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RESEARCH ARTICLE

# Mangrove species found in contrasting environments show differing phytohormonal responses to variation in soil bulk density

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**Abstract**

*Background and aims* Mangrove species respond to variation in soil bulk density (BD). However, very little is known about the regulatory mechanisms that trigger these responses.

*Methods* Endogenous concentrations of different phytohormones were measured in the roots of two mangrove species (*Avicennia marina* and *Rhizophora stylosa*) grown in low and high BD soils. The potential involvement of ethylene in regulating plant growth responses was tested by applying the ethylene biosynthesis inhibitors cobalt chloride ($CoCl_2$) and aminoisobutyric acid (AIB).

*Results* The two mangrove species responded differently to variation in soil BD. High BD decreased root growth of *R. stylosa*, but not *A. marina*. Soil BD had no effect on root phytohormone levels in *R. stylosa*, but loose soils increased 1-aminocyclopropane-1-carboxylic acid whilst decreasing salicylic acid and gibberellin in *A. marina*. Applying ethylene inhibitors enhanced *R. stylosa* root growth, while increasing indole-3-acetic acid but decreasing isopentenyl adenine levels. In contrast, AIB inhibited *A. marina* root growth, while increasing *trans*-zeatin levels. Ethylene inhibitors affected salicylic acid levels in both species.

*Conclusion* Salicylic acid is central to root growth responses to variation in BD in *A. marina*. Conversely, the interaction of ethylene and gibberellin drives responses in *R. stylosa.* Hormonal interactions involving ethylene potentially reflect the adaptations

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of the two species to differing conditions within the intertidal zone, with *A. marina* behaving like an aquatic species and *R. stylosa* behaving like a terrestrial species.

**Keywords** Adaptation · *Avicennia marina* · Ethylene · Inhibitors (AIB, $CoCl_2$) · *Rhizophora stylosa* · Roots

## Introduction

Mangroves occupy approximately 147 359 $km^2$ of tropical and subtropical coastline providing key ecosystem services to coastal communities (Bunting et al. 2022). These forests are frequently inundated by tides, which affect mangrove tree growth by influencing soil water content, nutrient availability, and salinity of the surface and soil water (Ball 1998). Australian mangroves often form characteristic zonation patterns parallel to the shore, where certain species (e.g. *Avicennia* spp.) are frequently found at the seaward fringe, while others (e.g. *Rhizophora* spp.) are often found further inland (Duke et al. 1998). Hydroperiod (i.e. the frequency and duration of tidal immersion) and salinity of the root zone are considered the main drivers of these species distribution patterns (Méndez Linares et al. 2007). However, soil physical characteristics such as soil bulk density (BD) also vary within mangrove communities, differentially influencing mangrove species growth, especially below-ground (Ola et al. 2020). Thus the development of roots under varying environmental conditions determines mangrove seedling establishment, species distributions, productivity and ecosystem service provision (Lee et al. 2014).

Variation in soil BD affects many soil physical, chemical and biological properties that influence plant growth, especially below-ground. Increases in soil BD and strength are inversely related to porosity (Nawaz et al. 2013). Decreases in macropore space are often associated with high water content at field capacity, which limits the diffusion of oxygen ($O_2$) into soils (Greacen and Sands 1980). Further, high soil BD often dramatically decreases microbial biomass, soil respiration and enzyme activity (Nawaz et al. 2013). For example, reductions in microbial biomass at BD of 1.7 g $cm^{-3}$ impeded carbon mineralization compared to soils with a low BD (Beylich et al. 2010). Plants respond to these complex interactions of abiotic and biotic factors that occur as soil BD varies, with soil compaction accounting for 20 to 37% and 16 to 22% of variation in the above-ground and below-ground traits of terrestrial woody plant species, respectively (Alameda and Villar 2009).

In terrestrial species, dense soils delay rooting (Tracy et al. 2015), restrict root elongation while increasing root diameter, and promote lateral root proliferation (Alameda and Villar 2012). Although mangrove soils are characterised by lower BD than terrestrial soils, ranging from 0.1 to 1.4 g $cm^{-3}$ (Ola et al. 2020), root traits in mangrove species are highly variable (Pi et al. 2009), with considerable plasticity in response to gradients in soil BD (Ola et al. 2020). Altering soil BD from 0.2 to 1.2 g $cm^{-3}$ affected root number, length, surface area, volume and dry weight (DW) of six months old *Avicennia marina* and *Rhizophora stylosa* seedlings (Ola et al. 2020). Interestingly, each species responded differently with *R. stylosa* root growth optimal at a BD of 0.4 g $cm^{-3}$, while root DW declined with increasing soil BD in *A. marina* (Ola et al. 2020). What regulates these responses is currently not known.

High BD causes many changes in root hormone status in terrestrial species. Increasing soil BD from 1.2 to 1.6 g $cm^{-3}$ had no effect on ABA levels in maize root tips, while indole-3-acetic acid (IAA) levels were 3.5 times higher (Lachno et al. 1982). Moreover, increasing soil BD from 1.2 to 1.6 g $cm^{-3}$ tended to increase ABA levels in whole root systems of tomato, with limited growth of an ABA-deficient mutant in dense soils suggesting that ABA counteracts negative impacts of soil BD on root growth (Tracy et al. 2015). In rice, soil compaction-induced ethylene accumulation increased ABA and IAA biosynthesis, with these downstream signals modulating root thickening and root elongation (Huang et al. 2022). Although these studies measured specific hormones in root tips (Lachno et al. 1982) and their spatial location in the roots using reporter genes (Huang et al. 2022), these techniques are not always possible in many other species including mangroves. However, multi-analyte hormone measurement techniques (Albacete et al. 2008; Šimura et al. 2018) could help unravel regulatory networks underlying root growth responses to soil BD.

