($P < 0.05$) between PST and IST for *B. attenuata* based on Welch's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant

flexuosa were separated along PC1 according to growth condition: those in IST extended towards the positive end (characterised by higher SRL), while those in PST were positioned towards the negative end, exhibiting more collaborative traits (Fig. 8b). The PC2 explained 25.3% of the variation, with positive loadings for TRL, Apase activity, and mycorrhizal colonisation (Fig. 8a; Table S2). Along PC2, *A. flexuosa* from the Mixed culture-PST treatment clustered at the negative end, characterised by low APase activity and AM colonisation rate, while *A. flexuosa* in IST aligned towards the positive end of PC2, reflecting higher TRL and APase activity (Fig. 8c).

Discussion

Effects of soil pasteurisation

Supporting our first hypothesis, our findings reveal that soil pasteurisation led to higher resin [P] and greater total plant biomass under PST than under IST in our target species *A. flexuosa*. This greater

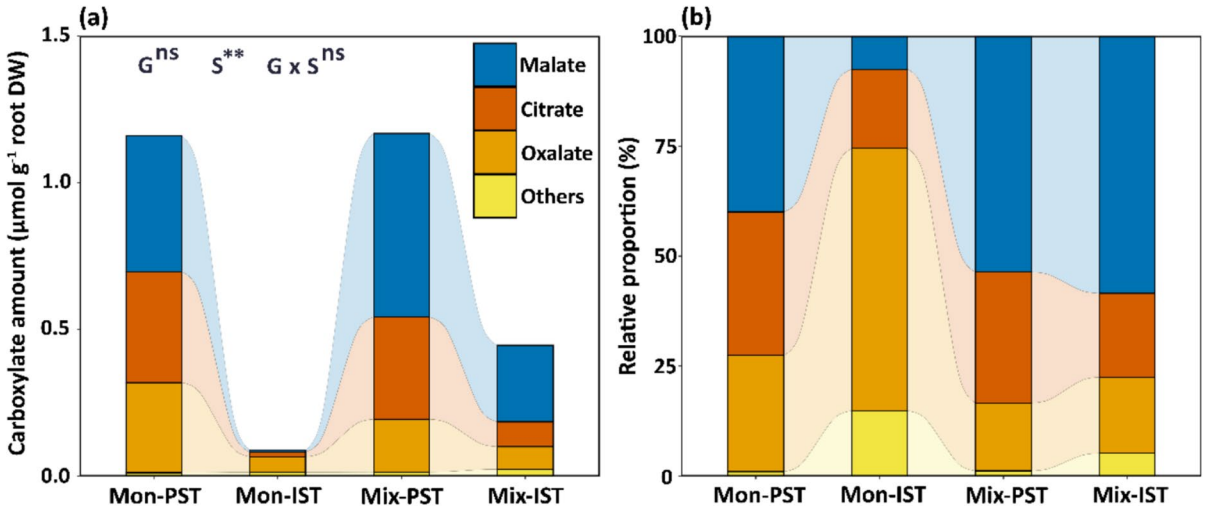


Fig. 5 Effect of growth condition (G) and soil treatment (S) on total carboxylate amount extracted from rhizosheath soil of *Agonis flexuosa*. (**a**) The carboxylate amount and (**b**) relative contributions of individual carboxylates are shown. The four treatment combinations for plant growth were Monoculture Pasteurisation (Mon—PST), Monoculture Inoculation (Mon—IST), Mixed culture Pasteurisation (Mix—PST), and Mixed culture Inoculation (Mix—IST). Data represent individual plants (monoculture: $n=20$; mixed culture: $n=10$). Different uppercase letters indicate significant differences in total carboxylates among treatments based on estimated marginal means from linear mixed-effects models, followed by Tukey's post hoc tests ($P<0.05$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant

