The effects of petroleum hydrocarbon contamination on selected intertidal macrophytes and meiofauna

by

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As the candidate's supervisor I have/have not approved this thesis for submission

Professor G. Naidoo	**Date**
(Supervisor)	
Dr. Y. Naidoo	**Date**
(Co-supervisor)	

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal from March 2010 to December 2014 under the supervision of Professor G. Naidoo and Dr. Y. Naidoo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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Krishnaveni Naidoo

DECLARATION: PLAGIARISM

I, _____

declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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Krishnaveni Naidoo

DEDICATION

This thesis would have not been possible without the love of Jesus in my life. Everything I have is because of Him, I therefore thank Him above all else.

I dedicate this thesis to my precious mum, Dhanam Naidoo, your unconditional love, wisdom, courage, and fighting spirit has inspired me to be a success in all areas of my life. And to my dad in heaven, thank you for instilling in my soul, a passion for nature and desire to pursue science.

To my husband, Evan Van Dieman, thank you for being a pillar of love, encouragement, motivation and support in every possible way and tirelessly assisting me throughout the PhD, I would not have been able to accomplish this without you.

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Thank you to all my treasured loved ones for always believing in me. I am grateful for your love and unwavering support in every facet of my life. You have helped shape me into the person I am.

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General abstract

The effects of bunker fuel oil on the growth of *A. marina*, *B. gymnorrhiza* and *R. mucronata* were investigated in glasshouse and field experiments. The effects of oil on community structure in micro-organisms were also investigated in microcosm glasshouse experiments. The differences in oil tolerance of the three mangroves were compared in propagule and sediment oiled treatments and growth monitored for 13 months under glasshouse conditions. In propagule oiled treatments, various portions of the propagule were coated with oil. In the sediment oiled treatments, 50ml oil were added to the sediment in each pot. In oiled treatments, plant height, number of leaves and chlorophyll content were significantly reduced in all species compared to the control. In *A. marina* and *R. mucronata*, oiling resulted in growth malformations such as abnormal phyllotaxy and deformity of leaves and stems.

The effects of oil on root growth were investigated in rhizotrons for 245 and 409 days respectively. In oiled treatments, root growth rate, length and volume were significantly reduced in all species. In *A. marina* and *B. gymnorrhiza* oil increased root diameter.

In another series of experiments, PAH accumulation in roots and leaves of the three species were determined in one year old seedlings subjected to oiling for 21 days. The concentrations of 15 PAHs in roots and leaves were determined by gas chromatography / mass spectrometry. The highest total concentration of PAHs was accumulated in oiled roots of *A. marina* (44,045.9µg/kg), followed by *B. gymnorrhiza* (10,280.4µg/kg) and *R. mucronata* (6,979.1µg/kg). In oiled treatments, the most common PAHs in roots of all species were fluorene and acenaphthene (two rings), phenanthrene and anthracene (three rings), pyrene and chrysene (four rings) and benzo[a]pyrene (five rings). In the leaves of all species in oiled treatments, the common PAHs were naphthalene and acenapthene (two rings) and phenanthrene (three rings).
To test for living and dead root tip cells and to compare the effects of oil on cell ultrastructure in roots and leaves of the three species, one year old seedlings were subjected to a control and sediment oiled treatments for seven days. Control root tips, stained with fluorescein diacetate, exhibited green fluorescence in living cells of the meristematic and conducting tissue in all species. Oiled root tips, stained with propidium iodide, exhibited red fluorescence, indicating cell death or dead cells.

Transmission electron micrographs revealed that oil damaged cell ultrastructure in root tips and leaves in all species. Anatomical changes induced by oil included, disorganization of cells in the root cap, epidermis and meristem. Oil also induced loss of cell contents and destruction of organelles in root tissue. Oil damaged chloroplasts and cell organelles in spongy mesophyll and palisade cells of leaves.

To compare the effects of oil on the ability of the three species to tolerate salinity, healthy one year old seedlings were subjected to 10% and 50% seawater in control and sediment oiled treatments for 12 months. In the oiled treatments, 200ml oil were added to the soil in each pot. Oil significantly reduced growth in the 50% seawater treatment in all species. Results suggested that oil reduces salt tolerance in the three species.

The effects of oil on salt secretion in *A. marina* were investigated by subjecting one year old seedlings to sediment oiling treatments at 0%, 10% and 50% seawater for three weeks. Sodium accumulated in the leaves of oiled seedlings at 10% and 50% seawater. The effects of oil on salt secretion in *A. marina* in the light and dark were compared in one year old seedlings subjected to oiling treatments for seven days. Sodium accumulated in the leaves of oiled seedlings in the light and dark within 11 hours. Oil reduced secretion rates of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} in all treatments.

The effects of oil on species abundance, richness and community structure of soil micro-organisms were determined by subjecting microcosms to oiling treatments with or without fertiliser for four weeks. In the oiled treatments, 15ml oil and 5ml/L fertiliser were added to 200g soil. Fertiliser consisted of 4% N, 2% P and 5% K. Nematodes were extracted after the experimental period and identified to genus or species level. Oil significantly reduced species abundance and richness. Oil also eliminated sensitive species and altered the abundance of dominant species thereby altering the free living nematode community structure. Addition of fertiliser increased richness and dominant species in oiled treatments.

The effects of oil coating on leaves and internodes on growth of the three mangroves were investigated in field experiments for 48 weeks. Oiling of the leaves resulted in leaf abscission and decreased leaf production in all species. The effects of sediment oiling (at a dose of $5Lm^{-2}$) on the three species were also investigated in a field study for 53

weeks. In *A. marina*, oil caused adventitious roots to develop on the stem, about 10-15 cm above the soil surface after 38 weeks of treatment. In oiled treatments, plant mortality occurred after 53 weeks in all three species.

The ability of *B. gymnorrhiza* and *R. mucronata* to exclude PAHs from sensitive root tissues probably accounted for the higher oil tolerance than *A. marina*. The capacity of the species to adapt to residual oil contamination by increasing root diameter (*A. marina* and *B. gymnorrhiza*), producing adventitious roots (*A. marina*), increasing root/shoot ratio (*R. mucronata*) and abscising oiled leaves (all species) probably contributed to oil tolerance.

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General Introduction

Mangrove habitats are plant-dominated ecosystems that occur between marine and terrestrial environments (Zhang *et al*., 2007a; Lewis *et al*., 2011). Mangroves comprise 70 tropical and subtropical species, 28 genera, and 19 families (Duke *et al*., 1998; Field, 1998). These halophytic macrophytes occupy a narrow ecological range and are adapted to high temperatures, fluctuating salinities and anoxic soils (Paliyavuth *et al*., 2004; Parida and Das, 2005). They protect shorelines from floods, wave action and erosion (Duke and Wolanski, 2001; Zahed *et al*., 2010; Gedan *et al*., 2011).

Mangrove ecosystems play an important role in the export of carbon and nutrients to the coastal zone (Kristensen *et al*., 2008; Adame and Lovelock, 2011). The highly productive mangrove habitats serve as nurseries for a wide variety of fauna (Holguin *et al.,* 2001; Feller *et al*., 2010). They are currently regarded as one of the most threatened marine environments (Bayen, 2012). Many mangrove species are at a high risk of becoming extinct (Polidoro *et al*., 2010).

Oil contamination has been recognized as one of the most serious threats to marine environments (Tam *et al*., 2005). Mangroves are particularly sensitive to the impacts of oil spills (Proffitt *et al*., 1995; Duke *et al.*, 2000; Duke and Watkinson, 2002). Exposed shores can recover faster from oil spills than sheltered areas which are often completely inundated with oil (Kingston, 2002; Anderson *et al*., 2008). Oil can persist in the marine environment and effects are measurable after decades (Kingston, 2002). Oil persisted for more than 25 years after the Metula oil spill in Tierra del Fuego, Chile (Owens *et al*., 1999).

Oil contamination occurs by spills from ships, leakage of fuel from refineries, improper disposal and discharge of used motor and lubricating oil (McNicoll and Baweja, 1995; Tam *et al*., 2005). Petroleum hydrocarbons are the toxic components of oil and are highly persistent organic pollutants in the environment (Moreira *et al*., 2011). Oil spills adversely affect a wide variety of natural resources and services in marine environments (Dsikowitzky *et al*., 2011). The severity of oil spills depends on the season, the discharge volume, type and location and environmental conditions at the time of occurrence (Moreira *et al*., 2011). Polluted mangrove habitats exhibit low

biodiversity, genetic abnormalities and reduced ecosystem function (Duke and Wolanski, 2001; Duke and Watkinson, 2002). Oil pollution disrupts trophic relationships between organisms (Lee, 1995).

As marine ecosystems are dynamic, the state to which an environment returns after oil spill damage is usually unpredictable (Kingston, 2002). The biological recovery of marine ecosystems is dependent on robust colonizing organisms that can tolerate residual oil contamination in sediments (Baker *et al*., 1990). Re-colonization of oil-impacted ecosystems depends on the time of year, the availability of colonizing organisms, biological interactions and climate (Carman *et al*., 1997; Kingston, 2002).

Oil pollution in South Africa poses a threat to coastal marine ecosystems (Orr *et al*., 2008; Whitfield and Cowan, 2010). The South African coastline is one of the world's busiest oil-shipping routes making it prone to tanker accidents, resulting in spills (Moldan and Jackson, 2005). Since 1987, 82 000 tonnes of oil were discharged into South African coastal waters (Strydom and King, 2009). The South African Maritime Safety Authority facilitates the clean-up of major oil spills along our coastline according to the Marine Pollution Control Act (No. 6 of 1981) (SAMSA, 2013). However, most spills are small (less than seven tonnes), occur during loading, discharging and bunkering and originate in or near ports (Taylor *et al.*, 2003; O'Brien, 2006). Ecosystems such as mangroves associated with ports are at high risk from oil pollution (Taylor *et al.*, 2003; O'Brien, 2006).

Oil spills result in loss of mangrove area cover in South Africa (Taylor *et al*., 2003). In South Africa, the total mangrove area is 1631.7 ha, with the largest cover occurring in the KwaZulu-Natal Province (1391.1 ha) (Hoppe-Speer, 2012). A loss of 6.5 per cent of mangroves per year is estimated along the South African coast (Taylor *et al*., 2003). As a result, *Bruguiera gymnorrhiza* and *Rhizophora mucronata* are listed in the Protected Tree list (DWAF, 2010).

Oil pollution research is limited in South Africa (Wolfaardt *et al*., 2009; Naidoo *et al*., 2010). Oil pollution studies have concentrated on the impact of spills on penguins (Underbill, 2000; Wolfaardt *et al*., 2009). Marine ecotoxicology research in South Africa faces limitations such as the high costs involved in organic contaminant analysis and the necessary equipment to conduct routine analyses (Wepener and Degger, 2012).

The assessment and monitoring of pollution effects are important (Whitfield and Cowan, 2010; Wepener and Degger, 2012) in maintaining the high degree of biodiversity in the relatively pristine marine ecosystems of South Africa (Griffiths *et al*., 2010).

Chapter 1

Literature Review

1. 1 Introduction

Population growth and industrialisation have led to anthropogenic pollution in many coastal regions (Iturbe *et al*., 2007; Inckot *et al*., 2011; Oyo-Ita *et al*., 2013). Oil pollution in particular, contributes to the reduction of biodiversity and productivity of coastal ecosystems worldwide (Dsikowitzky *et al*., 2011; Moreira *et al*., 2011). Many studies have documented the devastating effects of oil pollution on coastal marshes (Kennish, 1992; Lin and Mendelssohn, 1996; Dowty *et al*., 2001) and mangroves (Nansingh and Jurawan, 1998; Tam *et al*., 2005; Naidoo *et al*., 2010) around the world.

Mangroves are prone to contamination from oil spills because they occupy sheltered bays that are inundated by tides (Proffitt *et al*., 1995; Mille *et al*., 2006). The results of prolonged oil pollution are reduced ecosystem function, low biodiversity and genetic abnormalities (Lee, 1995; Duke and Watkinson, 2002). Oil adheres to and soaks into the sediments and covers exposed trunks, foliage, prop roots and pneumatophores (Getter *et al*., 1985; Duke *et al*., 1998). This leads to oxygen deficiency, suffocation, growth irregularities and plant mortality (Garrity *et al*., 1994; Duke and Burns, 2003; Naidoo *et al*., 2010). The intensity of oil toxicity is dependent on the type and quantity of the oil, the period of exposure, climatic conditions, sediment type and plant species (Lin *et al*., 2002; Ke *et al*., 2011a).

1.2 Factors influencing oil toxicity

1.2.1 Characteristics of oil

Oil is a naturally occurring substance (Kingston, 2002). Hydrocarbons (the components of oil) can be either biogenic, petrogenic or pyrogenic in origin (Guo *et al*., 2005; Lima *et al*., 2005; Yu *et al*., 2005). Biogenic sources of hydrocarbons are biosynthesized by living organisms and include plant alkanes, sterols, fatty acids and waxes (Wang *et al*., 2009). Petrogenic sources include petroleum which is comprised of saturates, olefins, alkanes, cyclohexanes, asphaltenes and polycyclic aromatic hydrocarbons (PAHs)

(Luz *et al*., 2010; Naidoo *et al*., 2010). Pyrogenic sources are formed through rapid, high temperature combustion and include motor, shipping and power plant fuels (Lima *et al*., 2005). PAHs are highly toxic and have carcinogenic and mutagenic properties (Luan *et al*., 2006; Cavalcante *et al*., 2009; Li *et al*., 2010). Substances that are added to oil such as amines, phenols, benzene, sulphur and lead are less biodegradable than the base oil (Thompson *et al*., 2007). These additives increase the toxicity of oil (Haus *et al*., 2001; Drzyzga, 2003; Powell *et al*., 2005).

The rate of oil degradation is determined by physical and chemical factors such as oil thickness and components (Kingston, 2002). Biological factors such as temperature, sediment grain size, microbial communities and availability of nutrients also influence degradation rates (Colombo *et al*., 2005; Santos *et al*., 2010). During the first 24 hours of a spill, up to 40% of the volatile components of oil start to evaporate (Shigenaka, 2011). Thick oils have a longer evaporation time (Durako *et al*., 1993; Ralph and Burchett, 1998). Oil is broken up by wave action into small droplets that are degraded by bacteria (Ramsay *et al*., 2000; Yuan *et al*., 2001; Soares-Gomes *et al*., 2010). Lighter oils can have a greater degree of penetration and persistence in soils (Vandermeulen *et al*., 1981; Gundlach *et al*., 1993).

1.2.2 Sediment type

The accumulation and retention of oil in sediments is determined by the proportions of sand, clay and organic matter and the size of soil particles (Li *et al*., 2010; Paixão *et al*., 2011). Sandy soils that have larger pore spaces allow a greater penetration of oil into the deeper root zones (Pezeshki *et al*., 2000; Kingston *et al*., 2003). PAHs are hydrophobic and adsorb onto organic matter and fine clay particles (Chen and White, 2004; Yu *et al*., 2005; Dsikowitzky *et al*., 2011). The waterlogging features of mangrove sediments reduce microbial breakdown of PAHs (Pereira *et al*., 2002). PAHs therefore accumulate and persist in muddy mangrove sediments causing long term exposure of plants to oil contamination (Burns *et al*., 2000; Taylor and Jones, 2001).

Oil persistence decreases sediment permeability, soil pH, dissolved oxygen concentrations, redox potentials and salinity of the interstitial water (Suprayogi and Murray, 1999; Pereira *et al*., 2002). Decreased oxygen levels in sediments reduce root respiration, disrupt ion and water relations and ultimately result in reduced plant growth

(Gilfillan *et al*., 1989; Pezeshki *et al*., 2000). The biodegradation of oil by microorganisms can also lead to oxygen depletion in sediments (Li *et al*., 2009). There is a higher concentration of bacteria in oil contaminated than in non-contaminated sediments (Espinosa *et al*., 2005). An increase in bacterial density increases the rate of oxygen depletion resulting in the creation of anaerobic niches (Pereira *et al*., 2002). This leads to the proliferation of anaerobic bacteria and products of degradation such as hydrogen sulphide that contributes to a slower oil degradation rate (Li *et al*., 2009).