Ethylene is also involved in moderating plant root growth responses to variations in soil BD and in

waterlogged conditions. While ethylene-induced root thickening has traditionally been interpreted as an adaptive response to facilitate root growth in dense soil (Masle 2002; Okamoto et al. 2008), roots of ethylene-insensitive mutants of rice were better able to penetrate dense soil than wild-type plants (Pandey et al. 2021). Variable effects of mechanical stresses on ethylene evolution may be due to differing experimental systems, species, and/or the developmental stage of the experimental tissue (Clark et al. 1999). As with increased soil BD, waterlogging reduces gas diffusion leading to the accumulation of gases including ethylene in root tissues (Liu et al. 2022). In terrestrial plants, waterlogging stimulates ethylene production, thereby inhibiting root growth or causing root tip death (Liu et al. 2022), but ethylene enhances growth of semi-aquatic plants when water table depths fluctuate widely (e.g. Visser et al. 1996). Responses of mangroves to variation in ethylene status has not been investigated, despite the importance of ethylene in plant responses to hypoxia and in the formation of adventitious roots (Vidoz et al. 2016; Visser et al. 1996), which occur in mangroves (Rasmussen et al. 2019).

This study used multi-analyte techniques to assess root tissue hormone levels accompanying root growth responses to variation in soil BD in the early developmental stages of the mangrove species *A. marina* and *R. stylosa*. Additionally, the impact of ethylene, which is synthesized from its precursor methionine and the immediate precursors S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC), on root tissue development and other root hormones were studied using the biosynthesis inhibitors cobalt chloride ($CoCl_2$) and aminoisobutyric acid (AIB), both of which inhibit ACC oxidase (Satoh and Esashi 1980). High soil BD was hypothesized to inhibit root growth, while applying $CoCl_2$ and AIB was predicted to alleviate this root growth inhibition. Since different mangrove species occur in contrasting intertidal zones, species differences in ethylene response were postulated to be of adaptive importance.

## Materials and methods

*Avicennia marina* and *Rhizophora stylosa* were chosen for this study, as they are the most common mangrove species in Australia (Alongi et al. 2000; Comley and McGuinness 2005).

### Experiment 1

Experiment 1 (comparing species responses to BD) was reported in Ola et al. (2020), using propagules that were collected in November 2015 at Myora, North Stradbroke Island, QLD, Australia (*R. stylosa*) and August 2016 at Nudgee Beach Reserve (Boondall Wetlands Park), QLD, Australia (*A. marina*). Only seedlings grown in soils with a BD of 0.2 (low BD) and 1.0 g $cm^{-3}$ (high BD) and with penetration resistance values of $4.0 \pm 0.7$ and $13.8 \pm 1.3$ kPa, respectively, were considered for this experiment. Variation in BD was achieved by altering the proportions of sand (BD: 1.5 g $cm^{-3}$) and perlite (BD: 0.056 g $cm^{-3}$; Chillagoe Perlite PTY Ltd, Mareeba, Australia) in the soil mix, assuming that the volumes of the individual components are additive. The soil mixtures were described by Ola et al. (2018). Seedlings were watered to field capacity three times per week using a salt solution (Ocean-Nature Sea Salt, Aquasonic, Wauchope, Australia) with a concentration of 20‰ (20 g NaCl per 1 l of water). They were grown for 24.5 weeks. Cultivation conditions were detailed in Ola et al. (2020).

Upon harvest, the roots were washed with tap water to separate them from the growth substrate. Total root length and the number of root tips (representing the number of roots) were determined using the root image analysis software WinRhizo 2009 (Regent Instruments Inc., Québec, Canada). The root material was then oven dried for four days at 60 °C to determine the DW of the below-ground biomass.

### Experiments 2 and 3

Experiments 2 and 3 used propagules of *R. stylosa* that were collected in May 2018, while propagules of *A. marina* were collected in June 2018 at the same locations as for Experiment 1. Seedlings were grown in soils with a BD of 0.2 (low BD) and 1.0 g $cm^{-3}$ (high BD). Variation in BD was achieved as described for Experiment 1. As in Experiment 1, seedlings were watered using a salt solution with a concentration of 20‰. The physiological significance of root ethylene evolution has been frequently studied using compounds such as cobalt chloride ($CoCl_2$)

and aminoisobutyric acid (AIB) (Spollen et al. 2000), both of which inhibit ACC oxidase and therefore ethylene biosynthesis (Satoh and Esashi 1980). Concentrations of 20 mM AIB and 30 µM $CoCl_2$ were chosen based on a dose response test (data not shown) and on the findings of Vasudevan et al. (2006). They were applied with the watering solution to study the effect of ethylene inhibitors on root growth of mangrove seedlings in high and low BD soils with nine replicate propagules for each species x soil BD x treatment (including a control) combination.

Growing conditions (temperature and relative humidity, Table 1) differed between the two species and depended on the time of year propagules could be collected, but resembled the conditions that seedlings of these species encounter during establishment in the field. After a growth period of three weeks, the seedlings were harvested and the roots washed with tap water. Roots were then severed from the surface of the propagule for subsequent analysis of root traits and hormone concentrations.

Root traits (number of roots, total root length, root DW) were determined for five randomly selected plants from a total of nine replicate seedlings (the remaining seedlings were used in the multi-hormone analysis). Therefore the number of roots were counted and root lengths were measured using a ruler, while root DW was determined after oven-drying the root material for four days at 60 °C.

Severed (whole) roots from four replicate seedlings for each species x soil BD x treatment combination, with DW ranging from 0.007 to 0.109 g per sample, were placed in vials and preserved in liquid nitrogen prior to lyophilisation (ALPHA 1–2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Cytokinins (*trans*-zeatin (*t*Z), zeatin riboside (ZR), isopentenyl adenine (iP)), gibberellins (GA1, GA3 and GA4), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed according to a modified protocol from Albacete et al. (2008). Briefly, 10 mg of freeze-dried plant material were homogenized in liquid nitrogen and dropped in 1 ml of a cold (-20 °C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20 000 *g*, 15 min) and re-extracted for 30 min at 4 ºC in 1 ml of the same, fresh extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †$C_{18}$ cartridge (SepPak Plus, Waters, Milford, USA) to remove interfering lipids and parts of plant pigments, and subsequently evaporated at 40 ºC under vacuum either to near dryness or until the organic solvent was removed. The residue was dissolved in a 0.5 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. Dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, USA). Ten µl of filtered extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, USA). To quantify plant hormone concentrations, calibration curves were constructed for each analysed component (1, 10, 50, and 100 ng $l^{-1}$) and corrected for 10 ng $l^{-1}$ deuterated internal standards. Recovery ranged between 92 and 95%.