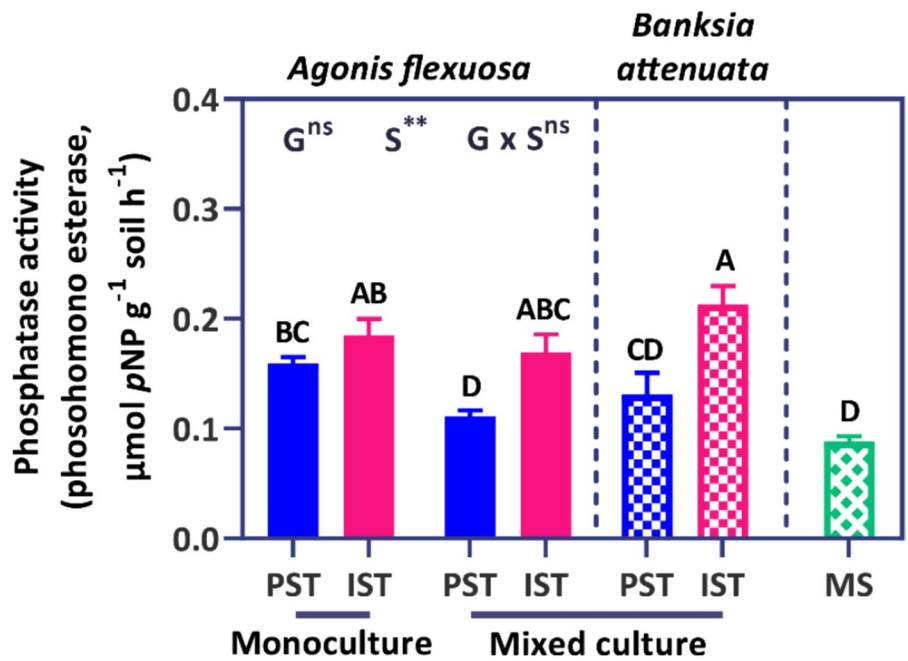


Fig. 6 Soil acid phosphomonoesterase activity in the rhizosheath soil of *Agonis flexuosa* (solid bars) in monoculture and in mixed culture with *Banksia attenuata* (stippled bars), under pasteurised soil treatment (PST, blue) and inoculated soil treatment (IST, red). Bars represent means ± SE (monoculture: $n=20$; mixed culture: $n=10$). "MS" (green stippled bar) refers to background phosphatase activity in a mixed bulk-soil sample from all treatments. Uppercase letters indicate significant differences among all treatments based on estimated marginal means from linear mixed-effects models, followed by Tukey's post hoc tests ($P<0.05$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant

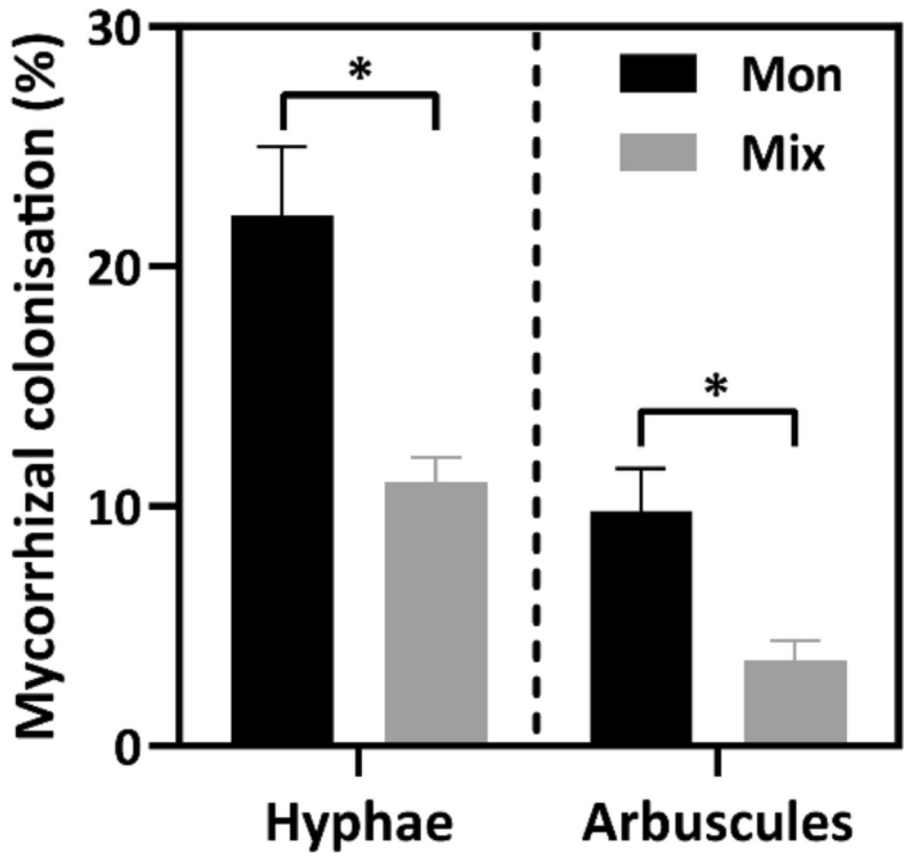


Fig. 7 Arbuscular mycorrhizal colonisation of roots from *Agonis flexuosa* grown in monoculture (Mon, black bars) and in mixed culture (Mix, grey bars) under inoculated soil treatment. Bars represent means ± SE (monoculture: $n = 20$; mixed culture: $n = 10$). Asterix indicates a significant difference at $P < 0.05$ (Welch's t-test)