1.2.3 Salinity

Mangroves have adapted to high salinity by developing salt tolerance mechanisms to regulate the salt concentrations in the plant (Naidoo and von Willert, 1995). *Kandelia obovata* (L.) and *Rhizophora mucronata* Lam. resist salt stress by excluding salt from entering the plant (Naidoo, 1986). *Aegiceras corniculatum* (L.) Blanco. and *Avicennia marina* (Forsk.) Vierh. secrete salt through salt glands present in the leaves (Naidoo, 1987; Naidoo and Chirkoot, 2004; Ye *et al*., 2005). *Bruguiera gymnorrhiza* (L.) Lam. stores excess salt in vacuoles or in older leaves, which are then shed (Tam and Wong, 2000; Youssef and Ghanem, 2002). Mangroves reduce the amount of salt that is transported to the foliage by regulating water loss through stomatal control (Naidoo and von Willert, 1995). This prevents salt accumulation in tissues (Parida and Das, 2005). High salinity can reduce the metabolism of plant cells thereby limiting growth (Lovelock *et al*., 2004; Parida and Jha, 2010). Naidoo *et al*. (2011) reported that *A. marina* under hypersaline conditions had reduced photosynthesis and exhibited swelling and disintegration of cell organelles such as chloroplasts, mitochondria and nuclei.

Oil impairs the salt secreting mechanism of *A. marina* (Youssef and Ghanem, 2002). PAHs damage root membranes, adversely affecting the ionic balance of plants and their ability to tolerate salinity (Gilfillan *et al*., 1989; Zhang *et al*., 2007a). The combination of high salinity and oil aggravates the toxicity of PAHs thereby increasing oil damage to plants (Ibemesim, 2010; Ke *et al*., 2011b). High salinity in combination with oil reduced germination in salt marsh grasses (Youssef, 2002). High soil salinity reduces the metabolism of microorganisms thereby reducing degradation of PAHs in the sediment (Ke *et al*., 2011b).

1.2.4 Tidal cycles and waterlogging

Mangroves occupying various coastal habitats endure different tidal cycles that are affected by sea level rise (Lacerda *et al*., 2002). During continuous tidal flooding the interstitial spaces between soil particles become saturated with water, reducing the supply of oxygen to plants (Ke *et al*., 2011b). Mangroves have well-developed aerenchyma that provides efficient transport of atmospheric oxygen to roots in anoxic soils (Armstrong *et al*., 2009). However, tidal inundation and waterlogging can cause severe oxygen stress reducing the growth of plants and microorganisms (Ye *et al*., 2003). Oxygen deficiency leads to decreased nutrient uptake thereby inhibiting ATP synthesis (Steffens *et al*., 2005). Nutrient deficiency can weaken plant resistance to pollutants and slow down microbial degradation of pollutants (Romantschuk *et al*., 2000). Oxygen depletion enables the accumulation and persistence of oil in the sediment and decreases the tolerance of mangroves to PAH toxicity (Li *et al*., 2009; Ke *et al*., 2011b).

1.2.5 Mangrove characteristics

Studies on the responses of mangroves to salinity and waterlogging are abundant (Naidoo, 1985; Patel *et al*., 2010; Naidoo *et al*., 2011). The effects of nutrients and heavy metals on mangroves have been widely reported (MacFarlane *et al*., 2003; Naidoo, 2009; Harish and Murugan, 2011). In comparison, studies on oil contamination have been few (Ye and Tam, 2007; Naidoo *et al*., 2010). The degree of oil toxicity in mangroves depends on the type, dosage and frequency of oiling treatment (Pezeshki *et al*., 2001; Ke *et al*., 2011a; Shigenaka, 2011). The effects of different oil types, such as Bunker fuel (Naidoo *et al*., 2010); No.2 fuel (Getter *et al*., 1985); No.6 fuel (Proffitt and Devlin, 1998); Kuwait crude and North West Shelf Condensate (Suprayogi and Murray, 1999) on mangroves have been reported. In addition, the effects of fresh (Proffitt *et al*., 1995) and used lubricating oil (Ye and Tam, 2007; Zhang *et al*., 2007a) have also been reported.

Oil is drawn up into roots and translocated to the foliage by transpiration (Getter *et al*., 1985). PAHs accumulate in roots, leaves and stems (Suprayogi and Murray, 1999; Meudec *et al*., 2006; 2007). When oil migrates into deeper soil sediments, it leads to mortality of the roots and ultimately the plant itself (Tam *et al*., 2005). The

morphological and physiological characteristics of plants can determine the degree of oil toxicity (Ye and Tam, 2007; Pi *et al*., 2009; Naidoo *et al*., 2010). Differences in leaf transpiration and root uptake determine the survival rates of plants exposed to oil (Getter *et al*., 1985). Therefore, oil can severely affect plant community composition depending on the sensitivity of individual species to toxicity (Proffit *et al*., 1995; Lin and Mendelssohn, 1996).

Short term negative effects of oil include inhibition of germination, leaf wilting, defoliation and abnormal growth morphology (Duke *et al*., 1997; Naidoo *et al*., 2010). Long term effects include reduced growth and biomass, genetic mutations and increased plant mortality (Duke and Watkinson 2002; Zhang *et al*., 2007a; Ke *et al*., 2011a). Oil that coats the leaves, blocks stomata and reduces transpiration, increases leaf temperature and restricts CO_2 entry, reducing photosynthesis (Pezeshki *et al*., 1997; 2000; Lin *et al*., 2002; Naidoo *et al*., 2010). Oil accumulation within the plant damages tissues and cells (Watts *et al*., 2006; Kang *et al*., 2010).

Oil contamination accelerates the production of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl free radicals (Proffitt *et al*., 1995; Zhang *et al*., 2007a). ROS disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Parida and Das, 2005). This adversely affects biological membranes and metabolic processes (Ke *et al*., 2011a) resulting in an inhibition of plant growth leading to mortality (Takemura *et al*., 2000). ROS-scavenging antioxidant enzymes, including superoxide dismutase (SOD), perioxidase (POD) and catalase (CAT), increase after exposure to oil (Parida *et al*., 2004; Zhang *et al*., 2007a). These enzymes are biomarkers for the recognition of damage caused by pollution (Caregnato *et al*., 2008). In addition, lipid peroxidation (oxidative degradation of lipids) caused by ROS produces malondialdehyde (MDA) that is used as an indicator of oxidative stress (Yadav, 2010; Harish and Murugan, 2011). Physiological damage which is caused by the direct contact of organelles with oil, leads to the visible physical effects on plants (Watts *et al*., 2006; Kang *et al*., 2010).

1.3 Oil effects between species

Avicennia marina, *B. gymnorrhiza* and *R. mucronata* exhibit distinct zonation patterns in Durban, South Africa. *Avicennia marina* occupies the seaward portions of swamps whereas the latter two species occur at the mid-tidal level (Naidoo, 1989). *Avicennia marina*, a pioneer species, possesses salt glands and is highly salt-tolerant (Naidoo, 1987; Patel *et al*., 2010). *Bruguiera gymnorrhiza* and *R. mucronata* are both salt excluders (Tam and Wong, 2000; Hoppe-Speer *et al*., 2011). *Avicennia marina*, *B. gymnorrhiza* and *R. mucronata* plants treated with oil exhibit leaf wilting, defoliation, decreased photosynthesis, reduced biomass and increased mortality (Ye and Tam, 2007; Naidoo *et al*., 2010; Ke *et al*., 2011a).

Oil dosage is important in determining the degree of damage to plants (Levings and Garrity, 1995; Profitt and Devlin, 1998). The degree of oil coverage on the propagule determines the rate of germination (Proffitt *et al*., 1995; Zhang *et al*., 2007a). Complete oil coating inhibits germination, for example, in *Rhizophora mangle* L. (Proffitt and Devlin, 1998). *Rhizophora mangle* exhibited a decrease in shoot length and number of leaves at heavily oiled compared to light sites (Levings *et al*., 1997). *Avicennia germinans* (L.) and *R. mangle* exhibited similar results as the previous study when exposed to single heavy and multiple, smaller doses of oil (Proffitt *et al*., 1995). The single heavier oil dose caused greater adverse effects, including, severe reduction in growth and survival rate compared to the latter treatment. A single high oiling dose to *B. gymnorrhiza* caused greater toxic effects than weekly oiling at lower doses (Ke *et al*., 2011a). Mangroves can adapt to oil toxicity over time and become more tolerant to multiple, smaller oiling doses (Ke *et al*., 2011a).

PAHs that enter the roots are drawn up and travel apoplastically with the transpiration stream via the xylem (Wild *et al*., 2005). *Avicennia marina* has well-developed xylem and phloem that allows for greater bioaccumulation of PAHs within roots, stems and leaves (Pi *et al*., 2009; Ke *et al*., 2011a). *Avicennia marina* can accumulate between two and six times more hydrocarbons in its leaves compared to other mangroves, including *R. mucronata* (Suprayogi and Murray, 1999). Lubricating oil damages the xylem vessels of fine roots of *A. marina* (Ye and Tam, 2007). PAHs disrupt cellular lipid membranes in the conducting tissue which leads to growth reduction (Gilfillan *et al*., 1989; Zhang *et al*., 2007a).

At sub-lethal doses, mangroves could change growth rates and develop morphological adaptations to survive the physical and chemical effects of oil contamination (Hoff, 2002). Bunker fuel oil applied to the base of debarked stems of *A. marina* and *B. gymnorrhiza* resulted in the production of adventitious roots in the former species but not in the latter (Naidoo *et al*., 2010). The study concluded that adventitious root production is an adaptive response after damage to the phloem tissue. *Bruguiera gymnorrhiza* exhibited a greater tolerance to oil compared to other oil-treated mangroves (Ke *et al*., 2011a). The root epidermis of *B. gymnorrhiza* consists of highly suberized cells that enable a high binding tendency of lipophilic substances such as oil (Ke *et al*., 2003). This characteristic restricts PAHs in the roots, reducing negative effects and increasing oil tolerance (Ke *et al*., 2011a).

Rhizophora mangle had a higher survival rate when exposed to oil compared to *A. germinans*. This was attributed to differences in root uptake and leaf transpiration (Getter *et al*., 1985). The differences in the effects of oil on *A. marina*, *R. mucronata* and *R. mangle* are also associated with differences in tolerance of anaerobic conditions in oil-saturated sediments (Dicks, 1986). High mortality of *A*. *marina* occurred in heavily oiled sediment with low oxygen levels and low soil redox potentials (Dicks and Westwood, 1987). *Avicennia germinans* is more sensitive to highly reduced soil conditions compared to *R*. *mangle* (Pezeshki *et al*., 1997). The roots of *R. mangle* (Joner *et al*., 2005) and *R. mucronata* (Holmer *et al*., 1999) exude compounds such as carbohydrates and amino acids. These compounds stimulate bacterial degradation of contaminants thereby increasing oil tolerance (Moreira *et al*., 2011).

Ye and Tam (2007) found that lubricating oil applied to the canopy resulted in greater physical effects and increased mortality compared to base-oiling in *A. marina*. Their study showed that canopy and base-oiling resulted in greater leaf and root damage and increased mortality in *Avicennia marina* compared to *Aegiceras corniculatum.* They concluded that the dense trichomes on the abaxial surface of *A. marina* leaves promote oil adherence, contributing to a higher sensitivity. Several studies have shown *A. marina* to be more sensitive to oil than other mangroves (Proffitt *et al*., 1995; Suprayogi and Murray, 1999).

The salt secreting ability of some mangroves is another factor that determines differential oil sensitivity. *Avicennia marina* is susceptible to greater oil uptake because

it is a salt-secretor and is unable to efficiently exclude salt and PAHs compared to salt-excluders (Suprayogi and Murray, 1999). The salt glands of *A. marina* are important in controlling the ionic composition of photosynthesizing leaves (Waisel *et al*., 1986). *Avicennia marina* is more efficient at salt secretion compared to *A. corniculatum* (Ye *et al*., 2005). However, fumigation of *A. marina* with volatile fraction crude oil at high salinity decreased the rate of salt secretion (Youssef and Ghanem, 2002).

The rate of salt secretion increases when the salt content in the foliage increases and is naturally independent of stomatal behaviour (Waisel *et al*., 1986; Naidoo and von Willert, 1995). Yousseff and Ghanem (2002) demonstrated that oil fumes interfered with the plasma membranes of salt glands and slowed the secretion process. Their study found that this in turn affects leaf water potential and decreases stomatal conductance and transpiration. As a result of reduced stomatal conductance, plants decrease gas and nutrient uptake, leading to a reduction in growth (Lindau *et al*., 1999). Kuwait crude oil decreased the concentration of nutrients in *A*. *marina* but not in *R. mucronata* (Suprayogi and Murray, 1999).

Oil-induced damage to the salt secreting mechanism results in the excess accumulation of salt in plant tissues (Page *et al*., 1985). In *B. gymnorrhiza*, high salinity in combination with oil resulted in reduced growth and biomass and increased the concentration of MDA (Ke *et al*., 2011b). The roots of *R. mangle* can exclude salt and PAHs, decreasing the amount of oil taken up by the roots thereby increasing its oil tolerability (Suprayogi and Murray, 1999).

Oil causes the production of albino propagules (lack chlorophyll) in some mangroves (Chen *et al*., 1996). Chlorophyll deficient propagules are unable to photosynthesise and appear white, yellow, pink or red in colour in *A. marina* and *R. mangle* (Handler and Teas, 1983; Duke and Watkinson, 2002). Newly established seedlings of these mutant propagules produce chlorophyll deficient leaves and therefore do not survive (Chen *et al*., 1996). Albino propagules are more frequent in sediments with higher levels of PAHs (Klekowski *et al*., 1994; Proffitt and Travis, 2005). The presence of PAHs in sediments is indicative of genetic deterioration of the mangrove habitat (Duke and Watkinson, 2002).

1.4 Oil contamination in meiofauna

Many studies have investigated the harmful effects of oil on meiofauna in field experiments (Ansari and Ingole, 2002; Thompson *et al*., 2007) and experimental microcosms (Suderman and Thistle, 2003; Boufahja *et al*., 2011). Benthic meiofauna are organisms that pass through a 500-1000 µm mesh and are retained on a 63-44 µm mesh (Gyedu-Arabio and Baird, 2006; Giere, 2009). Meiofauna play a vital role in the benthic food web (Sundbäck *et al*., 2010) and are involved in sediment bioturbation (reworking of the soil) and recycling of organic matter (Lindgren *et al*., 2012). Juvenile fish in intertidal habitats feed on meiofauna (Coull *et al*., 1995).

Effects of oil on nematodes

Among benthic meiofauna, nematode communities are commonly used in microcosm studies (Austen *et al*., 1994; Mahmoudi *et al*., 2005) because they are small, numerous, easy to maintain (Suderman and Thistle, 2003) and are sensitive to contaminants (Santos *et al*., 2010). There are many factors that contribute to the negative effects of PAHs on nematode communities, including, type of oil and dosage, morphological and physiological differences and sedimentary conditions (Di Toro *et al*., 1991; Carman and Todaro, 1997; Louati *et al*., 2001). Lubricants produce more intense toxic effects on nematode assemblages than other oils (Thompson *et al*., 2007). An increase in oil dosage leads to an increase in the intensity of toxicity (Mahmoudi *et al*., 2005).

PAHs decrease nematode abundance and species richness (Powell *et al*., 2005; Moreno *et al*., 2009; Lv *et al*., 2011). In addition, oil can cause the increase, decrease or elimination of certain species (Mahmoudi *et al*., 2005; Beyrem *et al*., 2007). Thus community composition is altered resulting in reduced biodiversity (Schratzberger *et al*., 2003; Beyrem *et al*., 2010) which in turn alters nematode community structure (Beyrem *et al*., 2010). Nematode communities that live in oil contaminated environments become more tolerant to PAHs over time (Carman *et al*., 2000).

PAHs decrease soil permeability (Langston and Spence, 1994; Pereira *et al*., 2002). The hydrophobic nature of PAHs restricts the number of habitable spaces between soil particles (Gao *et al*., 1998). PAHs decrease dissolved oxygen concentrations in the

sediment that leads to oxygen stress (Carman *et al*., 2000; Neira *et al*., 2001). Oil enhances the retention of other toxins such as heavy metals (Millward *et al*., 2004). Combinations of heavy metals and PAHs further reduce nematode assemblages (Kennicutt *et al*., 1996; Beyrem *et al*., 2007). The food sources of nematodes that include bacteria are negatively affected by PAHs (Austen and McEvoy, 1997; Blakely *et al*., 2002). In addition, PAHs promote the growth of microalgae that produce mucous exo-polymers (Carman *et al*., 1997). These exo-polymers have an affinity for heavy metals and are consumed by nematodes, resulting in greater toxic effects (Decho and Lopez, 1993).