The effect of soil BD (Low, High) on root traits was determined using one-way ANOVAs except 'Number of roots', which was analysed using a Poisson Generalized Linear Model (GLM). If the assumption of normality was not met by the residues of the variables even after data transformation, the Kruskal-Wallis test was performed. Welch's-t-test was performed if the assumption of homoscedasticity was violated. One-way ANOVAs were also used to assess the effect of soil BD on hormone levels of the root tissue. Two-way ANOVAs were used to assess the effect of ethylene inhibitors and soil BD on root traits within a species except 'Number of roots', which was

**Table 1** Glasshouse conditions (temperature, °C and relative humidity, %) during the trial periods for each mangrove species studied

| Species | Temperature (°C) | | | Relative humidity (%) | | |
|---|---|---|---|---|---|---|
| | Min | Max | Mean | Min | Max | Mean |
| *Avicennia marina* | 11.6 | 26.6 | 18.6 | 19.0 | 93.4 | 66.5 |
| *Rhizophora stylosa* | 12.8 | 26.6 | 12.8 | 23.5 | 88.6 | 64.7 |

analysed using a GLM. The effects of ethylene inhibitors (AIB, $CoCl_2$, Control) and soil BD (Low, High) on hormone levels of the root tissue were determined using two-way ANOVAs. Post hoc analysis (Tukey's HSD) was applied to further explore significant differences among ethylene inhibitors. If the assumption of normality was not met by the residues of the variables even after data transformation, robust two-way ANOVAs were performed (Kloke and McKean 2012). Interaction terms were dropped from the model unless significant. All statistical tests were performed on both species separately, as the phytohormone profiles (i.e. the type of hormones detected in the root tissue) of the two species differed. The data was analysed using R (R Core Team: http://www.R-project.org/).

## Results

### Soil bulk density alters mangrove root traits (Experiment 1)

Assessing a subset of the data (only plants of *A. marina* and *R. stylosa* grown under BD 0.2 g $cm^{-3}$ for loose soil and 1.0 g $cm^{-3}$ for dense soil) collected by Ola et al. (2020) showed that root traits of *A. marina* and *R. stylosa* differed when grown in low and high soil BD (Fig. 1). Whereas high BD soil significantly decreased root length of *R. stylosa* by 62%, it increased root length of *A. marina* by 30% (Fig. 1a). Similarly, whereas high BD significantly decreased the number of roots in *R. stylosa* by 62%, it significantly increased the number of roots in *A. marina* by 13% (Fig. 1d). Mean root length (total root length/number of roots) was not affected by BD in either species (Fig. 1b) indicating that variation in root initiation had the greatest influence on root traits. Furthermore, high soil BD decreased root DW in *A. marina* by 37%, but had no significant impact in *R. stylosa* (Fig. 1c).

### Phytohormone assay (Experiment 2)

In total, seven different hormones were detected in the root tissue of *A. marina* and nine different hormones in the root tissue of *R. stylosa.* Abscisic acid, ACC, IAA, JA, SA, and *t*Z were present in tissue of both species. However, the gibberellin types differed between species with GA4 only occurring in *A. marina*, while GA1 and GA3 occurred in *R. stylosa.* Additionally, cytokinins of the iP-type were found exclusively in root tissue of *R. stylosa*.

In *A. marina*, soil BD affected root concentrations of several phytohormones (Fig. 2, S1), but no significant differences were detected in *R. stylosa*. Root ACC concentrations were significantly ($p = 0.005$) higher (by 75%) in *A. marina* roots grown in low BD soils than high BD soils ($10808 \pm 811$ ng $g^{-1}$ DW versus $6191 \pm 698$ ng $g^{-1}$ DW, respectively). In contrast, GA4 levels were significantly lower in roots grown in low BD soil ($0.15 \pm 0.09$ ng $g^{-1}$) than in high BD soil ($0.45 \pm 0.07$ ng $g^{-1}$, $p = 0.038$). Similarly, SA levels were significantly lower in roots grown in low BD soil ($93.8 \pm 6.6$ ng $g^{-1}$) than in high BD soil ($129.2 \pm 10.1$ ng $g^{-1}$, $p = 0.025$). In *R. stylosa* root tissue hormone levels were not affected by soil BD (Fig. 2, S2).

### Ethylene inhibitors (Experiment 3)

Biosynthesis inhibitors were applied to the substrate to determine the importance of ethylene in regulating

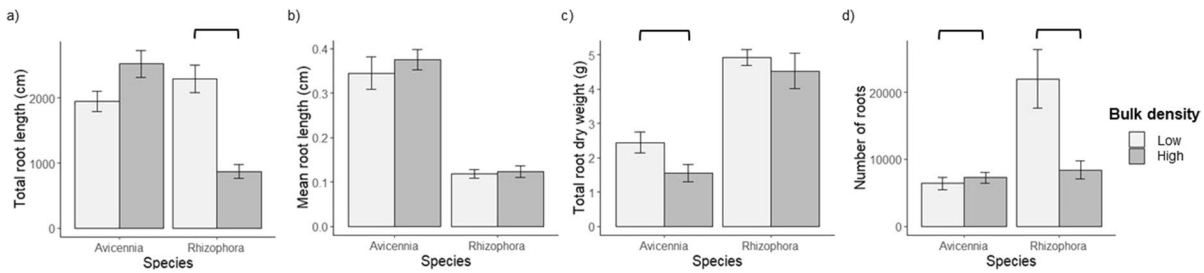


**Fig. 1** The effect of soil bulk density (low, high) on **a**) Total root length, **b**) Mean root length, **c**) Total root dry weight, **d**) Number of roots of *Avicennia marina* and *Rhizophora stylosa* seedlings based on Ola et al. (2020). Significant effects ($p < 0.05$) of BD within the species are indicated by brackets. Error bars show standard error of the mean