plant biomass was likely driven primarily by increased P availability, partly through the release of microbial P and reduced microbial competition for P. We found that the lower total biomass of *A. flexuosa* under IST than under PST, was accompanied by greater SRL and thinner roots. Such root trait responses may arise from either P limitation or pathogenic stress. However, we observed no symptoms of disease, such as root necrosis or foliar chlorosis, that are typically associated with pathogenic infection (Buhtz et al. 2017; Péret et al. 2014; Vitti et al. 2013), indicating that P limitation was responsible for the differences in root traits in IST plants.

We also observed higher leaf [P] in *A. flexuosa* under PST, likely driven by higher soil P availability than in IST. According to Huang et al. (2017), *A. flexuosa* shows substantial variation in leaf [P] across P supply levels, ranging from approximately 0.4 mg P g^{-1} leaf DW under severely P-depleted conditions to over 1.2 mg P g^{-1} leaf DW at the highest P supply (30 mg kg^{-1} soil). The leaf [P] we observed under IST, at 0.4 mg P g^{-1} leaf DW, closely matched extremely low-P conditions. Therefore, the higher leaf [P] under PST (1.1 mg P g^{-1}) likely reflected increased soil P availability.

The present results raise important questions about the effects of soil pasteurisation on soil P availability in experimental situations. Although the soil originated from a highly weathered region known for its extreme P impoverishment, soil resin [P] was 20% higher after pasteurisation. This increase can be explained mainly by the release of P from killed microbial cells, leading to an increase in P availability (Anderson and Magdoff 2005; McNamara et al. 2003). Additionally, pasteurisation may alter soil redox conditions, leading to the release of P from Mn-P and iron (Fe)-P complexes and potentially increasing the availability of both P and redox-sensitive elements such as Mn and Fe (Sonneveld and Voogt 1975). Although the change in soil resin [P] was only 0.3 mg kg^{-1} soil, if it was mainly released from microbial lysis, it would imply the release of a much greater amount of organic P (Bünemann et al. 2011). Moreover,