1.5 Remediation of oil contaminated sites

A clean environment is one that contains a level of PAHs that does not impact the functioning of an ecosystem (Kingston, 2002). Natural attenuation is the process by which oil is degraded in the sediment by microorganisms such as bacteria and fungi (Colombo *et al*., 2005). This process is controlled by physical and chemical factors such as composition of the microbial community, temperature, sediment grain size and nutrient availability (Lee and Lee, 2003; Smets and Pritchard, 2003; Tam *et al*., 2005). Degradation of individual PAHs occurs at different rates and is dependent on the structure of the PAH and the group of microorganism (Fedorak and Westlake, 1981; Cerniglia, 1992).

The manual removal of spilled oil facilitates natural attenuation (Ramsay *et al*., 2000; Kingston, 2002). However, removal of persistent PAHs from soils is difficult, slow and expensive (Huang *et al*., 2005; Moreira *et al*., 2011). Mechanical techniques include removal of vegetation and flushing with clean water that causes permanent habitat destruction (Pezeshki *et al*., 2000). Incineration of the site leads to a change in species dominance (Pezeshki *et al*., 2001). Dispersants and chemical cleaners are toxic to many organisms (Fingas, 2011; Mishra *et al*., 2012). Phytoremediation uses the planting of trees to enhance natural attenuation (Parrish *et al*., 2006). The by-products of natural attenuation are carbon dioxide and water (Lee *et al*., 1997) that are taken up by plant roots (Park *et al*., 2011).

Bioremediation is the addition of nutrients or fertiliser to enhance natural attenuation (Lin *et al*., 2002; Colombo *et al*., 2005; Xu *et al*., 2005). Fertilisers are effective in

biodegradation of PAHs because they maintain nutrient concentrations at high levels in oil-contaminated sediments (Xu *et al*., 2005). This provides sources of energy, carbon and nitrogen for microorganisms (Richmond *et al*., 2001). Nutrients stimulate the growth and activity of microorganisms thereby accelerating PAH degradation (Lee and Merlin, 1999; Ramsay *et al*., 2000; Mills *et al*., 2004). Nutrient addition accelerates the degradation process by up to 70% in mangrove sediments (Zhu *et al*., 2004). This process does not pose a threat to the environment and is efficient and cheap (Moreira *et al*., 2011).

1.6 Aims of study

Studies on oil contamination have mainly concentrated on the mass mortality of plants after large-scale crude oil spills (Lamparelli *et al*., 1997; Jones *et al*., 1998; Melville *et al*., 2009). Few studies have focussed on the effects of oil contamination on seedling establishment and growth of mangroves under sub-lethal concentrations (Proffitt *et al*., 1995; Zhang *et al*., 2007a). Studies on the responses of *A. marina*, *B. gymnorrhiza* and *R. mucronata* to oil contamination are few (Ye and Tam, 2007; Naidoo *et al*., 2010).

The three mangrove species were selected because they co-occur under similar environmental conditions with respect to salinity, tidal cycle, and substrate matrix. These species are taxonomically different – *A. marina* (family: Avicenniaceae), *B. gymnorrhiza* and *R. mucronata* (family: Rhizophoraceae). They possess different salinity adaptations. *Bruguiera gymnorrhiza* and *R. mucronata* are salt excluders whereas *A. marina* possesses leaf salt glands and is a salt secretor. These species have different tolerance levels to oil contamination (Zhang *et al*., 2007a; Naidoo *et al*., 2010; Ke *et al*., 2011a).

The degree of oil exposure is important in understanding plant responses to contamination (Suprayogi and Murray, 1999). In this study, different methods of oiling were used to characterise the responses of mangroves to oil contamination. Propagules (with and without pericarps in *A. marina*), leaves, internodes and sediments were oiled. Little is known about the accumulation of oil components in tissues and cells of roots and leaves and their contribution to oil-induced plant stress.

Little is known about the effects of oil on South African meiofauna. Field studies of oil contamination are few and restricted to the composition of beach communities after an oil spill (Hennig and Fricke, 1980; Fricke *et al*., 1981). There are no studies on the effects of oil on nematode community structure and species richness. Nematodes were selected because they are useful bio-indicators of contamination (Blakely *et al*., 2002; Gyedu-Arabio and Baird, 2006) and are sensitive to PAHs (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010).

The primary objective of this study was to determine the effects of spent bunker fuel oil on:

i. mature plants of three mangrove species, namely, *A. marina*, *B. gymnorrhiza* and *R. mucronata* in glasshouse and field experiments, and
ii. selected meiofauna, namely, nematodes in microcosm experiments.

Specifically, this study determined the effects of oil on:

- growth of seedlings and mature plants;
- root growth and development;
- salt secretion in *A. marina*;
- root and leaf tissues and cells;
- nematode assemblages, and,
- nematodes in fertiliser amended sediments.

Chapter 2

The effects of oil contamination on growth of *Avicennia marina*, *Bruguiera gymnorrhiza* and *Rhizophora mucronata* in glasshouse and field experiments

Abstract

The responses of the mangroves *A. marina*, *B. gymnorrhiza* and *R. mucronata* to bunker fuel oil in glasshouse and field experiments were investigated. In the glasshouse study, freshly picked propagules of all species were subjected to oiled treatments for 13 months and growth monitored. In *A. marina*, treatments included a control; sediment oiled; propagules completely oiled (with or without pericarps) and ½ of the propagule without a pericarp oiled. In *B. gymnorrhiza* and *R. mucronata*, treatments included a control; sediment oiled and ⅔ propagule oiled. Oiling reduced plant height, number of leaves and chlorophyll content in all species compared to their respective controls. In *A. marina*, plant height, number of leaves and chlorophyll content were highest in the control and significantly reduced in the propagule without pericarp oiled treatment, by 96%, 92% and 66% respectively. Fifty percent plant mortality occurred in the propagule without pericarp oiled treatment after eight months. In *B. gymnorrhiza*, plant height, number of leaves and chlorophyll content were highest in the control and significantly reduced in the sediment oiled treatment by 45%, 31% and 22% respectively. In *R. mucronata*, plant height, number of leaves and chlorophyll content were significantly reduced in the sediment oiled treatment by 56%, 89% and 69%, respectively, compared to the control. Field experiments were carried out in Beachwood Mangroves Nature Reserve and Isipingo estuary on healthy, naturally occurring one to two year old seedlings. Leaves and internodes of all species were oiled for 48 weeks and growth monitored. In all species, oiling of the leaves resulted in leaf abscission and considerable decreases in plant height and leaf production. All species were also subjected to a sediment oiled treatment (at a dose of 5 Lm^{-2}) for 53 weeks and growth monitored. In *A. marina*, adventitious roots developed on the stem, about 10-15 cm above the soil surface after 38 weeks of treatment. Sediment oiling resulted in 89%, 100% and 75% plant mortality in *A. marina*, *B. gymnorrhiza* and *R. mucronata*, respectively, after 53 weeks. These results indicate that bunker fuel oil adversely affects growth of the three mangrove species.

Keywords:

Growth, morphology, oil, propagules, sediment

2.1 Introduction

The demand for petroleum escalates with increasing population growth (Inckot *et al*., 2011). A rise in petroleum production has resulted in oil spills that contaminate many coastal ecosystems (Dsikowitzky *et al*., 2011; Moreira *et al*., 2011). Oil pollution has had devastating impacts on coastal marshes (Dowty *et al*., 2001; Ko and Day, 2004; Mishra *et al*., 2012) and mangroves (Ansari and Ingole, 2002; Tam *et al*., 2005; Cavalcante *et al*., 2009).

Mangroves are distributed within the intertidal zones of tropical and subtropical regions (Paliyavuth *et al*., 2004) and are highly vulnerable to oil spills as they are located in low wave energy sheltered bays (Duke *et al*., 2000; Mille *et al*., 2006). Spilled oil that enters mangrove ecosystems soaks into sediments and coats aerial and prop roots (Getter *et al*., 1985; Proffitt *et al*., 1995). Oil coating leads to oxygen deficiency, suffocation and growth irregularities in the short-term and mortality in the long-term (Duke *et al*., 1998; Naidoo *et al*., 2010).

PAHs are the components of oil that penetrate plant roots and consequently cause root membrane damage (Gilfillan *et al*., 1989; Pezeshki *et al*., 2000; Zhang *et al*., 2007a). The responses of mangroves to environmental conditions such as salinity, water-logging and inorganic pollutants such as nutrients and heavy metals have been widely reported (Caregnato *et al*., 2008; Harish and Murugan, 2011; Naidoo *et al*., 2011). Few studies have focussed on the effects of oil contamination on establishment and growth of mangroves (Zhang *et al*., 2007a; Naidoo *et al*., 2010).

The aim of this study was to determine and compare the effects of bunker fuel oil on the growth of three mangroves, *A. marina*, *B. gymnorrhiza* and *R. mucronata* in controlled glasshouse and field experiments. The effects of oiling of the propagules, leaves and sediments were also compared.

2.2 Materials and methods

Glasshouse study

2.2.1. Growth conditions

Propagules of *A. marina* and *B. gymnorrhiza*, were collected from the Beachwood Mangroves Nature Reserve (29° 48' S, 31° 02' E) and those of *R. mucronata* from the Isipingo estuary (29° 59' S, 30° 56' E) in March 2010. After collection, *A. marina* propagules were placed in water and pericarps allowed to shed naturally (24 hours). Propagules of *A. marina* were planted in 17 cm diameter x 15 cm height and those of *B. gymnorrhiza* and *R. mucronata* in 24 cm x 21 cm plastic pots. All pots contained a mixture of sand, potting soil and compost (1:2:1). Pots were watered daily with tap water and once monthly with 10% seawater. All pots were maintained in a glasshouse for 13 months. The temperature in the glasshouse during the experimental period was 25 °C (day) and 18 °C (night).

In this study, propagules of *A. marina*, with or without pericarps were subjected to one of five treatments:

i. control (C) – propagules with pericarps were planted in the sediment.
ii. SO – propagules without pericarps were planted in sediment to which 50 ml of oil were carefully poured onto the soil surface.
iii. PO1 – propagules with pericarps were completely dipped in oil and planted in sediment.
iv. PO2 – propagules without pericarps were completely dipped in oil and planted in sediment.
v. ½PO – ½ of the propagule without a pericarp was dipped in oil using a pair of forceps.

Propagules of *B. gymnorrhiza* (about 12.5 ± 0.5 cm in height), and *R. mucronata* (about 22 ± 2 cm in height) were subjected to one of three treatments:

i. control (C) – untreated propagules were planted in the sediment.

ii. SO – propagules were planted in sediment to which 50 ml of oil were carefully poured onto the soil surface.
iii. ⅔PO – ⅔ of the propagule from the base/radical end was dipped in oil using a pair of forceps.

There were four replications per treatment for all species. The bunker fuel oil used was obtained from FFS Refineries (Pty) Ltd, Durban. The properties of this oil are indicated in Table 2.1.

2.2.2 Plant growth measurements

Measurements were made of plant height and number of leaves monthly.

2.2.3 Chlorophyll content

Measurements of leaf chlorophyll content (five per replicate) began four months after commencement of treatments, and monthly thereafter. Chlorophyll content was determined with a hand-held chlorophyll absorbance meter (CCM-200, Opti Sciences, Tyngsboro, MA, USA). The CCM-200 is a cost-effective instrument that provides a non-destructive and reliable estimate of leaf chlorophyll content (Naidoo *et al*., 2010; Khaleghi *et al*., 2012; Flores-de-Santiago *et al*., 2013).

Table 2.1
Specifications of bunker fuel 150 (FFS Refiners, 2002)

	Unit	Value
Energy content (gross)	kJ kg^{-1}	43,400
Viscosity @ 50 °C	cSt	140
Total sulphur as S	mass%	3.2
Pour point	°C	-10
Flashpoint (PMCC)	°C	95
Water content	mass%	0.45
Density @ 20 °C	kg l^{-1}	0.98
Ash	mass%	0.05

Field study

Field investigations were undertaken in the Beachwood Mangroves Nature Reserve and Isipingo estuary. The average daily maximum and minimum temperatures for Beachwood were 26 °C and 17 °C and for Isipingo, 27 °C and 16 °C, respectively. The mean annual rainfall is 1228 mm for Beachwood and 1040 mm for Isipingo. Soil characteristics for Beachwood and Isipingo are indicated in Table 2.2. Selected plants were tagged for identification with plastic tape.

2.2.4 Leaf and internode oiling experiment

Healthy, young, naturally occurring, one year old seedlings of *A. marina* (about 46.5 ± 7 cm in height), *B. gymnorrhiza* (about 48.5 ± 8 cm in height) and *R. mucronata* (about 66.5 ± 9 cm in height) were selected for oiling experiments. Two study sites were used, Beachwood and Isipingo, based on the abundance of seedlings present for each species. *A. marina* (Fig. 2.12A) and *B. gymnorrhiza* (Fig. 2.13A) seedlings from Beachwood and *R. mucronata* seedlings from Isipingo (Fig. 2.14) were subjected to one of three treatments:

i. control (C) – plants were untreated.
ii. IO – the second internode from the base of the stem of *A. marina* (the first internode was too short for oil application) and the first internode from the base of the stem of *B. gymnorrhiza* and *R. mucronata* were oiled.
iii. LO – both surfaces of the last pair of mature leaves directly below the shoot tip of each plant were oiled.
Oil was applied with a paintbrush.

Internodes and leaves of *A. marina* and *B. gymnorrhiza* were oiled at the commencement of the experiment in October 2010 and again after 7, 15, 19, 27, 32, and 48 weeks. Initially, leaves were oiled on the adaxial surface at the commencement of the experiment and again after seven weeks. At 15 weeks until the end of the experiment, leaves were oiled on both the adaxial and abaxial surfaces. Internodes and leaves of *R. mucronata* were oiled at the commencement of the experiment in November 2010 and again after 5, 14, 18, 25, 30 and 48 weeks. Leaves were oiled on the adaxial surface at the commencement of the experiment and again after five

weeks. At 14 weeks until 48 weeks, leaves were oiled on both the adaxial and abaxial surfaces. There were seven replications per treatment for each species. Measurements of plant height and number of leaves were taken on the same days as the oiling.

2.2.5 Sediment oiling experiment

This investigation was undertaken in the Isipingo estuary. Healthy young, one to two year old seedlings of *A. marina* (about 110 ± 50 cm in height), *B. gymnorrhiza* (about 65 ± 6 cm in height) and *R. mucronata* (about 76 ± 8 cm in height) were selected for oiling experiments. Groups of seedlings of each species were enclosed by 0.5 m radius x 0.5 m height circular perspex sheets (Figs. 2.18 - 2.20). The sheets were pushed into the soil to a depth of 5 cm to prevent loss of oil as the tide receded. The height of the enclosures was adequate to prevent the escape of oil with the rising tide. Control plants were also enclosed like the treatment plots, but no oil was applied. There were nine replications per treatment for *A. marina* (because of the abundance of *A. marina* plants in the stand) and four for *B. gymnorrhiza* and *R. mucronata*, respectively. Dosage rate was 5 Lm^{-2} for all plots. Oil treatments were applied once in June 2011 and monitoring continued until June 2012. Measurements of plant height and number of leaves were taken at 16, 26, 38, 46 and 53 weeks after treatment.

2.2.6 Soil analysis

Soil samples were collected from the experimental sites in Beachwood and Isipingo, placed onto plastic sheets and left to air dry in the glasshouse. After one week, the samples were crushed using a wooden mallet and thereafter passed through a 1 mm sieve. Coarse materials (>1 mm) were discarded. Clay content was analyzed by near-infrared reflectance. Soil pH was measured using a gel-filled combination glass electrode placed in a potassium chloride solution (Manson and Roberts, 2000).

Concentrations of ions were determined by atomic absorption using mid-infrared spectroscopy (Bruker Tensor 27, FTIR Spectrometer with HTS/XT). The instrument detects light (2500 – 15000 nm) reflected by the sample. Carbon and nitrogen contents were analyzed by the Automated Dumas dry combustion method using a LECO CNS 2000 (Leco Corporation, Michigan, USA; Matejovic, 1996). The results of the soil analyses for Beachwood and Isipingo are indicated in Table 2.2

2.2.7 Data analyses

Means and standard errors were calculated for all measurements. Resulting data were tested for normality using the Kolmogorov-Smirnov test and subjected to one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparisons test ($P \leq 0.05$) using MINITAB version 16 (Minitab Statistical Software, MINITAB Inc. USA). Other data were subjected to unpaired *t*-tests to detect for differences between control and oiled treatments.

2.3 Results

2.3.1 Glasshouse study

A. marina

Plant height was highest in the control and significantly lower in the sediment oiled, propagule with pericarp oiled, propagule without pericarp oiled and ½ propagule oiled treatments by 67%, 86%, 96% and 61% respectively (Fig. 2.1A).