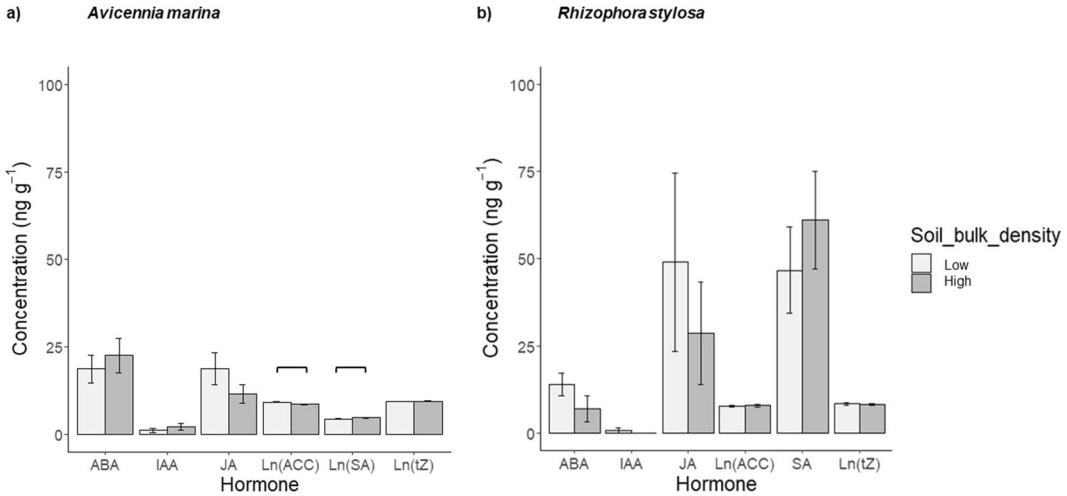


**Fig. 2** The effect of soil bulk density (low, high) on hormone levels (ABA, ACC, IAA, JA, SA, *t*Z) of root tissue of **a**) *Avicennia marina* and **b**) *Rhizophora stylosa*. Significant effects ($p < 0.05$) of soil bulk density are indicated by brackets. Error bars show standard error of the mean. ABA (abscisic acid); ACC (1-aminocyclopropane-1-carboxylic acid); IAA (indole-3-acetic acid); JA (jasmonic acid); SA (salicylic acid); *t*Z (cytokinin *trans*-zeatin)

root growth response to high soil BD. While soil BD had no effect on root traits of *A. marina* in Experiment 3 (Fig. 3; Table 2), the ethylene inhibitors affected all root traits of *A. marina* (Fig. 3; Table 2). Total root length of AIB-treated roots (9.2 ± 1.6 cm) was significantly shorter than total root length of the

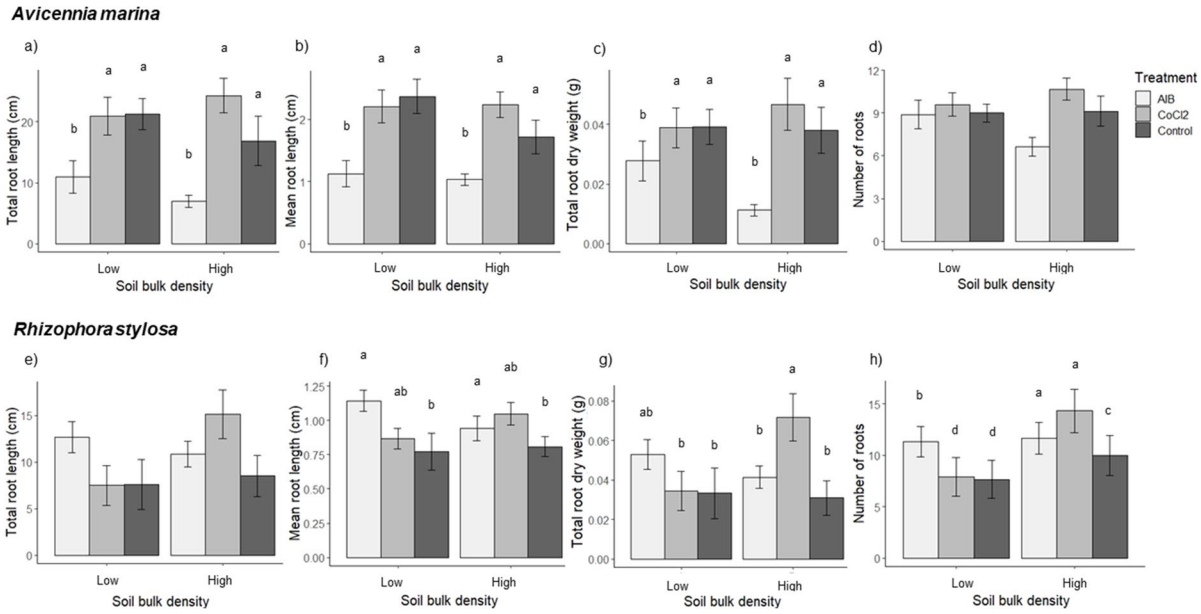


**Fig. 3** The effect of soil bulk density (low, high) and treatment (control, ethylene inhibitors AIB and $CoCl_2$) on root traits: **a**, **e**) Total root length; **b**, **f**) Mean root length; **c**, **g**) Root dry weight; and **d**, **h**) Number of roots of *Avicennia marina* (top) and *Rhizophora stylosa* (bottom). Significant differences ($p < 0.05$) are indicated by letters. Error bars show standard error of the mean

**Table 2** The effect of soil bulk density (BD, low vs. high) and treatment (ethylene inhibitor: AIB and $CoCl_2$, vs. Control) on root traits (mean root length, total root length, and root dry weight (DW)) of *Avicennia marina* and *Rhizophora stylosa* seedlings

| | | | Avicennia marina | | | Rhizophora stylosa | | |
|---|---|---|---|---|---|---|---|---|
| Root trait | Source of Variation | Df | MS | F-value | p | MS | F-value | p |
| Total length | BD | 1 | 19.9 | 0.272 | 0.61 | 79.86 | 1.494 | 0.23 |
| (cm) | Treatment | 2 | 881.9 | 12.022 | <0.001 * | 67.53 | 1.766 | 0.19 |
| Mean length | BD | 1 | 0.542 | 1.097 | 0.30 | 0.002 | 0.021 | 0.88 |
| (cm) | Treatment | 2 | 6.954 | 14.081 | <0.001 * | 0.282 | 3.569 | 0.036 * |
| Total DW | BD | 1 | <0.001 | 0.208 | 0.65 | 0.001 | 1.154 | 0.29 |
| (g) | Treatment | 2 | 0.003 | 6.121 | 0.004 * | 0.002 | 2.166 | 0.13 |
| | BD*Treatment | 2 | na. | na. | na. | 0.003 | 3.541 | 0.037 * |