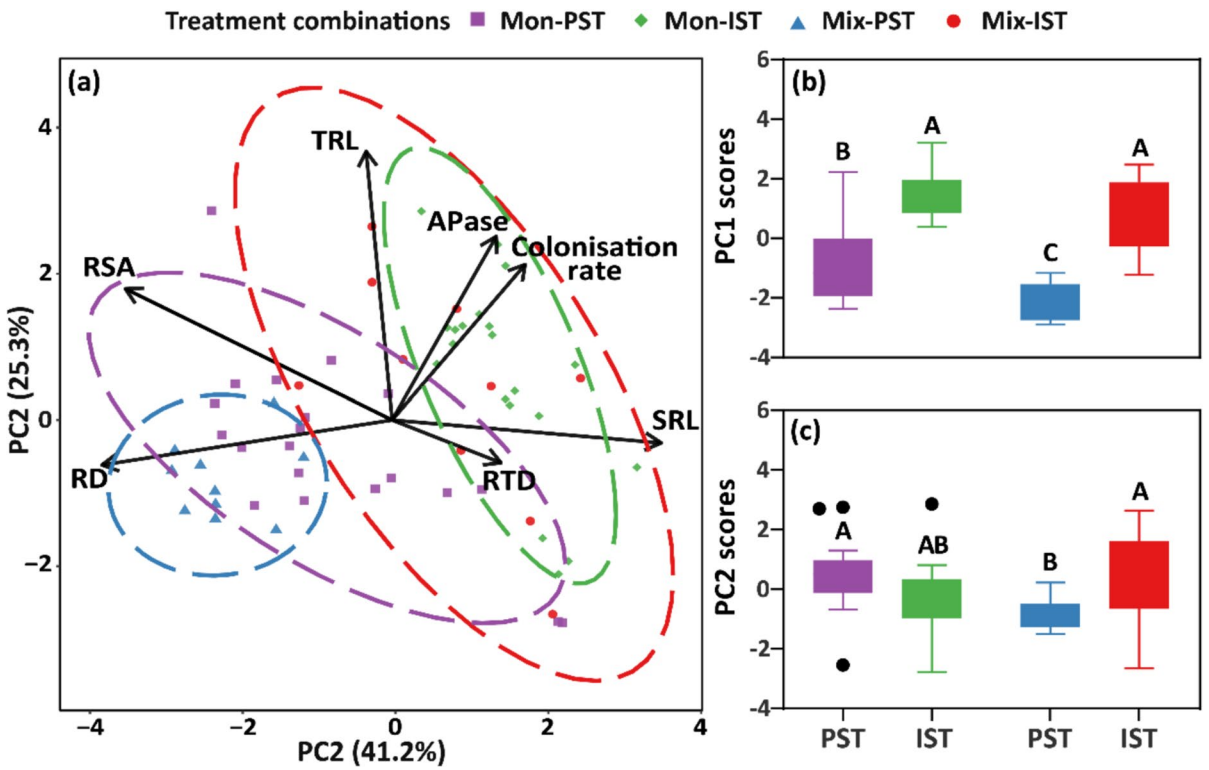


Fig. 8 Principal component analysis (PCA) of root functional traits of *Agonis flexuosa* under four treatment combinations. PCA biplot (**a**) shows the root functional trait distribution of *A. flexuosa* across treatments, with vectors for seven key traits, where each point is an individual plant. Boxplots show PC1 scores (**b**) and PC2 scores (**c**) for *A. flexuosa* under each treatment. The four treatment combinations were Monoculture—Pasteurisation (Mon—PST; purple squares), Monoculture—Inoculation (Mon—IST; green rhombus), Mixed culture—Pasteurisation (Mix—PST; blue triangles), and Mixed culture—Inoculation (Mix—IST; red circles). Percentage values on each axis indicate the proportion of variance explained by the corresponding principal component. In the biplot (a), ellipses are 95% confidence intervals for each treatment group. In the boxplots (b, c), boxes represent the 25^{th}-75^{th} percentiles and whiskers show a range up to 1.5 times the interquartile range. Data represent individual plants (monoculture: $n=20$; mixed culture: $n=10$). Uppercase letters indicate significant differences among treatments based on estimated marginal means from linear mixed-effects models, followed by Tukey's post hoc tests ($P<0.05$). TRL, total root length; RSA, root surface area; RD, root diameter; SRL, specific root length; RTD, root tissue density; APase, acid phosphatase activity

microbial competition for P likely also had an impact on our results. Hammer et al. (2011) demonstrated that fungal extraradical mycelium can contain very high P concentration (up to 2 mg g^{-1}) under severe carbon limitation. This evidence highlights that microbial immobilisation of P can represent a substantial and dynamic P pool, which should not be overlooked in extremely P-impoverished soils. Under IST, although two-thirds of the soil had been pasteurised and P availability was higher than that in the native soil, the leaf [P] of *A. flexuosa* remained low. This suggests that the reintroduced microbes competed with plants for the available P. In contrast, the complete removal of microbes under PST not only released microbial P but also eliminated competition.

Recently, Mou et al. (2025) reported high microbial biomass along the Warren chronosequence due to continuous organic matter inputs, in precisely the region from which we collected soil samples. Similarly, Zhong et al. (2025) identified appreciable levels of organic P in the same region, with microbial-derived phosphomonoesters accounting for 5.7 mg P kg^{-1}. These findings indicate that soils classified as severely P-limited with respect to plant-available P can still contain significant amounts of microbial P

that can be released upon pasteurisation, thus enhancing plant P availability. Our results are consistent with De Deyn et al. (2004), who showed that soil sterilisation substantially increases the availability of both nitrogen and P compared with non-sterilised soils. Furthermore, Manea et al. (2024) reviewed 68 studies, found that only 23% reported soil N or P both before and after sterilisation. Among these, half reported significant nutrient increases due to sterilisation, while the other half observed either no change or decreases. Although most studies used autoclaving, we employed milder pasteurisation and still observed that nutrient shifts may occur. Therefore, to avoid incorrectly attributing plant responses to direct microbial effects, it is essential to quantify changes in distinct soil P pools before and after pasteurisation and to assess soil microbial biomass.