In the sediment oiled, propagule with pericarp oiled, propagule without pericarp oiled and ½ propagule oiled treatments, number of leaves was significantly lower by 54%, 83%, 92% and 47% respectively, compared to the control (Fig. 2.1B).

In the sediment oiled, propagule with pericarp oiled, propagule without pericarp oiled and ½ propagule oiled treatments, chlorophyll content was significantly lower by 34%, 58%, 66% and 28% respectively, compared to the control (Fig. 2.1C).

There were no significant differences in plant height, number of leaves and chlorophyll content between the sediment oiled and ½ propagule oiled treatments and between the propagule with pericarp oiled and propagule without pericarp oiled treatments (Fig. 2.1A, B and C). After eight months, there was 25% and 50% plant mortality, respectively, in the propagule with pericarp oiled and propagule without pericarp oiled treatments.

Oiled treatments exhibited leaf wilting, senescence and defoliation. In the propagule with pericarp oiled and propagule without pericarp oiled treatments, leaves initially exhibited necrosis (yellow and brown spots) and eventually turned completely brown and withered (Fig. 2.3C). Although no measurements were made, internodes of oiled seedlings were shorter and leaves smaller compared to the controls.

Furthermore, the propagule without pericarp oiled treatment caused abnormal phyllotaxy or arrangement of leaves after two months. Instead of the normal opposite arrangement, leaves formed an abnormal whorled arrangement with three leaves per node (Fig. 2.2B) which continued throughout the duration of the experiment (Fig. 2.2C). Another abnormal feature of the oiled treatments was the development of two or three shoots from a single propagule (Fig. 2.3A). These additional shoots eventually died, leaving surviving plants with a single shoot.

B. gymnorrhiza

Plant height was highest in the control and significantly lower in the sediment oiled and ⅔ propagule oiled treatments by 45% and 36% respectively (Fig. 2.4A). Seedlings of oiled treatments exhibited stunted growth compared to those in the control (Fig. 2.5A and B).

In the sediment oiled and ⅔ propagule oiled treatments, number of leaves was significantly lower by 31% and 24% respectively, compared to the control (Fig. 2.4B).

In the sediment oiled and ⅔ propagule oiled treatments, chlorophyll content was significantly lower by 22% and 20% respectively, compared to the control (Fig. 2.4C). Leaves of seedlings in oiled treatments were healthy and green as those in the control and no abnormalities were observed.

There were no significant differences in plant height, number of leaves and chlorophyll content between the oiled treatments (Fig. 2.4A, B and C).

R. mucronata

Plant height was highest in the control and significantly lower in the sediment oiled and ⅔ propagule oiled treatments by 56% and 49% respectively (Fig. 2.6A). Seedlings of oiled treatments exhibited stunted growth compared to those in the control (Fig. 2.7A and 2.8A).

In the sediment oiled and ⅔ propagule oiled treatments, number of leaves was significantly lower by 89% and 70% respectively, compared to the control (Fig. 2.6B).

In the sediment oiled and ⅔ propagule oiled treatments, chlorophyll content was significantly lower by 69% and 56% respectively, compared to the control (Fig. 2.6C). Leaves of seedlings in oiled treatments were pale and light green in colour compared to the dark green leaves of those in the control (Fig. 2.7C).

There were no significant differences in plant height and number of leaves between the oiled treatments (Fig. 2.6A and B). Chlorophyll content was significantly higher by 30% in the ⅔ propagule oiled than in the sediment oiled treatment (Fig. 2.6C).

All seedlings in the sediment oiled (Fig. 2.7B) and 25% in the ⅔ propagule oiled treatments (Fig. 2.8B) did not shed their leaf caps after the formation of new leaves, whereas those in the control shed theirs two to three weeks after planting. Although there was no mortality, 25% of seedlings in the sediment oiled and 25% in the ⅔ propagule oiled treatments did not produce leaves for the duration of the experiment.

The leaves of seedlings in the oiled treatments were severely deformed (Fig. 2.7B and 2.8B). Leaf wilting, senescence and defoliation were observed in the oiled treatments (Fig. 2.7C). In addition, seedlings in the sediment oiled treatment exhibited necrosis on their propagules. The necrotic area initially appeared dark yellow to orange, then brown and eventually black. Seedlings in the oiled treatments exhibited yellowing and deformity of the stems (Fig. 2.8B).

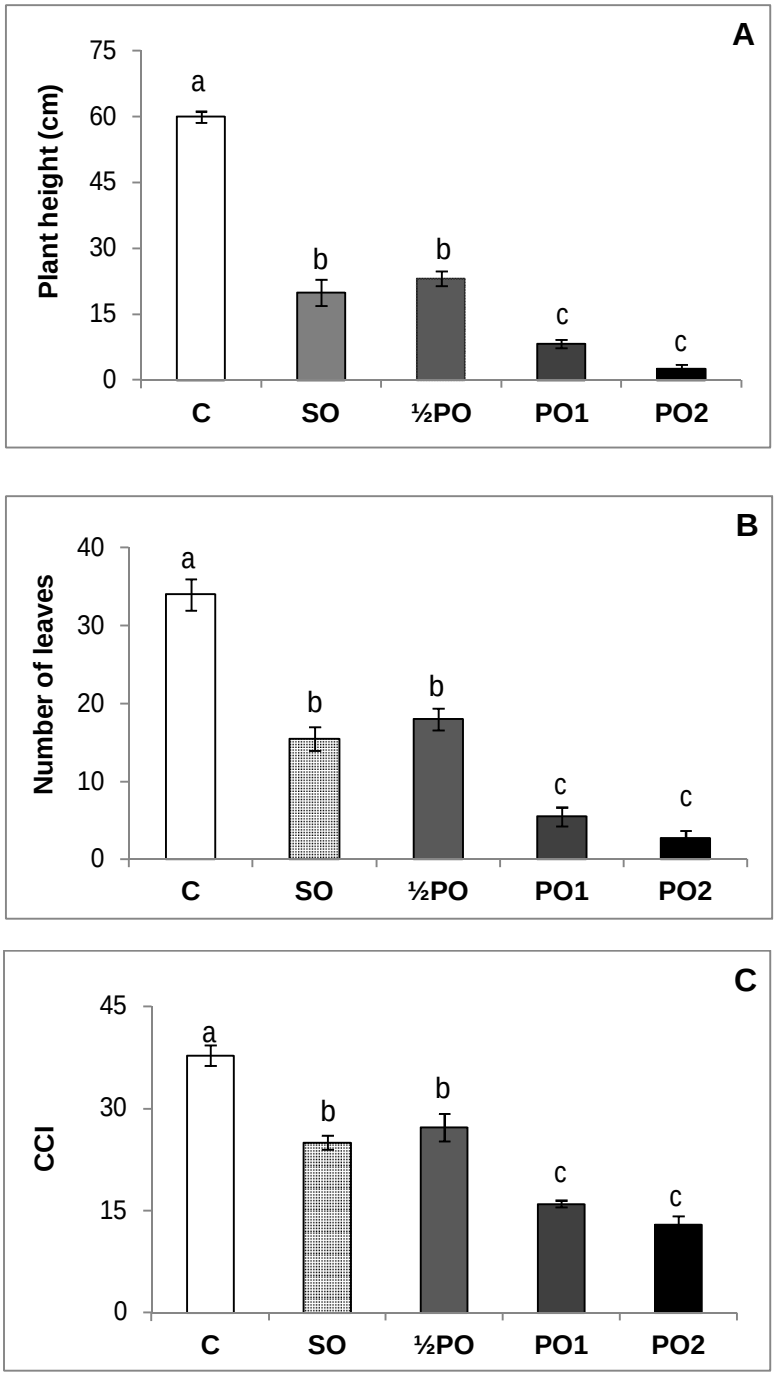

Fig. 2.1 Effects of oiling on plant height (A), number of leaves (B) and chlorophyll content index (CCI) (C) in *A. marina* in the glasshouse study. Measurements were taken every four weeks for 55 weeks after oiling treatment, C = control, SO = sediment oiled, ½PO = ½ propagule oiled, PO1 = propagule with pericarp oiled, PO2 = propagule without pericarp oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

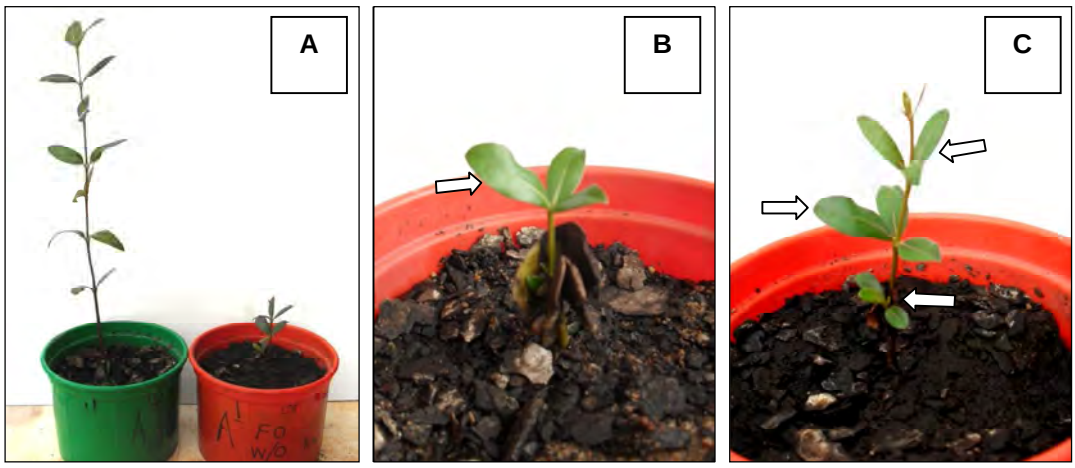

Fig. 2.2 *Avicennia marina* in the glasshouse study. (A) Control (left) and propagule without pericarp oiled treatment (right) after seven months. (B) Abnormal leaf arrangement in propagule without pericarp oiled treatment after two months (arrow). Note the whorled leaf arrangement. (C) Whorled leaf arrangement after seven months (arrows).

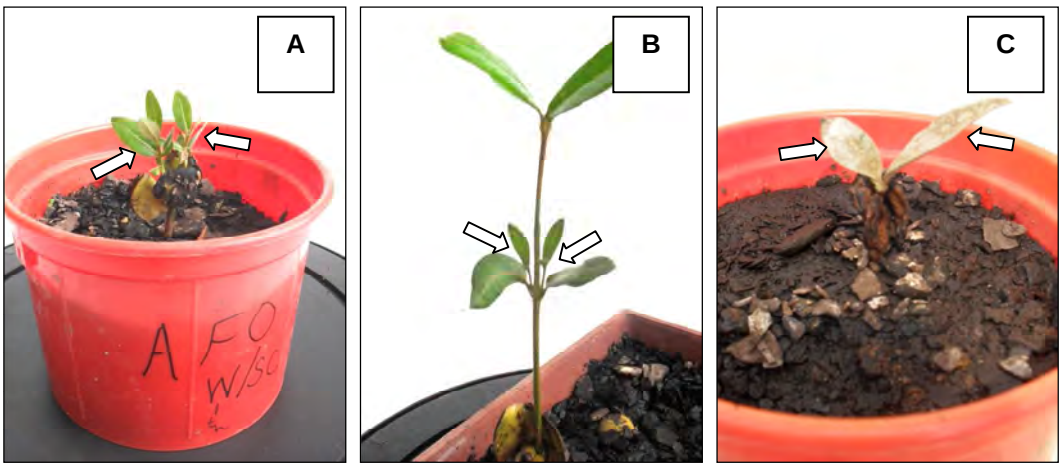

Fig. 2.3 *Avicennia marina* in the glasshouse study. (A) Abnormal shoot growth in propagule with pericarp oiled treatment after three months (arrows). Note two shoots growing from the propagule, a smaller third shoot is present but hidden from view. (B) Abnormal shoot growth in the ½ propagule oiled treatment after two months (arrows). (C) Dead seedling of propagule without pericarp oiled treatment after eight months. Note stunted shoot growth and brown leaves (arrows).

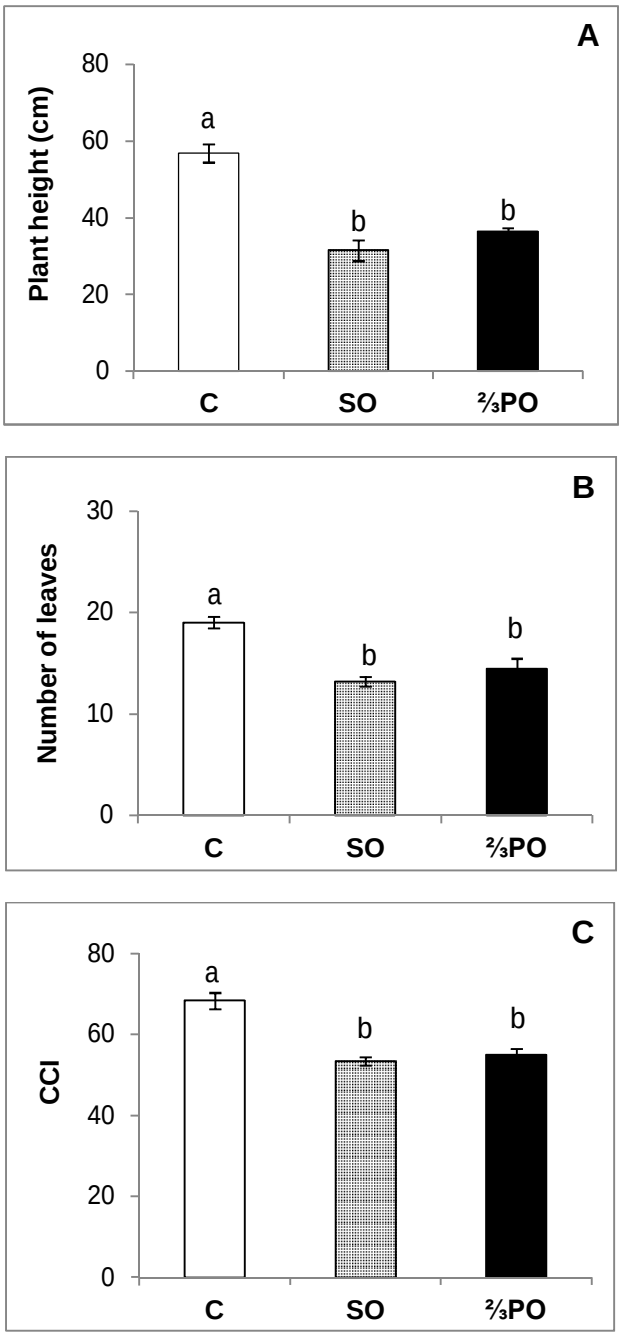

Fig. 2.4 Effects of oiling on plant height (A), number of leaves (B) and chlorophyll content index (CCI) (C) in *B. gymnorrhiza* in the glasshouse study. Measurements were taken every four weeks for 55 weeks after oiling treatment, C = control, SO = sediment oiled, ⅔PO = ⅔ propagule oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

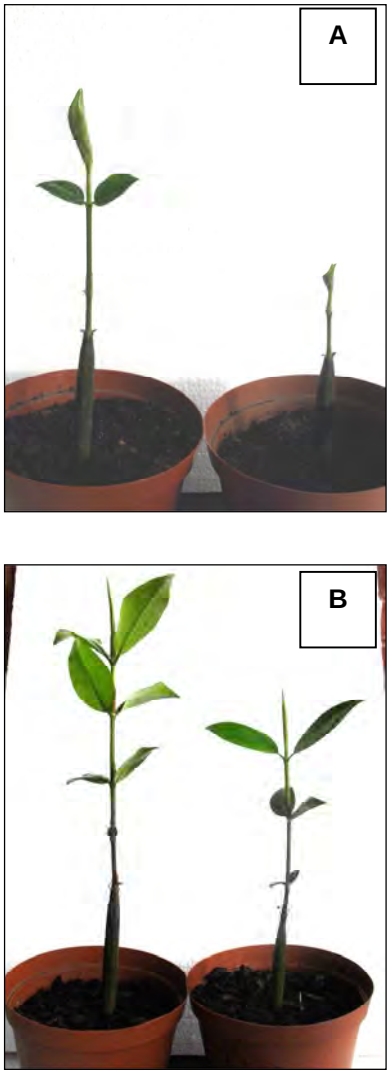

Fig. 2.5 *Bruguiera gymnorrhiza* seedlings in the glasshouse study. (A) Control (left) and sediment oiled (right) treatments after two months. (B) Control (left) and ⅔ propagule oiled (right) treatments after four months.