Insignificant interaction terms that were dropped from the model are indicated (na = not available). Significant effects ($p < 0.05$) are highlighted (*)

control (19.2 ± 2.3 cm, $p = 0.003$) or $CoCl_2$-treated roots (22.5 ± 2.1 cm, $p < 0.001$). Similarly, mean root length of AIB-treated roots (1.1 ± 0.1 cm) was significantly shorter than mean root length of the control (2.1 ± 0.2 cm, $p < 0.001$) or $CoCl_2$-treated roots (2.1 ± 0.2 cm, $p < 0.001$). Root DW followed the same pattern with significantly less biomass of AIB-treated roots (0.02 ± 0.00 g) than control (0.04 ± 0.00 g, $p = 0.029$) or $CoCl_2$-treated roots (0.04 ± 0.01 g, $p = 0.005$). However, the number of roots was neither affected by AIB (z = -1.197, $p = 0.23$) nor $CoCl_2$ (z = 1.012, $p = 0.31$). Thus AIB, but not $CoCl_2$, inhibited root growth of *A. marina* independently of BD.

In *R. stylosa*, ethylene inhibitors also significantly altered mean root length ($p = 0.036$, Fig. 3; Table 2) predominantly due to significantly greater mean root length of AIB-treated plants than the control ($p = 0.029$). Interestingly, both ethylene inhibitors increased total root DW of *R. stylosa* seedlings in dense soils ($p = 0.037$, Table 2). Additionally, the number of roots was significantly increased by AIB (z = 2.446, $p = 0.014$) and $CoCl_2$ (z = 2.063, $p = 0.039$), as well as in dense soils (z = -3.515, $p < 0.001$). Thus AIB, but also $CoCl_2$, promoted root growth of *R. stylosa*.

Higher soil BD significantly decreased ACC levels of *A. marina* roots compared to loose soil ($p < 0.001$, Fig. 4; Table 3) consistent with Experiment 2. Despite their mechanism of action, the ethylene inhibitors had no significant effects on root ACC concentrations. Nevertheless, the ethylene inhibitors (especially AIB) increased root *t*Z concentrations irrespective of soil BD in *A. marina* (Table 3). Additionally, ethylene inhibitors significantly increased root SA levels in loose soil ($p < 0.001$, Fig. 4; Table 3), as indicated by the interaction of ethylene inhibitors and soil BD. Thus applying ethylene inhibitors also affected root concentrations of other hormones in *A. marina*.

Soil BD had no effect on *R. stylosa* root tissue hormone levels. However, ethylene inhibitors (especially AIB) decreased GA3 levels in roots grown in loose soil, while the opposite occurred in dense soil, as indicated by a significant interaction of ethylene inhibitors and soil BD ($p = 0.032$, Table 4). Applying $CoCl_2$ significantly increased root SA levels when compared to AIB ($p = 0.01$) or the control ($p = 0.004$). Moreover, the ethylene inhibitors increased root IAA levels 6-fold (AIB and $CoCl_2$: 3.2 ± 1.0 ng $g^{-1}$, versus control: 0.5 ± 0.5 ng $g^{-1}$) but decreased root iP levels by 75 to 90% (AIB: 2.1 ± 0.9 ng $g^{-1}$, and $CoCl_2$: 5.1 ± 3.8 ng $g^{-1}$, versus control: 20.6 ± 8.3 ng $g^{-1}$, Table 4). Thus applying ethylene inhibitors also affected root concentrations of other hormones in *R. stylosa*.

## Discussion

Dense soil generally inhibited root growth of *R. stylosa*, but not *A. marina* (Experiment 1, Fig. 1). Similarly to *R. stylosa*, dense soil (i.e. relative increase in soil BD of 0.3 and 0.4 g $cm^{-3}$ respectively) decreased root length of *Eucalyptus nitens* by 31% (Misra and Gibbons 1996) and two *Corymbia* tree species by 60% (Smith et al. 2001). In *R. stylosa* contact of aerial roots with dense soils markedly decreased growth