Responses of root traits to soil pasteurisation

Supporting our second hypothesis, the increased soil P availability due to pasteurisation of extremely P-impoverished soil induced coordinated changes in root traits. First, our results confirmed that pasteurisation effectively reduced soil microbes, including those potentially beneficial to plant growth, as evidenced by the absence of AM colonisation under PST. Second, the rhizosheath carboxylate amounts associated with *A. flexuosa* were higher under PST than under IST. While species such as *Lupinus albus* and *Arabidopsis thaliana* generally reduce root carboxylate exudation as P availability increases (Ryan et al. 2012; Veneklaas et al. 2003), *A. flexuosa* exhibits the opposite pattern, with carboxylate release increasing under higher P supply (Huang et al. 2017). In this context, while reduced microbial consumption of carboxylates under PST cannot be excluded, the observed increase in carboxylate abundance is more likely to reflect enhanced exudation by *A. flexuosa* in response to increased P availability. Previous studies have confirmed that certain plant species exhibit increased carboxylate exudation under elevated P supply, which is interpreted as a metabolic "overflow" strategy to dispose of surplus carbon (Lambers et al. 2013; Prescott et al. 2020). This response prevents internal carbon accumulation and sustains rapid photosynthetic rates, and many also offer benefits, including enhanced acquisition of other nutrients (e.g., nitrogen, zinc and Fe) and the stimulation of beneficial microbial communities through rhizosphere-mediated interactions (Gerke 2015; Korenblum et al. 2022; Prescott 2022; Richardson et al. 2022).

Under PST, *A. flexuosa* occupied a position primarily within the 'collaboration' region of the RES, characterised by greater RD and RSA, but lower SRL. This root trait response contrasts with the conventional expectation that, in the absence of microbes, plants repress collaboration-based root traits due to the lack of microbial partners (Bergmann et al. 2020; Ma et al. 2023). However, this apparent contradiction further supports the conclusion that pasteurisation enhanced soil P availability. It suggests that, rather than responding to the absence of microbial partners, *A. flexuosa* matched its root traits with the greater P availability. In this context, thicker but less-dense roots (indicated by reduced RTD) likely represent a strategy suitable for greater P availability, favouring short-lived, fast-cycling roots over long-term structural investment. A similar response was reported by Sun et al. (2022), who observed marked differences in root morphology, including increased RD and projected root area, under conditions of sufficient P supply even in the absence of AM inoculation. This observation reinforces emerging evidence that root trait responses along the collaboration gradient are not only governed by microbial associations but can also shift in response to nutrient availability (Guilbeault-Mayers et al. 2026).

In summary, pasteurisation significantly increased soil P availability in an extremely P-impoverished soil, driving notable changes in *A. flexuosa* in terms of plant growth, sustained rhizosphere carboxylate accumulation, and strategic shifts in root morphology. This pattern is consistent with previous observations in other species, reinforcing the critical role of P availability in shaping root traits (Kumar et al. 2019; Lambers 2022; Zhang et al. 2020). These findings highlight that, under elevated P availability, plants may prioritise enhancing root absorptive efficiency over investing in additional root length, even in the absence of microbial partners.

Interaction with *B. attenuata*

Facilitation, defined as the enhancement of the growth, survival, or fitness of one plant by another (Callaway 1995; Fletcher et al. 2016), and P facilitation is increasingly recognised as an important strategy for nutrient acquisition (Muler et al. 2014; Shen

et al. 2024). Interestingly, with respect to our third hypothesis, soil pasteurisation promoted the growth of *A. flexuosa* when grown with *B. attenuata*, with a greater growth response observed under PST than under IST. The greater biomass of *A. flexuosa* in mixture with *B. attenuata* is therefore consistent with a facilitative effect but might also reflect reduced intraspecific competition or a competitive advantage of *A. flexuosa* in interspecific interactions. Notably, *B. attenuata* cluster roots not only exude carboxylates but are also capable of secreting phosphatases that mineralise organic P into forms absorbable by plants (Lambers et al. 2006; Wasaki et al. 2003). Hence, under PST, plants likely exploited larger inorganic and organic P pools, amplifying facilitative effects.