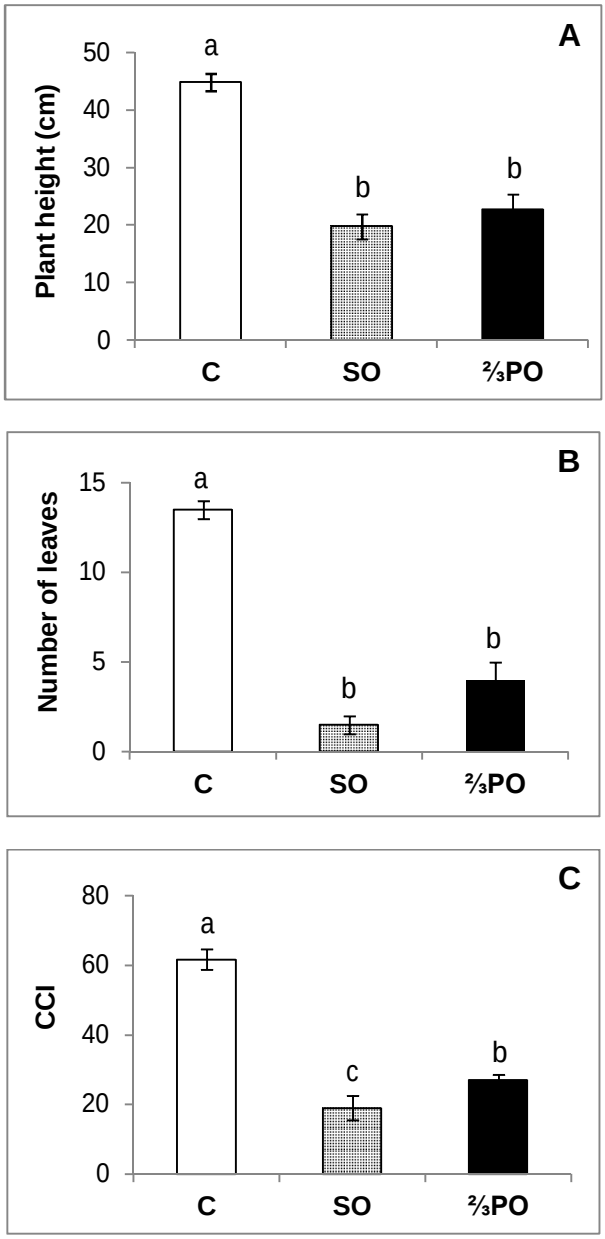

Fig. 2.6 Effects of oiling on plant height (A), number of leaves (B) and chlorophyll content index (CCI) (C) in *R. mucronata* in the glasshouse study. Measurements were taken every four weeks for 55 weeks after oiling treatment, C = control, SO = sediment oiled, ⅔PO = ⅔ propagule oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

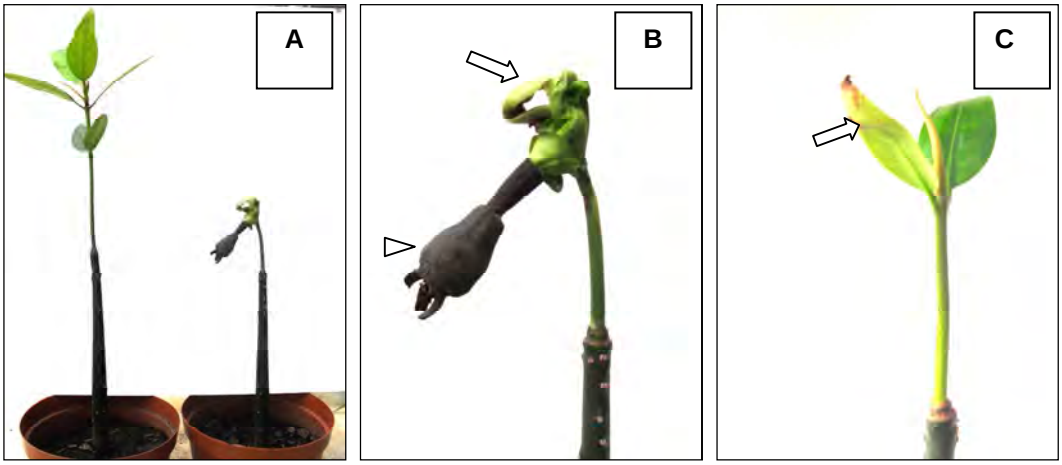

Fig. 2.7 *Rhizophora mucronata* in the glasshouse study. (A) Control (left) and sediment oiled (right) treatments after seven months. (B) Leaf cap that has not fallen off in the sediment oiled treatment after seven months (arrowhead) despite the growth of new leaves. Note that new leaves are deformed (arrow) (C) Light green leaves in the sediment oiled treatment after 11 months. Note wilting and senescence of the leaf on the left (arrow).

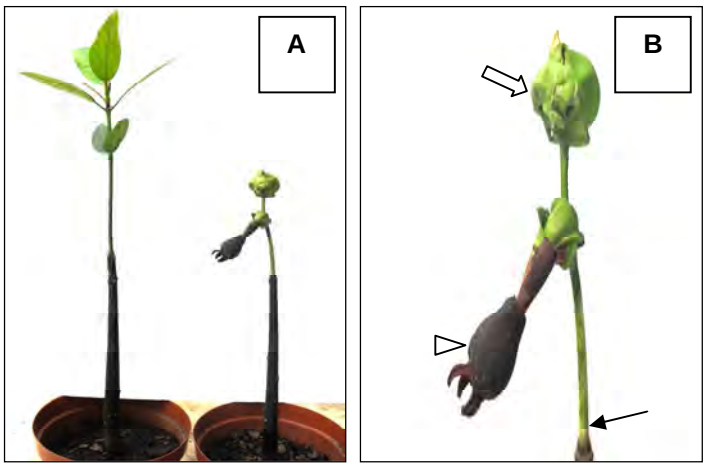

Fig. 2.8 *Rhizophora mucronata* in the glasshouse study. (A) Control (left) and ⅔ propagule oiled treatments (right) after seven months. (B) New leaves are deformed (arrow) and leaf cap (arrowhead) has not fallen off. Note the yellowing stem (black arrow).

Table 2.2

Soil characteristics of the study sites at Beachwood Mangroves Nature Reserve and Isipingo estuary.

		Beachwood	Isipingo
Salinity (psu)		19 ± 3	26 ± 4
Clay content %		27.7 ± 2	23.3 ± 3
pH		6.2 ± 0.2	6.6 ± 0.4
P	gl^{-1}	0.06 ± 0.003	0.03 ± 0.001
K		0.8 ± 0.02	0.5 ± 0.01
Ca		1.3 ± 0.01	1.8 ± 0.02
Mg		1.7 ± 1.9	1.2 ± 0.05
Zn		0.08 ± 0.002	0.32 ± 0.003
Mn		0.05 ± 0.002	0.03 ± 0.001
Cu		0.01 ± 0.001	0.02 ± 0.003
N		4.7 ± 0.1	5.4 ± 0.05
C		47.0 ± 4.3	51.7 ± 3.8

2.3.2 Leaf and internode oiling experiment

A. marina

Plant height increment (final plant height – initial) was highest in the control and significantly lower in the internode oiled and leaf oiled treatments by 62% and 83% respectively (Fig. 2.9A). Plant height increment was significantly higher by 54% in the internode oiled than in the leaf oiled treatment.

Change in number of leaves (final number of leaves – initial) was highest in the control and significantly lower in the internode oiled and leaf oiled treatments by 88% and 109% respectively (Fig. 2.9B). The negative value for the leaf oiled treatment indicated a decrease in the number of leaves after 48 weeks of leaf oiling compared to that at the commencement of the experiment. Leaves abscised in oiled plants and fewer new

ones were produced. Change in number of leaves was significantly higher by 100% in the internode oiled than in the leaf oiled treatment (Fig. 2.9B).

Oiled seedlings exhibited stunted growth and old shoot tips died while new shoots grew adjacent to the dead ones. In addition to leaf wilting, senescence and defoliation, new leaves that formed above the oiled leaves were deformed after 19 weeks. Plants in the internode oiled treatment were more susceptible (50%) to faunal feeding activity compared to those in the control (25%) and leaf oiled (10%) treatments. Leaves that were oiled were monitored at each of the sites to determine whether they remained on the plants or were abscised (Fig. 2.12B). The percentage of abscised leaves in the leaf oiled treatments is indicated in Table 2.3.

Table 2.3
Percentage of abscised leaves in leaf oiled treatments in *A. marina* over the experimental period, AD = adaxial and AB = abaxial surface, n = 7.

Leaf oiled treatments		**% abscission**
7 weeks:	- AD oiled on commencement	36
15 weeks:	- AD oiled on commencement	71
	- AD oiled after 7 weeks	50
19 weeks:	- AD on commencement	100
	- AD oiled after 7 weeks	64
	- AD+AB after 15 weeks	79
27 weeks:	- AD oiled after 7 weeks	100
	- AD+AB oiled after 15 weeks	100
	- AD+AB oiled after 19 weeks	86
32 weeks:	- AD+AB oiled after 19 weeks	100
	- AD+AB oiled after 27 weeks	100
48 weeks:	- AD+AB oiled after 32 weeks	100

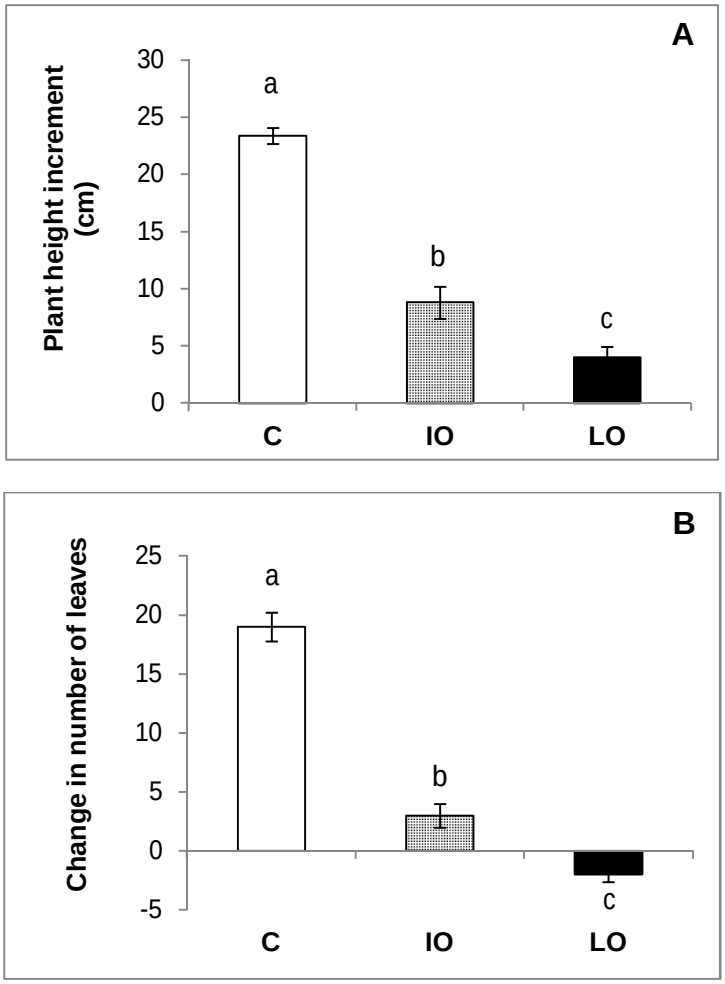

Fig. 2.9 Effects of oiling on plant height increment (final plant height – initial) (A) and change in number of leaves (final number of leaves – initial) (B) in *A. marina* in the field study at Beachwood. Measurements were taken 7, 15, 19, 27, 32, and 48 weeks after oiling treatment, C = control, IO = internode oiled, LO = leaf oiled. Means ± standard error are given, n = 7. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

B. gymnorrhiza

Plant height increment was highest in the control and significantly lower in the internode oiled and leaf oiled treatments by 56% and 78% respectively (Fig. 2.10A). Plant height increment was significantly higher by 51% in the internode oiled than in the leaf oiled treatment.

Change in number of leaves was highest in the control and significantly lower in the leaf oiled treatment by 133% (Fig. 2.10B). Leaves abscised in oiled plants and fewer new ones were produced. There were no significant differences in number of leaves between the control and internode oiled treatments. Change in number of leaves was significantly higher by 159% in the internode oiled than in the leaf oiled treatment.

Plants in the internode oiled and leaf oiled treatments exhibited stunted growth while new leaves that formed above the oiled leaves were not deformed as in *A. marina* and *R. mucronata*.

Plants in the internode oiled treatment were more susceptible (50%) to faunal feeding activity compared to those in the control (25%) and leaf oiled (10%) treatments, similar to *A. marina.*

Leaves that were oiled were monitored at each of the sites to determine whether they remained on the plants or were abscised (Fig. 2.13B). The percentage of abscised leaves in the leaf oiled treatments is indicated in Table 2.4.

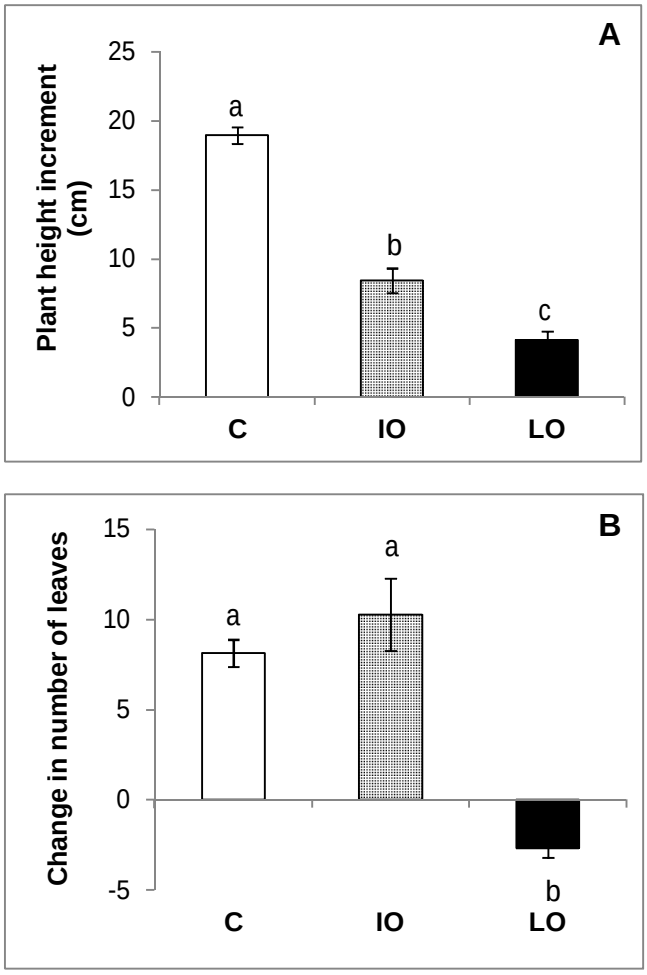

Fig. 2.10 Effects of oiling on plant height increment (final plant height – initial) (A) and change in number of leaves (final number of leaves – initial) (B) in *B. gymnorrhiza* in the field study at Beachwood. Measurements were taken 7, 15, 19, 27, 32, and 48 weeks after oiling treatment, C = control, IO = internode oiled, LO = leaf oiled. Means ± standard error are given, n = 7. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

Table 2.4

Percentage of abscised leaves in leaf oiled treatments in *B. gymnorrhiza* over the experimental period, AD = adaxial and AB = abaxial surface, n = 7.

Leaf oiled treatments		**% abscission**
7 weeks:	- AD on commencement	0
15 weeks:	- AD on commencement	0
	- AD oiled after 7 weeks	0
19 weeks:	- AD on commencement	43
	- AD oiled after 7 weeks	29
	- AD+AB oiled after 15 weeks	86
27 weeks:	- AD on commencement	64
	- AD oiled after 7 weeks	57
	- AD+AB oiled after 15 weeks	90
	- AD+AB oiled after 19 weeks	64
32 weeks:	- AD on commencement	82
	- AD oiled after 7 weeks	71
	- AD+AB oiled after 15 weeks	100
	- AD+AB oiled after 19 weeks	72
	- AD+AB oiled after 27 weeks	29
48 weeks:	- AD on commencement	100
	- AD oiled after 7 weeks	79
	- AD+AB oiled after 15 weeks	100
	- AD+AB oiled after 19 weeks	100
	- AD+AB oiled after 27 weeks	93
	- AD+AB oiled after 32 weeks	86

R. mucronata

Plant height increment was highest in the control and significantly lower in the internode oiled and leaf oiled treatments by 72% and 85% respectively (Fig. 2.11A). Plant height increment was significantly higher by 100% in the internode oiled than in the leaf oiled treatment (Fig. 2.11A).