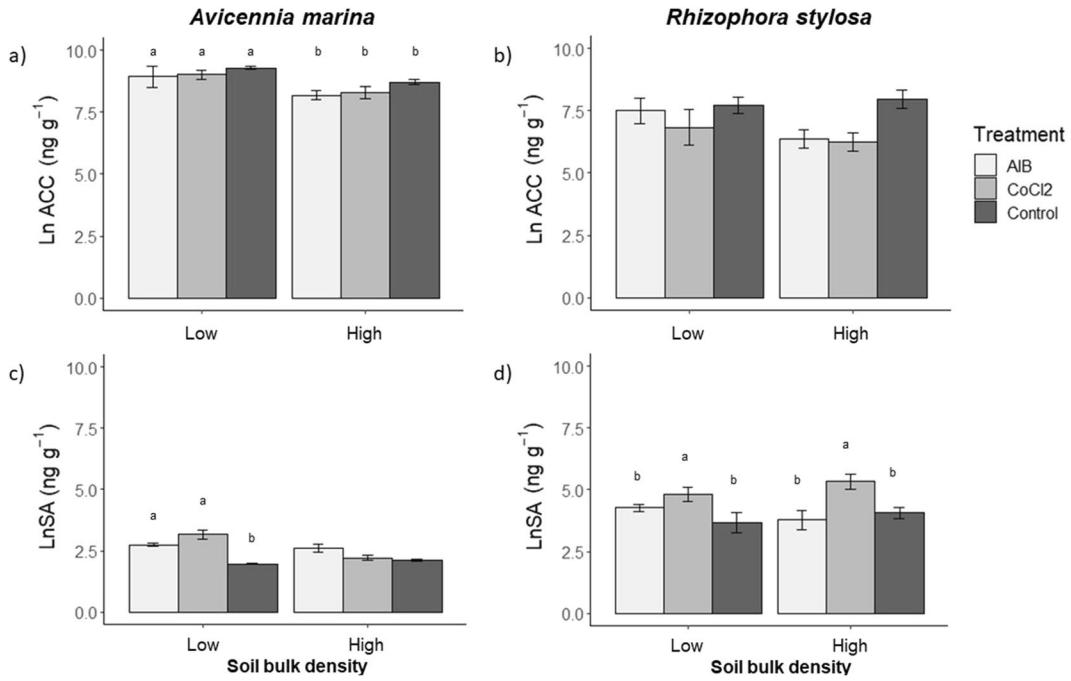


**Fig. 4** The effect of soil bulk density (low, high) and treatment (control, ethylene inhibitors AIB and $CoCl_2$) on **a**, **b**) 1-aminocyclopropane-1-carboxylic acid (LnACC) and **c**, **d**) salicylic acid (LnSA) in root tissue of two mangrove species **a**, **c**) *Avicennia marina*, and **b, d**) *Rhizophora stylosa*. Significant differences ($p < 0.05$) are indicated by letters. Error bars show standard error of the mean

**Table 3** *Avicennia marina*: The effect of soil bulk density (BD, low vs. high) and treatment (ethylene inhibitors: AIB and $CoCl_2$, vs. Control) on hormone levels (ABA, Ln(ACC), GA4, IAA, JA, Ln(SA), Ln(*t*Z)) of root tissue of *Avicennia marina* seedlings

| Plant hormone | Factor | Df | MS/MRD | F-value | p |
|---|---|---|---|---|---|
| ABA | BD | 1 | 508.9 [a] | 2.743 | 0.09 |
| | Treatment | 2 | 0.2 [a] | 0.001 | 0.97 |
| Ln(ACC) | BD | 1 | 116,182,950 [a] | 19.302 | < 0.001 * |
| | Treatment | 2 | 12,062,651 [a] | 2.004 | 0.16 |
| GA4 | BD | 1 | 0.25 [b] | 3.1 | 0.10 |
| | Treatment | 2 | 0.18 [b] | 2.263 | 0.10 |
| IAA | BD | 1 | 1.59 [b] | 1.512 | 0.24 |
| | Treatment | 2 | 0.5 [b] | 0.476 | 0.63 |
| JA | BD | 2 | 5.27 [a] | 0.073 | 0.93 |
| | Treatment | 1 | 4.32 [a] | 0.06 | 0.81 |
| Ln(SA) | BD | 2 | 3035.86 [b] | 24.771 | < 0.001 * |
| | Treatment | 1 | 2109.05 [b] | 17.209 | < 0.001 * |
| | BD*Treatment | 2 | 1885.88 [b] | 15.388 | < 0.001 * |
| Ln(*t*Z) | BD | 1 | 779.76 [b] | 0.617 | 0.44 |
| | Treatment | 2 | 29021.26 [b] | 22.972 | < 0.001 * |

Insignificant interaction terms were dropped from the model. Significant effects ($p < 0.05$) are highlighted (*). ABA (abscisic acid); ACC (1-aminocyclopropane-1-carboxylic acid); GA (gibberellin); IAA (indole-3-acetic acid); JA (jasmonic acid); SA (salicylic acid); *t*Z (cytokinin *trans*-zeatin)

[a] ANOVA (MS)

[b] robust ANOVA (MRD)

**Table 4** *Rhizophora stylosa*: The effect of soil bulk density (BD, low vs. high) and treatment (ethylene inhibitors: AIB and $CoCl_2$, vs. Control) on hormone levels (ABA, Ln(ACC), Ln(GA1), Ln(GA3), IAA, iP, JA, Ln(SA), Ln(*tZ*)) of root tissue of the *Rhizophora stylosa* seedlings

| Plant hormone | Factor | Df | MS/MRD | F-value | p |
|---|---|---|---|---|---|
| ABA | BD | 1 | 300.18 [a] | 4.119 | 0.06 |
| | Treatment | 2 | 8.83 [a] | 0.121 | 0.89 |
| Ln(ACC) | BD | 2 | 2.05 [a] | 2.326 | 0.14 |
| | Treatment | 1 | 2.92 [a] | 3.312 | 0.06 |
| Ln(GA3) | BD | 1 | 0.64 [a] | 0.582 | 0.46 |
| | Treatment | 2 | 5.96 [a] | 5.409 | **0.015** * |
| | BD*Treatment | 2 | 4.69 [a] | 4.254 | **0.032** * |
| IAA | BD | 1 | 0.81 [b] | 0.683 | 0.42 |
| | Treatment | 2 | 4.17 [b] | 3.534 | **0.05** * |
| iP | BD | 1 | 0.07 [b] | 0.018 | 0.90 |
| | Treatment | 2 | 14.42 [b] | 3.6 | **0.05** * |
| JA | BD | 1 | 35.92 [b] | 3.533 | 0.08 |
| | Treatment | 2 | 28.37 [b] | 2.79 | 0.09 |
| Ln(SA) | BD | 1 | 0.18 [a] | 0.436 | 0.52 |
| | Treatment | 2 | 3.4 [a] | 8.388 | **0.002** * |
| Ln(*t*Z) | BD | 1 | 3,211,580 [a] | 0.717 | 0.41 |
| | Treatment | 2 | 2,324,136 [a] | 0.519 | 0.60 |

Insignificant interaction terms were dropped from the model. Significant effects ($p < 0.05$) are highlighted (*). ABA (abscisic acid); ACC (1-aminocyclopropane-1-carboxylic acid); GA (gibberellin); IAA (indole-3-acetic acid); iP (cytokinin isopentenyl adenine); JA (jasmonic acid); SA (salicylic acid); *t*Z (cytokinin *trans*-zeatin)

[a] ANOVA (MS)

[b] robust ANOVA (MRD)

of these roots below-ground compared to growth in less dense soils (Ola et al. 2019). Decreased total root length in *R. stylosa* seedlings may therefore be attributed to the restricted growth of the main roots. Indeed, increasing BD from 0.8 to 1.2 g $cm^{-3}$ increased mean lateral root length of Brazilian pine *(Araucaria angustifolia)*, while main root and total root length decreased (Mósena and Dillenburg 2004). Moreover, high BD modestly (13%) increased root number of *A. marina*, while it substantially (62%) decreased root number of *R. stylosa* (Fig. 1b). The concomitant decrease in total root length suggests that dense soil inhibited root initiation in *R. stylosa*. Conversely, *A. marina* invests more biomass in fewer roots under loose soils and vice versa under dense soils, but root systems of this species are initially more fibrous like cereal root systems (Ola et al. 2020; Pi et al. 2009). However, these effects were less pronounced when root traits were measured in the experiments that assayed phytohormones (Fig. 3), possibly since experimental duration was shorter. Thus dense soils have different effects on root growth according to the root type of the mangrove species (Ola et al. 2018, 2020; Pi et al. 2009).