Our results show that leaf [Mn] did not differ significantly between monoculture and mixed culture under PST. However, the values had already reached approximately 4 mg kg^{-1}, which is close to the maximum recorded value for this species (Huang et al. 2017). Previous studies indicate that once rhizosphere Mn surpasses a certain threshold, further uptake is constrained by internal regulation (Lambers et al. 2021). This suggests that, although both the carboxylates released by *B. attenuata* roots and the redox changes induced by pasteurisation may jointly affect Mn availability in soil, these effects may not be directly reflected in the leaf [Mn] of *A. flexuosa*. Nevertheless, we cannot exclude the possibility that some of the leaf Mn may have been influenced by increased soil Mn availability during pasteurisation, which highlights that caution is needed when using pasteurised soil in mineral nutrition studies, especially when Mn acquisition is of particular interest.

We found that *A. flexuosa* showed greater RD and lower SRL in the mixed culture under PST, suggesting greater P availability and a reduced need for carbon investment in fine roots. In addition, the lower activity of excreted APase in the rhizosheath suggests that the plants may have down-regulated their phosphatase release at the lower internal P demand. This is a typical plant response that reduces energetically costly enzyme investments at higher P availability (Touhami et al. 2020). We speculate that plants show divergent carbon-investment strategies for phosphatases and carboxylates under higher P availability because carboxylate release may provide multiple simultaneous benefits, but further phosphatase production offers little additional benefit once P demand is met. In addition, each acid phosphatase enzyme contains approximately 550 amino acids, corresponding to more than 1500 carbon atoms, whereas citrate contains only six carbons (Johnson et al. 1994). Thus, the carbon investment required to produce and export a single phosphatase monomer is equivalent to that needed to synthesise many citrate molecules. In contrast, under IST living microbes can immobilise and take up P for their growth, and are additional P competitors in the rhizosphere (Hinsinger et al. 2015). In this context, the net effect of the P-mobilising strategies of *B. attenuata* on neighbouring plants may be diminished, as the accessible P pool was reduced by microbial uptake.

Although PST increased soil P availability, the additional growth observed in mixed culture indicates that *A. flexuosa* continued to benefit from carboxylate-mediated P mobilisation by *B. attenuata*. This sustained growth response is supported by large-scale trials, where *A. flexuosa* reached maximum biomass under the highest P addition of 30 mg P kg^{-1} soil, without any signs of toxicity (Handreck 1997). By increasing soil P availability and simultaneously eliminating microbial competitors that typically immobilise P, soil pasteurisation created a favourable environment that enabled the cluster-rooted *B. attenuata* to facilitate P acquisition of *A. flexuosa*. However, how strongly this facilitative effect responds to changes in P availability remains unclear. For future research, examining the interaction between the two species across a gradient of soil P supply, combined with controlled microbial inoculation, would help clarify how P availability and microbial context jointly shape facilitative effects.

Under IST, *A. flexuosa* showed significantly lower AM colonisation when co-planted with *B. attenuata* than in its absence. Huang et al. (2017) reported that *A. flexuosa* maintains high levels of AM colonisation (> 30% of root length) across all P levels tested, with no significant reduction even under very high P availability. Therefore, the lower colonisation rate we observed was unlikely due to increased soil P availability. Instead, it might be due to the release of isoflavonoids by *B. attenuata* (Beeck 2017), some of which suppress AM colonisation. This mechanism is supported by studies showing that cluster root-forming plants like white lupin (*Lupinus albus* L.) release isoflavonoids that reduce microbial activity in the rhizosphere, thereby protecting carboxylates such as citrate

from microbial degradation (Akiyama et al. 2010; Weisskopf et al. 2006a, 2006b).

Conclusions

Our study revealed that soil pasteurisation can substantially affect abiotic conditions in extremely P-impoverished soils to an extent that influences plant growth. Specifically, the combination of pasteurisation-induced release of microbial P, desorption of P from complexes (Fe–P and Mn-P) and reduction in microbial competition for P triggered *A. flexuosa* to strategically adjust its root morphology and thus efficiently capitalise on the increased P availability. Moreover, our results highlight the complexity of plant interactions in severely P-impoverished ecosystems. When the ability of *B. attenuata* to mobilise both organic and inorganic P via cluster roots was combined with a decrease in microbial competitors following soil pasteurisation, growth of *A. flexuosa* in association with *B. attenuata* was further enhanced. These interactions may also involve chemical signalling, such as the release of certain isoflavonoids that have been reported to suppress AM colonisation. Future studies should explicitly quantify both microbial- and nutrient-mediated effects of soil pasteurisation on plant growth to achieve a clearer understanding of experimental results.

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Data availability Data available from the UWA Profiles and Research Repository: https://doi.org/10.26182/03a2-jd45.

Declarations

Competing interests None that need to be declared.

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