Change in number of leaves was highest in the control and significantly lower in the internode oiled and leaf oiled treatments by 78% and 110% respectively (Fig. 2.11B). Leaves abscised in oiled plants and fewer new ones were produced. Change in number of leaves was significantly higher by 100% in the internode oiled than in the leaf oiled treatment (Fig. 2.11B).

Plants in oiled treatments exhibited stunted growth and after 48 weeks, there was 29% and 57% mortality respectively, in the internode oiled and leaf oiled treatments. In addition, shoot tips of leaf oiled plants died while new shoots grew adjacent to the dead ones. Some plants in the leaf oiled treatment developed two new shoots on either side of the dead one.

In addition to leaf wilting, senescence and defoliation in oiled plants, new leaves that formed above the oiled ones were deformed after 18 weeks. After 48 weeks, all new leaves of oiled plants were deformed.

Control plants were more susceptible to faunal feeding activity (40%) compared to those in the internode oiled (10%) and leaf oiled (20%) treatments.

Leaves that were oiled were monitored at each of the sites to determine whether they remained on the plants or were abscised. The percentage of abscised leaves in the leaf oiled treatments is indicated in Table 2.5.

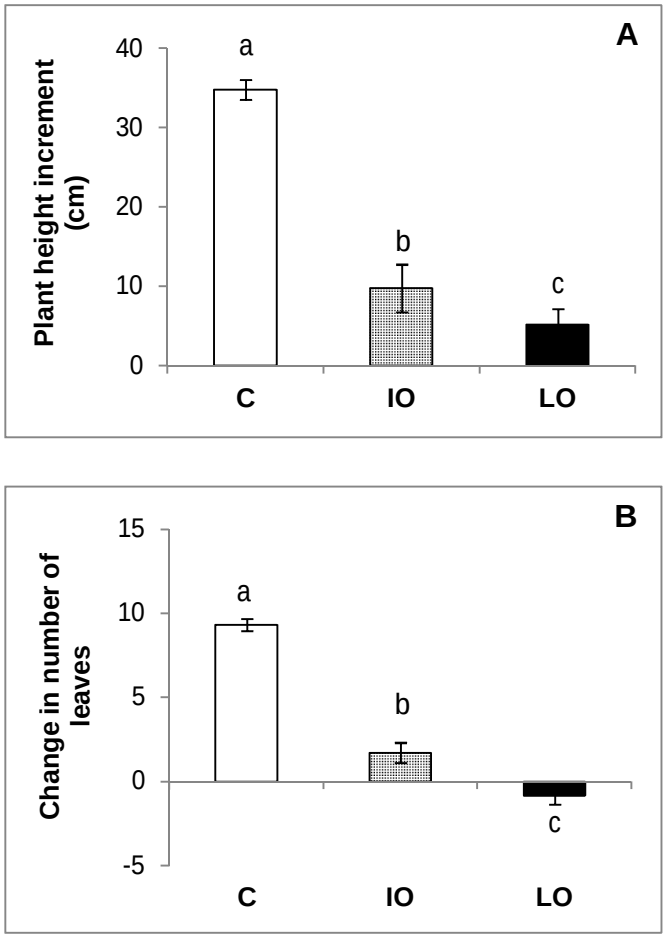

Fig. 2.11 Effects of oiling on plant height increment (final plant height – initial) (A) and change in number of leaves (final number of leaves – initial) (B) in *R. mucronata* in the field study at Isipingo. Measurements were taken 5, 14, 18, 25, 30, 48 weeks after oiling treatment, C = control, IO = internode oiled, LO = leaf oiled. Means ± standard error are given, n = 7. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

Table 2.5

Percentage of abscised leaves in leaf oiled treatments in *R. mucronata* over the experimental period, AD = adaxial and AB = abaxial surface, n = 7.

Leaf oiled treatments		**% abscission**
5 weeks:	- AD on commencement	86
14 weeks:	- AD on commencement	100
	- AD oiled after 5 weeks	100
18 weeks:	- AD+AB oiled after 14 weeks	93
25 weeks:	- AD+AB oiled after 14 weeks	100
	- AD+AB oiled after 18 weeks	100
30 weeks:	- AD+AB oiled after 27 weeks	79
48 weeks:	- AD+AB oiled after 27 weeks	100
	- AD+AB oiled after 32 weeks	100

Fig. 2.12 *Avicennia marina* in the leaf and internode oiling experiment. (A) Individually tagged plants in the internode oiled treatment (arrows). (B) Individually tagged plant in the leaf oiled treatment. Note the dead oiled leaves (arrows).

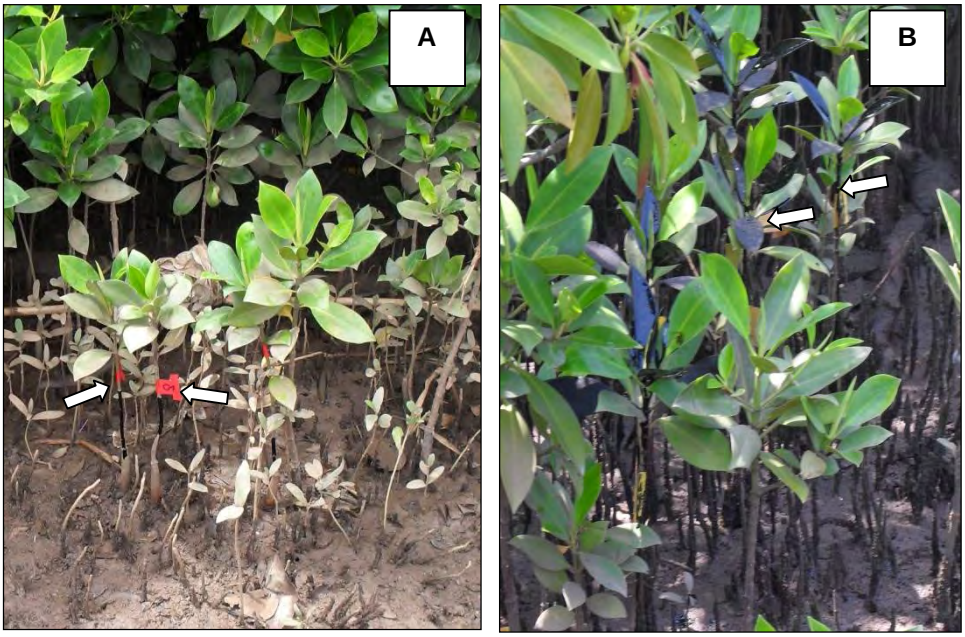

Fig. 2.13 *Bruguiera gymnorrhiza* in the leaf and internode oiling experiment. (A) Individually tagged plants in the internode oiled treatment (arrows). (B) Individually tagged plants in the leaf oiled treatment (arrows).

Fig. 2.14 Individually tagged *R. mucronata* seedlings showing internode oiled plants (arrow) in the field study at Isipingo.

2.3.3 Sediment oiling experiment

A. marina

Plant height increment was highest in the control and significantly lower in the sediment oiled treatment by 97% (Fig. 2.15A). Change in number of leaves was highest in the control and significantly lower in the sediment oiled treatment by 106% (Fig. 2.15B).

After 16 weeks, there was 78% plant mortality in the sediment oiled treatment. After 38 weeks, adventitious roots developed on the stem, about 10-15 cm above the soil surface in surviving plants (22%). After 53 weeks, these adventitious roots (11) were about 4-12 cm in length and ± 0.8 cm in width and grew towards the substrate (Fig. 2.18C). There was 89% mortality in the sediment oiled treatment after 53 weeks. Ten seedlings from newly fallen propagules grew in the control, while in the sediment oiled treatment, 19 new seedlings established after 53 weeks (Fig. 2.18B).

B. gymnorrhiza

Plant height increment was highest in the control and significantly lower in the sediment oiled treatment by 100% (Fig. 2.16A). Change in number of leaves was highest in the control and significantly lower in the sediment oiled treatment by 100% (Fig. 2.16B).

After 26 weeks, there was 50% mortality in the sediment oiled treatment and after 53 weeks all plants died. Four seedlings from newly fallen propagules grew in the control while in the sediment oiled treatment five new seedlings established after 53 weeks (Fig. 2.19C).

R. mucronata

Plant height increment was highest in the control and significantly lower in the sediment oiled treatment by 95% (Fig. 2.17A). Change in number of leaves was highest in the control and significantly lower in the sediment oiled treatment by 108% (Fig. 2.17B). After 16 weeks, there was 75% plant mortality in the sediment oiled treatment (Fig. 2.20). New seedlings were absent because there was no source of propagules (Fig. 2.20).

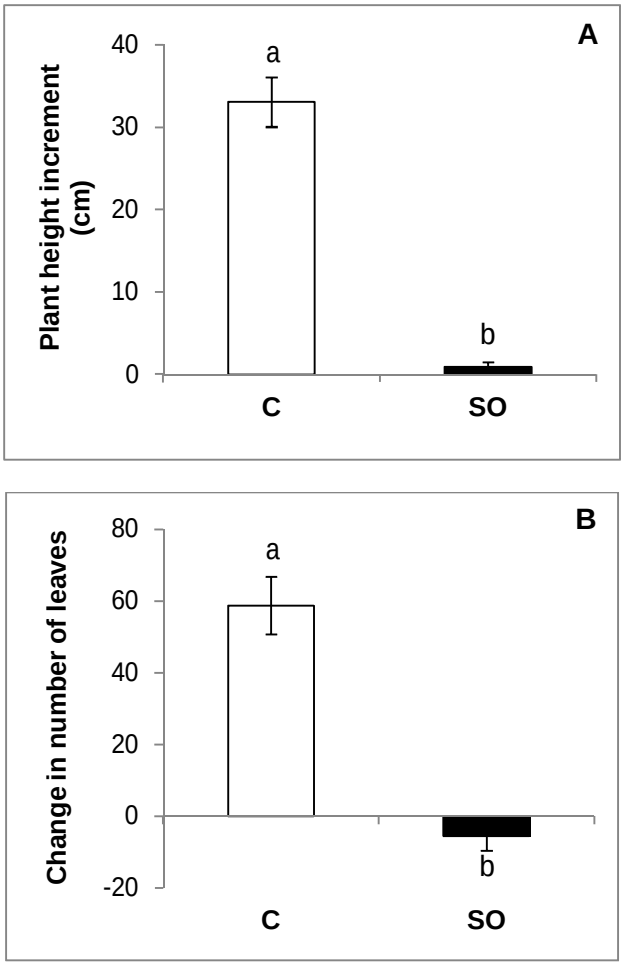

Fig. 2.15 Effects of oiling on plant height increment (final plant height – initial) (A) and change in number of leaves (final number of leaves – initial) (B) in *A. marina* in the field study at Isipingo. Measurements were taken 16, 26, 38, 46 and 53 weeks after treatment, C = control, SO = sediment oiled. Means ± standard error are given, n = 9. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

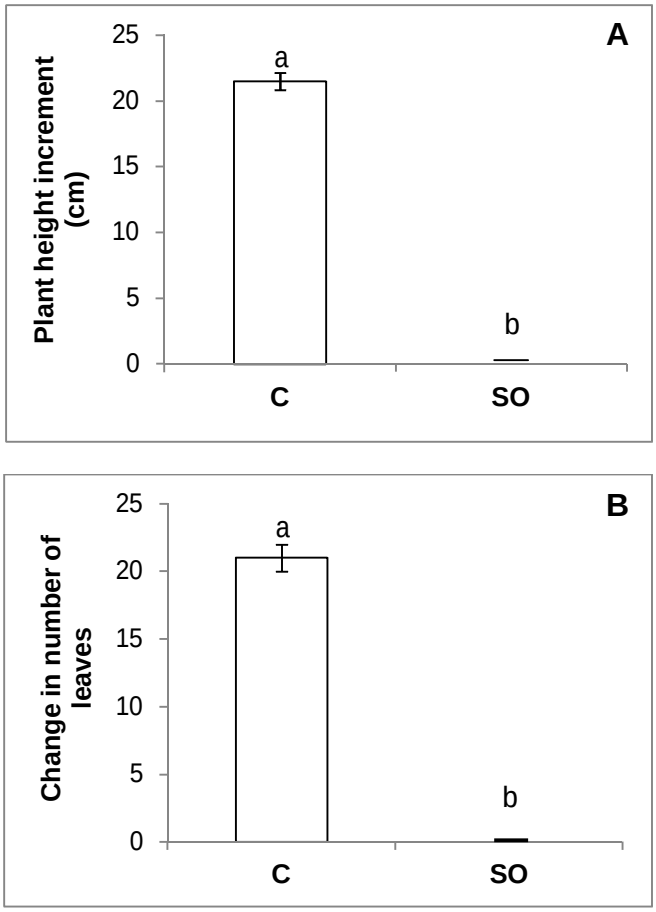

Fig. 2.16 Effects of oiling on plant height increment (final plant height – initial) (A) and change in number of leaves (final number of leaves – initial) (B) in *B. gymnorrhiza* in the field study at Isipingo. Measurements were taken 16, 26, 38, 46 and 53 weeks after the commencement of treatments, C = control, SO = sediment oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

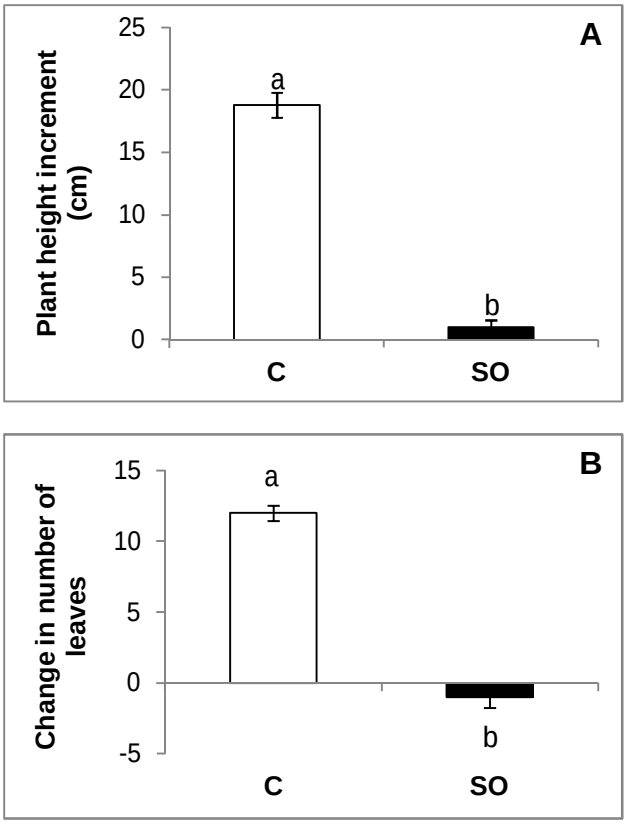

Fig. 2.17 Effects of oiling on plant height increment (final plant height – initial) (A) and change in number of leaves (final number of leaves – initial) (B) in *R. mucronata* in the field study at Isipingo. Measurements were taken 16, 26, 38, 46 and 53 weeks after treatment, C = control, SO = sediment oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

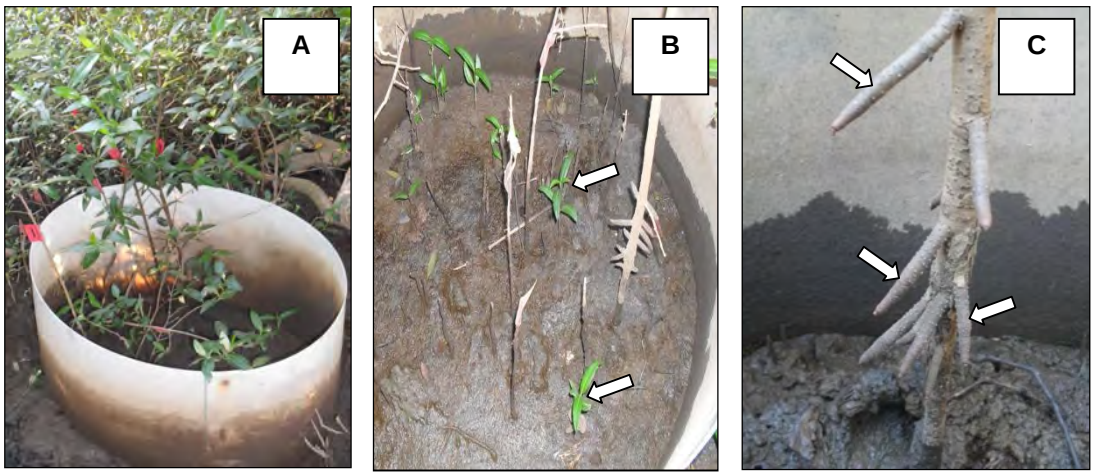

Fig. 2.18 *Avicennia marina* in the sediment oiled treatment in Isipingo. (A) Control plants at the commencement of the experiment. (B) Sediment oiled plants 53 weeks after oiling. Note the new seedling growth from recently fallen propagules (arrows). (C) Adventitious root development in a sediment oiled plant after 53 weeks (arrows).