Soil BD also differentially affected root phytohormone concentrations of the two mangrove species (Experiment 2, Fig. 2). Soil BD did not affect root phytohormone concentrations of *R. stylosa*, whilst *A. marina* was strongly affected. Lower ACC levels of *A. marina* growing in high BD soils suggest enhanced ACC oxidase activity and thus ethylene synthesis, as in impeded maize roots (He et al. 1996; Sarquis et al. 1992). Interestingly, SA levels were also higher in *A. marina* roots in dense soil with many environmental stresses increasing root SA levels (Bagautdinova et al. 2022). Salicylic acid blocks ethylene synthesis (e.g. Zhu et al. 2020) and may be upregulated in dense soils. Salicylic acid can also be produced by the deami-Natesphenylalanine (PAL) pathway leading to production of trans-cinnamic acid, a precursor for the biosynthesis of various phenolic compounds such as lignin (Miura and Tada 2014). Lignification of root

tissue has been implicated in plant growth responses to physical impedance (Schneider et al. 2021) including in mangroves (Ola et al. 2019), suggesting SA may modulate mangrove root trait responses in dense soils. Abiotic stresses including compaction often decrease endogenous GA levels (e.g. Colebrook et al. 2014) unlike increased GA levels in *A. marina* root tissue in dense soils reported here. Although effects of mechanical impedance *per se* on tissue GA levels have been poorly studied, hypoxia (a common side effect of compaction e.g. Day and Bassuk 1994) increased GA levels in leaves and roots of two *Malus* species (Bai et al. 2011). However, further research is needed to understand the role of individual phytohormones in the growth responses of mangroves to variation in soil BD and associated abiotic stresses.

Ethylene inhibitors had variable effects on root growth of mangrove species (Experiment 3). In *R. stylosa*, ethylene inhibitors promoted root growth in agreement with observations that ethylene inhibits root growth by downregulating root cell elongation in terrestrial species (Masle 2002; Pandey et al. 2021). Hence, the effect of both inhibitors and soil BD on the number of roots and root DW suggests that ethylene evolution modulates root growth response of *R. stylosa* to variation in soil BD. In *A. marina*, typically located on the seaward site of the intertidal zone, the ethylene inhibitor AIB restricted root growth, possibly by increasing *t*Z cytokinin, which can prolong mitotic cycles and inhibits root growth in many species (Ivanov and Filin 2018). Thus *A. marina's* growth response may be unrelated to ethylene signalling. Decreased ethylene action in the more seaward mangrove species may be for similar reasons that the ethylene signalling pathway has been lost in the seagrasses *Zostera muelleri* (Golicz et al. 2015) and *Zoestera marina* (Olsen et al. 2016), where ethylene accumulation is not beneficial in aquatic plants (Li et al. 2022). Thus ethylene inhibitors produced divergent root growth responses in the seaward and landward mangrove species investigated in this study.

Adding ethylene inhibitors altered the levels of other hormones and again responses differed between species. For *A. marina* roots, especially AIB increased root *t*Z cytokinin concentrations irrespective of soil BD, which may partially inhibit root growth (Ivanov and Filin 2018). The interaction between ethylene and *t*Z cytokinin may occur via decreased ethylene receptor (ETR1 and ERS1) activities restricting *t*Z oxidase activity (Liu et al. 2017). Additionally, the inhibitors affected SA levels, particularly in loose soil, as in Experiment 2. The ethylene and SA pathway interact antagonistically with ethylene transcriptionally regulating SA modifying genes (Goda et al. 2008) and altering activities of SA modulating enzymes (e.g. Forouhar et al. 2005; Song et al. 2009) thereby limiting SA accumulation. Although dense soil decreased ACC levels in Experiments 2 and 3, inhibitors had no effect on root ACC levels despite their known mechanism of action. This suggests a feedback loop prevents further ACC production if the conversion to ethylene is inhibited. Further molecular and genetic studies are needed to confirm the effect of ethylene precursors on levels of ACC (and ethylene), SA and *t*Z cytokinin in root tissue and their proposed role in regulating growth of *A. marina* when BD varies (Fig. 5).

Ethylene inhibitors affected levels of other hormones in *R. stylosa* root tissue. While ethylene inhibitors increased root IAA levels 6-fold in *R. stylosa*, ACC treatment decreased endogenous IAA levels of pea root tips (Bertell and Eliasson 1992) via an antagonism between ethylene and IAA (Grossmann 2007). However, IAA accumulation at the stem base has been associated with adventitious root formation under hypoxia in several terrestrial species (Grichko and Glick 2001), which may explain the importance of IAA signalling in *R. stylosa*. The inhibitors may also directly affect IAA levels independent of ethylene synthesis (Soeno et al. 2010). Moreover, ethylene inhibitors decreased levels of the cytokinin iP by 75 to 90% in *R. stylosa* root tissue, possibly because the increased IAA levels inhibited cytokinin accumulation (Eklöf et al. 1997). Furthermore, inhibitors and/or growth response to variation in soil BD affected GA levels in root tissue of *R. stylosa*. Especially AIB caused low GA3 levels in roots grown in loose soil and high GA3 levels in dense soil. Repressing ethylene signalling upregulated GA metabolism in tomato (Shinozaki et al. 2015), suggesting that inhibiting ethylene in *R. stylosa* roots grown at high BD may increase GA levels. Taken together, applying ethylene inhibitors may suggest an IAA-ethylene-iP cytokinin crosstalk in *R. stylosa* root tissue, while ethylene and its interaction with GA regulate root growth response to soil BD (Fig. 5).