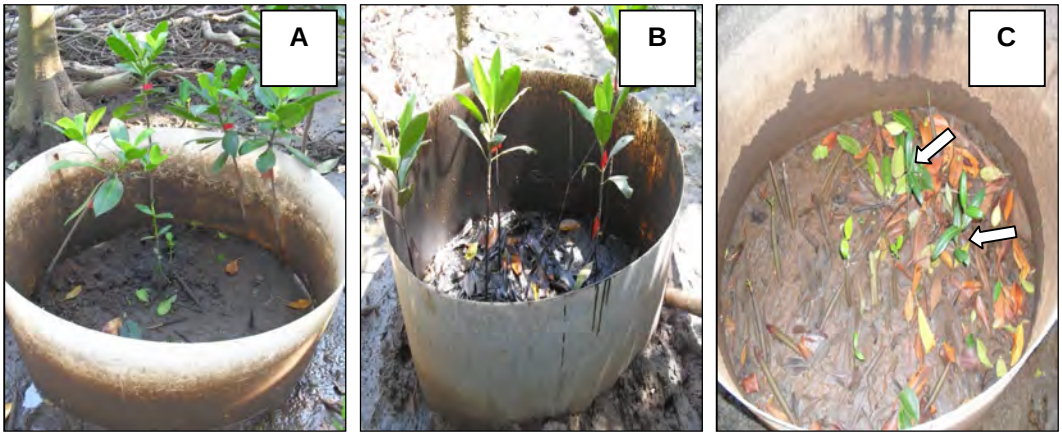

Fig. 2.19 *Bruguiera gymnorrhiza* in the sediment oiled treatment in Isipingo. (A) Control plants at the commencement of the experiment. (B) Sediment oiled plants at the commencement of the oiling treatment. (C) Sediment oiled plants 53 weeks after treatment. Note abscised leaves on the sediment and new seedlings from recently fallen propagules (arrows).

Fig. 2.20 *Rhizophora mucronata* in the sediment oiled treatment in Isipingo after 53 weeks. Note the single surviving plant on the left (arrow).

2.4 Discussion

This study has demonstrated that bunker fuel oil produces adverse effects, such as leaf wilting, necrosis, defoliation, reduced growth and plant mortality in the three species. Abnormal growth morphology particularly in *A. marina* and *R. mucronata* were caused by the mutagenic properties of PAHs (Guo *et al*., 2005; Luan *et al*., 2006; Cavalcante *et al*., 2009).

In the glasshouse study, the growth of all species was significantly reduced by oiled treatments. Oil did not inhibit the establishment of propagules in all species. Propagules of *A. marina*, *B. gymnorrhiza* and *R. mucronata* were coated with oil and anchored vertically into the substratum, the position in which seedling establishment and growth takes place in the natural environment (Zhang *et al*., 2007a). Oil coating on the radical end of the propagule probably caused PAHs to enter the developing primary root resulting in reduced growth and morphological deformities in stems and leaves. Sediment oiling enabled PAHs to enter the entire root system over the experimental period. Oil entering the roots and its translocation to above ground parts of the plant probably caused reduced growth and deformity. Previous studies have demonstrated that the primary mechanism of PAH toxicity is related to oil that enters roots and is drawn up the stems and leaves by transpiration (Getter *et al*., 1985; Suprayogi and

Murray, 1999). Oil damage to conducting tissues (Youssef and Ghanem, 2002; Ye and Tam, 2007) and disruption of cellular lipid membranes in roots (Zhang *et al*., 2007a) probably caused reduced growth.

In *A. marina*, complete oiling of propagules resulted in fewer new leaves, defoliation, deformity, necrosis, abnormal arrangement of leaves, reduced growth and plant mortality. In *R. mucronata*, oil caused necrosis in propagules, deformity of the stems and leaves and the inability to shed the leaf cap when flag leaves were produced. These oil effects are probably a result of the translocation of oil from the roots to the foliage (Getter *et al*., 1985; Duke *et al*., 1997). Deformity and reduced growth were probably caused by oil disrupting biochemical processes and plant metabolism (Zhang *et al*., 2007a; Ke *et al*., 2011a). Physiological stress can eventually lead to plant mortality (Takemura *et al*., 2000). Tissue necrosis was probably caused by oil accumulation which led to the death of cells (Suprayogi and Murray, 1999; Meudec *et al*., 2006; 2007). Previous studies have shown that oil accumulation in the roots and leaves resulted in organelle and tissue damage in the grasses, *Spartina alterniflora* Loisel. (Watts *et al*., 2006) and *Lolium multiflorum* Lam. (Kang *et al*., 2010). Leaf deformity (Duke and Watkinson, 2002; Tam *et al*., 2005) and tissue necrosis (Ye and Tam, 2007) caused by oil have been reported before.

In *A. marina*, reduced growth and mortality occurred when the pericarp was intact or removed before oiling. The pericarp did not appear to protect the endosperm and embryo from oil effects. Oil could have penetrated the developing embryo when propagules were completely coated, causing deformity, reduced growth and mortality. There was no mortality in oiled *B. gymnorrhiza* and *R. mucronata* seedlings possibly because propagules are thicker and more dense (De Ryck *et al*., 2012), which probably restricted the degree of oil penetration.

Oiling significantly reduced chlorophyll content in all species. Oil probably damaged chloroplasts resulting in reduced chlorophyll content. Previous studies have demonstrated that PAHs negatively affect the photosynthetic apparatus of chloroplasts (Naidoo *et al*., 2010; Yin *et al*., 2011) and break down chlorophyll (Huang *et al*., 1996). Reduced chlorophyll content probably decreased photosynthetic performance (Naidoo *et al*., 2010; Khaleghi *et al*., 2012; Flores-de-Santiago *et al*., 2013) which resulted in reduced growth.

Growth was significantly reduced in all species when internodes and leaves were oiled in the field. Leaf oiling resulted in abscission of leaves in all species within seven weeks. Abscission was probably due to the carcinogenic properties of PAHs (Luan *et al*., 2006; Li *et al*., 2010). Ethylene, for example, is a hydrocarbon known to be a natural constituent of plants and serves as a growth regulator that promotes leaf abscission (Yang and Hoffman, 1984; Zakrzewski, 2002). However, Jackson and Osborne (1970) demonstrated that an increase in the rate of ethylene production after the onset of sensitivity to this hydrocarbon can be closely correlated with the time of abscission. Excess ethylene (Zakrzewski, 2002) and PAHs (Luan *et al*., 2006) are carcinogenic plant toxins (Guo *et al*., 2005; Cavalcante *et al*., 2009). Sensitivity to and toxicity of PAHs could have resulted in premature leaf abscission in this study. Additionally, aromatic hydrocarbons in vehicle emissions (e.g. benzene and xylene) cause cuticle degradation in leaves (Sauter and Pambor, 1989). Furthermore, oil passed through intercellular spaces and aerenchyma channels in leaves of *Phragmites australis* (Cav.) Trin. ex Steud. whereas water normally does not (Armstrong *et al*., 2009). Therefore another reason for leaf abscission could be that oil penetrated the leaf cells resulting in death of chloroplasts and other organelles (Watts *et al*., 2006; Kang *et al*., 2010) causing early senescence.

In *A. marina* and *R. mucronata*, leaf and internode oiling resulted in leaf wilting, senescence and defoliation. These adverse effects could have been caused by increased leaf temperature because of blocked transpiration pathways (Pezeshki *et al*., 2000). In *A. marina* and *R. mucronata*, new leaves that formed above the oiled ones were deformed after 19 and 18 weeks respectively. After 48 weeks, all new leaves of *R. mucronata* were deformed. Oil that entered the leaves could have been translocated to new shoots and leaves (Meudec *et al*., 2006; 2007) causing deformity by altering plant physiology (Zhang *et al*., 2007a; Ke *et al*., 2011a). Previous studies have reported that PAHs in tissues increase reactive oxygen species (Proffitt *et al*., 1995; Zhang *et al*., 2007a) that disrupt normal metabolism by damaging lipids, proteins and nucleic acids (Parida and Das, 2005). Damage to vital constituents of cells drastically affects biological membranes and metabolic processes (Ke *et al*., 2011a) which probably resulted in deformity and reduced growth. *Rhizophora mucronata* failed to produce new leaves after oiled leaves abscised and this eventually (27 weeks) led to plant mortality. Refined oil such as Bunker C was also reported to inhibit the formation of new leaves in *S. alterniflora* leading to plant mortality (Pezeshki *et al*., 1995). A similar study with

Aegiceras corniculatum and *Avicennia marina* showed that mortality occurred as a result of direct contact of leaves with oil (Ye and Tam, 2007).

Field sediment oiling caused mass mortality of seedlings in all species. This was probably related to the high oil dosage (5 Lm^{-2}). Sediment oiling probably decreased soil permeability, pH, dissolved oxygen concentrations, redox potentials and salinity of the interstitial water (Suprayogi and Murray, 1999; Pereira *et al*., 2002). Oil probably reduced growth by smothering and eventually killing roots (Suprayogi and Murray, 1999; Tam *et al*., 2005; Zhang *et al*., 2007a). The high dose of oil in the sediment probably led to the death of roots, which in turn resulted in rapid plant mortality in all species, as reported previously for *A. marina* and *B. gymnorrhiza* (Proffitt and Devlin, 1998; Ke *et al*., 2011a). The three species were more tolerant to lower oil doses in the glasshouse study, enabling them to survive residual oil contamination, as documented previously for *A. germinans*, *B. gymnorrhiza* and *R. mangle* (Proffitt *et al*., 1995; Ke *et al*., 2011a).

Adventitious roots developed at the base of the stem in *A. marina* plants in the sediment oiled field experiment as described previously (Naidoo *et al*., 2010). The formation of adventitious roots is an adaptive response after damage to the phloem tissue (Naidoo *et al*., 2010). Plants of *A. marina* that formed adventitious roots survived residual oil contamination. There was no adventitious root development in *B. gymnorrhiza* and *R. mucronata* after 53 weeks of treatment probably because these species lack this adaptive capacity.

Some mangroves can survive the physical and chemical effects of oil contamination by developing morphological adaptations (Hoff, 2002) such as adventitious roots in *A. marina*. Plants of all species adapted to leaf oiling by abscising oiled leaves. In addition, new shoots grew adjacent to the dead apices in *A. marina* and *R. mucronata* plants after leaf oiling.

Species variation in the effects of oil (see Table 2.6) is probably due to morphological and physiological differences (Lamparelli *et al*., 1997; Pi *et al*., 2009; Naidoo *et al*., 2010). In this investigation, *B. gymnorrhiza* did not exhibit necrosis or morphological deformity when leaves and internodes were oiled. *Bruguiera gymnorrhiza* exhibited minimal oil effects when propagules and sediments were oiled in the glasshouse. The

higher tolerance of *B. gymnorrhiza* to oil (see Table 2.6) could be due to its suberized root epidermal cells which bind PAHs thus minimizing the amount of oil entering the plant (Ke *et al*., 2003). Furthermore, although oiled leaves were abscised in *B. gymnorrhiza* plants, new leaves were produced and there was no plant mortality, compared to *A. marina* and *R. mucronata*. The leaves of *B. gymnorrhiza* appeared to have a thicker waxy cuticle than *A. marina* and *R. mucronata*, which probably decreased oil accumulation in the leaves and translocation of PAHs to other plant organs.

Although *R. mucronata* and *A. marina* exhibited similar adverse oil effects (see Table 2.6), the latter was the least tolerant of the three species to PAH contamination. The lower tolerance of *A. marina* to oil was probably due to morphological and physiological characteristics. The roots of *A. marina* can synchronously take up salt and PAHs whereas those of *R. mucronata* and *B. gymnorrhiza* can exclude salt and oil (Suprayogi and Murray, 1999; Ye and Tam, 2007) which probably resulted in differences in tolerance. Furthermore, the leaves of *A. marina* possess dense trichomes on the abaxial surface which causes oil adherence (Ye and Tam, 2007). Oil adherence probably contributed to a greater penetration of oil into leaf cells thereby increasing translocation of PAHs throughout the plant. This work supports those of others (Suprayogi and Murray, 1999; Ye and Tam, 2007) that *A. marina* is more sensitive to oil contamination than *B. gymnorrhiza* and *R. mucronata*.

Table 2.6

Oil effects in *A. marina*, *B. gymnorrhiza* and *R. mucronata* in glasshouse and field experiments. *asterisk indicates oil effect exhibited in species.

Oil effects	***A. marina***	***B. gymnorrhiza***	***R. mucronata***
Plant height	*	*	*
Shorter internodes	*		
Abnormal shoot growth	*		*
Death of shoot tips	*		*
Stem deformity			*
Yellowing of stems			*
Necrosis of propagules			*
Number of leaves	*	*	*
Necrosis of leaves	*		
Smaller leaves	*		
Pale leaves			*
Fewer new leaves	*	*	*
Leaf deformity	*		*
Leaf wilting	*		*
Leaf abscission	*	*	*
Senescence	*		*
Defoliation	*		*
Abnormal phyllotaxy	*		
Chlorophyll content	*	*	*
Adventitious roots	*		
Plant mortality	*	*	*

Chapter 3

The effects of oil on morphological characteristics in *Avicennia marina*, *Bruguiera gymnorrhiza* and *Rhizophora mucronata* in rhizotrons under glasshouse conditions

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Abstract

This study investigated the effects of oil on morphological characteristics of *A. marina*, *B. gymnorrhiza* and *R. mucronata* grown in rhizotrons in the glasshouse. Freshly picked propagules of all species were collected from the field and subjected to oiled treatments for 245 and 409 days. Oil was applied to propagules or sediments. After the experimental period, plant parts were separated, measured and weighed. In propagule oiled treatments, root diameter increased in *A. marina* and *B. gymnorrhiza* by 31% and 27%, respectively, compared to those in the control. In the propagule oiled treatment, *R. mucronata* produced numerous lateral roots just beneath the soil surface. Oiled propagules in *R. mucronata* resulted in 67% mortality after two months. Sediment oiling reduced root biomass, growth rate, length and volume of seedlings in all species compared to the control. In the sediment oiled treatment, there was 67% plant mortality in *A. marina* and 67% in *B. gymnorrhiza* after four weeks. Sediment oiling reduced shoot length, number of leaves, leaf area, chlorophyll content and biomass accumulation of seedlings in all species. This study demonstrated that oiling of propagules and sediments altered growth patterns and root morphology.

Keywords:

Biomass, growth, oil, propagules, root morphology

3.1 Introduction

Plants possess various adaptations to acquire, allocate and store resources for growth (Chapin *et al*., 1990). Biomass production and accumulation, particularly belowground, are important in contributing to vertical plant growth (Sánchez, 2005). Roots have heterogeneous morphological traits and physiological functions (Eissenstat *et al*.,

2000). Mangroves have specialized adaptations such as pneumatophores (*A. marina*), knee (*B. gymnorrhiza*) and prop (*R. mucronata*) roots (Kathiresan and Bingham, 2001) for survival in anoxic soils (Hoff, 2002).

Studies in belowground activities are limited due to the difficulty of observing roots (Sánchez, 2005). Soil contaminants are absorbed by the roots and either stored or metabolised by the plant (Evangelou *et al*., 2007; Park *et al*., 2011). The use of plants to remove, degrade or inactivate contaminants in soil has been the focus in numerous studies (Parrish *et al*., 2006; Inckot *et al*., 2011; Sodré *et al*., 2013). There are also many studies on the effects of heavy metals on root growth (Zhang *et al*., 2007b; Kopittke *et al*., 2009; Naidoo *et al*., 2014).

Many studies on oil contamination in mangroves have focussed on the aboveground component (Proffitt *et al*., 1995; Duke *et al*., 1997; Youssef and Ghanem, 2002). Few studies have focussed on the effects of oil on roots (Ye and Tam, 2007; Zhang *et al*., 2007a). The toxic effects of PAHs on root growth have been reported previously (Jiao *et al*., 2007; Kechavarzi *et al*., 2007; Reynoso-Cuevas *et al*., 2008). However, most studies have focussed on graminoids and agricultural crop plants. Studies on the effects of oil on mangrove root growth are limited.