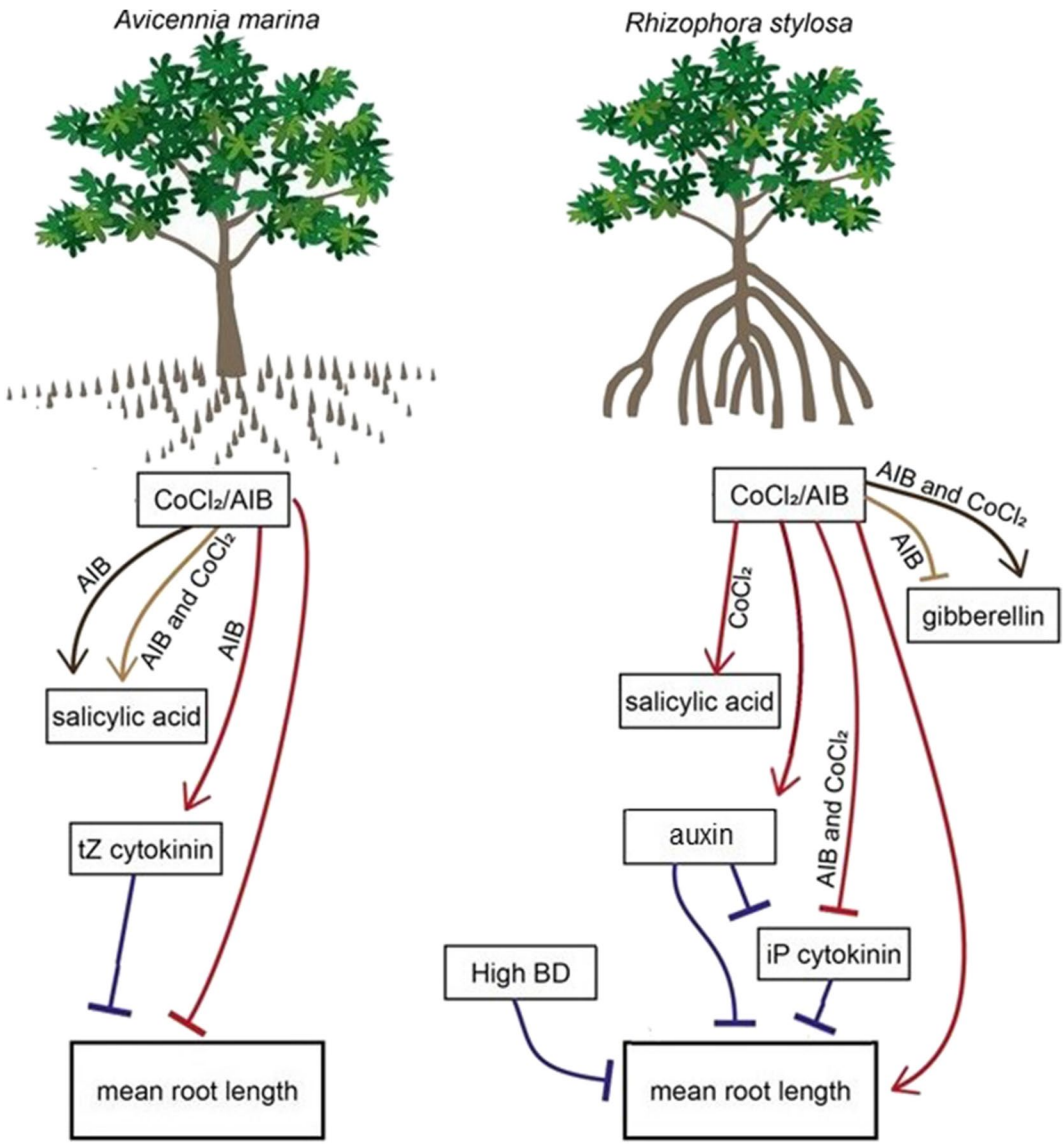


**Fig. 5** Proposed hormone networks affecting root growth response of the mangrove species *Avicennia marina* (left) and *Rhizophora stylosa* (right) to variation in soil bulk density (BD) and ethylene inhibitors (cobalt chloride ($CoCl_2$) and aminoisobutyric acid (AIB)) with red: levels tested in this study; dark brown: the response to ethylene inhibitors under high BD; light brown: the response under low BD; blue: published interactions. Briefly, blocking ACC oxidase/ethylene biosynthesis with these inhibitors is postulated to inhibit root growth. In both mangrove species, inhibitors increased salicylic acid levels, however in *A. marina* the effect depended on soil BD. Additionally, AIB increased *t*Z cytokinin levels in *A. marina* root tissue with possible root growth inhibition. Conversely, inhibitors dramatically increased auxin (IAA) levels in *R. stylosa* with possible negative effects on iP cytokinin levels. Further, inhibitors affect gibberellin (GA3) depending on soil BD in *R. stylosa*

In conclusion, dense soils inhibited root growth of *R. stylosa* but not *A. marina*, possibly due to differences in root types of these two mangrove species. In *A. marina* SA seems to regulate root growth responses to soil BD, possibly by affecting the biosynthesis of various phenolic compounds such as lignin that reinforce the root. Moreover, ethylene appears to be of little importance in *A. marina*. Conversely, ethylene and its interaction with GA regulate *R. stylosa* growth responses to soil BD. Further, the responses elucidated

by applying ethylene inhibitors may reflect adaptations to differing conditions within the intertidal zone, as *A. marina* behaves like an aquatic species, while *R. stylosa* behaves like a terrestrial species. These findings improve our understanding of the regulatory mechanisms underlying the response of these mangrove species to environmental drivers such as soil BD, which may inform mangrove production in nurseries for, and the design of, restoration projects, which often fail due to inappropriate or unsuitable growth environments (Lovelock et al. 2022). Ultimately, these findings may help to develop molecular techniques that facilitate the establishment of plants on soils with suboptimal BD conditions.

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**Declarations**

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**Competing Interests** The authors have no competing interests to declare.

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