Studies on the effects of oil on root growth have been limited to biomass i.e. quantitative characteristics (Hou *et al*., 2001; Lin *et al*., 2002). Qualitative characteristics are often neglected although they provide more insight into the functioning of the root system than biomass alone (Blouin *et al*., 2007). The structure of the root system influences the ability of a plant to exploit nutrient resources (Lynch, 1995).

Root morphology is described by characteristics such as weight, length, volume and ratios among these traits (Boot, 1989; Eissenstat, 1991). Root biomass is defined as plant investment in belowground construction and maintenance (Bouma *et al*., 1996; 2000; Sánchez, 2005). Length determines the capacity of the root system to acquire water and nutrients (Bouma *et al*., 2001; Blouin *et al*., 2007) which is more important than mass (Boot and Mensink, 1990). Length, diameter and volume of roots are important characteristics for describing and comparing belowground components (Bouma *et al*., 2000).

There is limited information on the effects of oil on root morphological characteristics in general (Merkl *et al*., 2005; Kechavarzi *et al*., 2007). In this study, the effects of oil applied to propagules and sediments, on length, diameter, volume and biomass of roots were investigated in three mangrove species. As far as we are aware, this is the first study to use rhizotrons to investigate the effects of oil on root growth and development in mangroves.

3.2 Materials and methods

3.2.1 Growth conditions

First rhizotron study

Propagules of *A. marina*, *B. gymnorrhiza* and R. *mucronata* were collected as described in Chapter 2.2.1. After collection, *A. marina* propagules were placed in water and pericarps allowed to shed naturally (24 hours). Root growth was monitored by growing plants in perspex rhizotrons with dimensions of 50 cm height x 31 cm length x 3.6 cm width. Rhizotrons were filled with a mixture of sand, potting soil and compost (1:2:1) and covered with black plastic to exclude light. Propagules of *A. marina*, *B. gymnorrhiza* and *R. mucronata* were inserted into the soil to about 5 mm, 3 cm and 7 cm, respectively. The rhizotrons were tilted at an angle of 30° from the horizontal and watered daily with tap water and once monthly with 10% seawater. The rhizotrons were maintained in a glasshouse for 245 days. The temperature in the glasshouse during the experimental period was about 25 °C (day) and 18 °C (night).

Propagules of *A.marina* without pericarps were subjected to one of three treatments:

i. C – control propagules were planted in the sediment.
ii. ½O – 50% of the propagule was dipped in oil using a pair of forceps.
iii. O – propagules were completely dipped in oil and planted in the sediment.

Propagules of *B. gymnorrhiza* (about 15.5 ± 2 cm in height), and *R. mucronata* about 22 ± 0.9 cm in height) were subjected to one of two treatments:

i. C – control propagules were planted in the sediment.
ii. ⅔O – 67% of the propagule from the base/radical end was dipped in oil using a pair of forceps.

The properties of the bunker fuel oil used in this study are indicated in Table 2.1, Chapter 2. There were four replications per treatment for *A. marina* and three for *R. mucronata*. One oiled propagule of *B. gymnorrhiza* was not viable and excluded, so that there were four replications in the control and three in the oiled treatment.

Second rhizotron study

Propagules of the three species were collected from the Isipingo estuary (29° 59' 59" S, 30° 56' 42" E). Root growth was monitored by growing *A. marina* and *B. gymnorrhiza* in perspex rhizotrons with the same dimensions as in the first study. *Rhizophora mucronata* was grown in rhizotrons with dimensions of 50 cm height x 31.2 cm length x 7 cm width. The experimental set-up was identical to the first study. The rhizotrons were maintained in a glasshouse for 409 days. The second study concentrated on sediment oiling in all species. In *A. marina*, the completely oiled propagule treatment was replicated as there was 100% mortality in the first study.

Propagules of *A. marina* without pericarps were subjected to one of three treatments:

i. C – control propagules were planted in the sediment.
ii. SO – propagules were planted in sediment to which 200 ml of oil were carefully poured onto the soil surface.
iii. O – propagules were completely dipped in oil and planted in the sediment.

Propagules of *B. gymnorrhiza* (about 17 ± 2.5 cm in height), and *R. mucronata* (about 22 ± 1.2 cm in height) were subjected to one of two treatments:

i. C – control propagules were planted in the sediment.
ii. O – propagules were planted in sediment to which 200 ml of oil were carefully poured onto the soil surface.

The sediment was oiled at the commencement of the experiment. There were three replications per treatment for *A. marina* and *B. gymnorrhiza* and two for *R. mucronata*.

3.2.2 Plant growth measurements

Measurements of shoot and internode length and number of leaves were determined after the experimental period.

3.2.3 Root growth and harvesting of plant parts

Root growth was monitored weekly by tracing new growth onto clear plastic transparencies attached to the outside of the rhizotron. At the end of the treatment (245 and 409 days, respectively), plants were carefully removed from rhizotrons and washed with water to remove soil. Plants were measured and separated into leaves, stems and roots. Plant parts were weighed and dried in an oven to constant mass at 70 °C for three days.

3.2.4 Leaf area and chlorophyll content

Leaf areas were determined by photocopying fresh leaves and scanning into a computer using image analysis software, SIS Pro Softward, version 3:1. Chlorophyll content was determined as described in Chapter 2.2.3.

3.2.5 Root/shoot ratio and relative root growth rate

Dry mass of roots and shoots were determined by weighing on a scale (Mettler Toledo AG 204, accuracy ± 0.1 mg, Mettler Toledo products, Switzerland). Root/shoot ratio was determined on a dry mass basis. Relative root growth rate (RRGR) expressed in grams per day, was calculated according to Sánchez (2005) using the equation:

$$RRGR = DM / t$$

where DM = root dry mass, t = duration of the experiment in days, assuming that the initial root mass was zero.

3.2.6 Root volume and morphology

Root volume was determined using the Archimede's principle (Harrington *et al*., 1994). Plant roots were suspended in a known volume of water in a measuring cylinder. Root volume was approximated to that of the water displaced. Specific root volume (SRV) was calculated according to Merkl *et al*. (2005) and is defined as the ratio of volume (V) per unit dry mass (DM):

$$SRV = V / DM$$

where V = root volume, DM = root dry mass.

Roots were separated into coarse (>2 mm) and fine (<2 mm) diameter. Root diameter (RD) was measured from cross sections using an ocular graticule (Muthukumar *et al*., 2003). Root length was determined by scanning traced transparencies into a computer using image analysis software, SIS Pro Softward, version 3:1. Specific root length (SRL) was calculated according to Bouma *et al*. (2001) and is defined as the ratio of length (L) per unit dry mass (DM):

$$SRL = L / DM$$

where L = root length, DM = root dry mass.

3.2.7 Data analyses

Means and standard errors were calculated for all measurements. Resulting data were tested for normality using the Kolmogorov-Smirnov test and subjected to one-way ANOVA and Tukey-Kramer multiple comparisons test ($P \leq 0.05$) using MINITAB version 16 (Minitab Statistical Software, MINITAB Inc., USA). Other data were subjected to two-way ANOVA and Tukey's multiple comparisons test ($P \leq 0.05$) using GraphPad Prism Version 6.05 (GraphPad Software, Inc., USA).

3.3 Results

3.3.1 First rhizotron study

3.3.1.1 Shoot growth and morphology

A. marina

There was 100% plant mortality in the completely oiled propagule treatment. In the ½ propagule oiled treatment, shoot length was significantly lower by 29% compared to the control (Fig. 3.2A). In the control and ½ propagule oiled treatments, the second internode was the longest compared to the others (Fig. 3.1). In the ½ propagule oiled treatment, the length of the second and fifth internodes were significantly lower by 38% and 75% respectively, compared to their counterparts in the control. A sixth internode was present in 75% of seedlings in the control but absent in the ½ propagule oiled treatment.

Number of leaves (Fig. 3.2B), leaf area (Fig. 3.2C) and chlorophyll content (Fig. 3.2D) were highest in the control and significantly lower in the ½ propagule oiled treatment by 40%, 30% and 31% respectively.

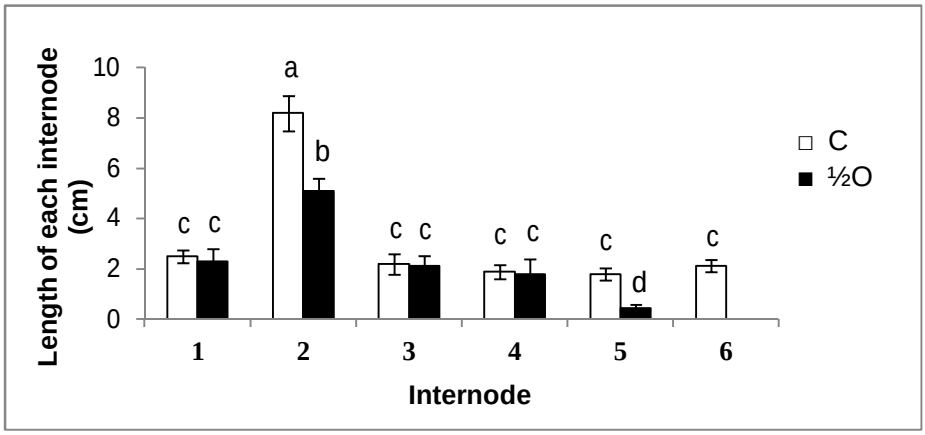

Fig. 3.1 Effects of oiling on length of each internode in *A. marina*. Measurements were taken after 245 days, C = control, ½O = ½ propagule oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

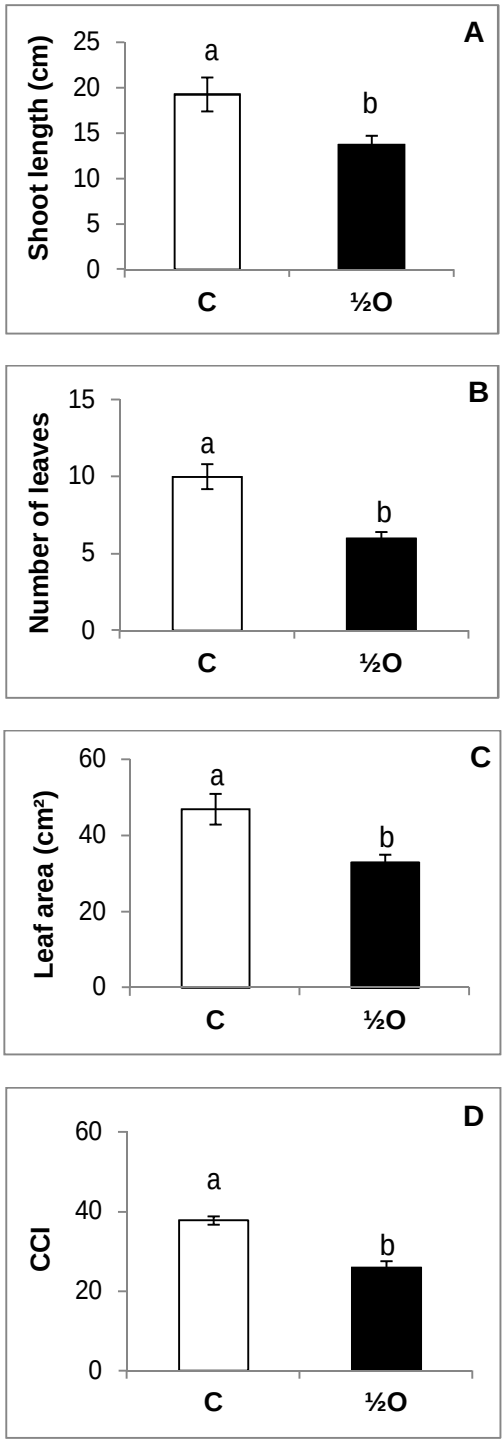

Fig. 3.2 Effects of oiling on shoot length (A), number of leaves (B), leaf area (C) and chlorophyll content index (CCI) (D) in *A. marina*. Measurements were taken after 245 days, C = control, ½O = ½ propagule oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

B. gymnorrhiza

There was no significant difference in shoot length between treatments (Fig. 3.4A).

In the oiled treatment, the length of the first internode was significantly lower by 42% compared to the control (Fig. 3.3). However, the length of the fourth and fifth internodes were significantly higher in the oiled treatment by 44% and 85%, respectively, compared to their counterparts in the control.

There were no significant differences in number of leaves (Fig. 3.4B), leaf area (Fig. 3.4C) or chlorophyll content (Fig. 3.4D) between treatments.

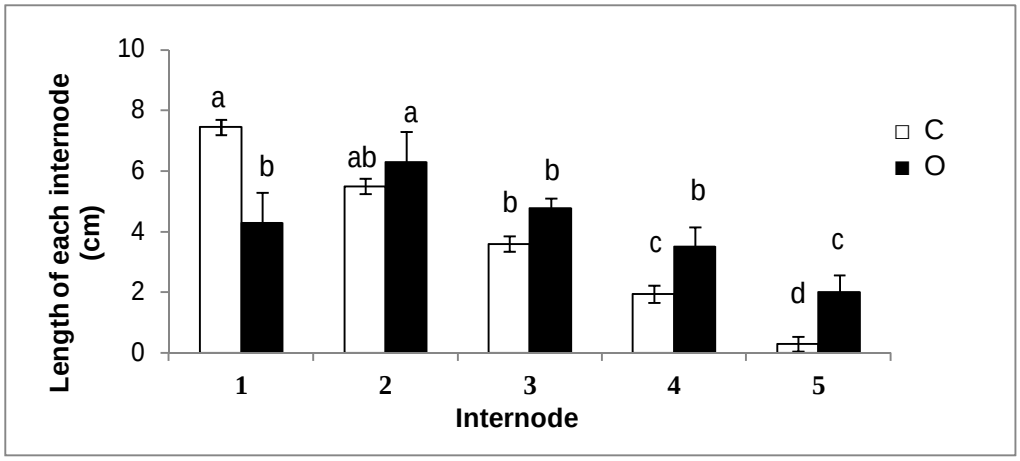

Fig. 3.3 Effects of oiling on length of each internode in *B. gymnorrhiza*. Measurements were taken after 245 days, C = control, O = propagule oiled. Means ± standard error are given, n = 4 - control; 3 – propagule oiled. Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

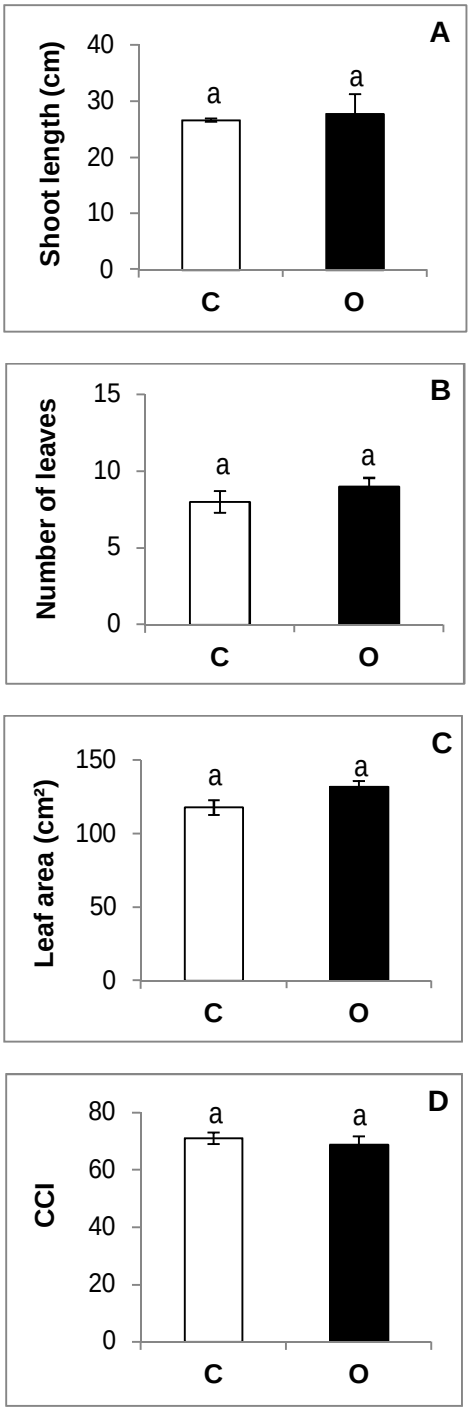

Fig. 3.4 Effects of oiling on shoot length (A), number of leaves (B), leaf area (C) and chlorophyll content index (CCI) (D) in *B. gymnorrhiza*. Measurements were taken after 245 days, C = control, O = propagule oiled. Means ± standard error are given, n = 4 - control; 3 – propagule oiled. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

R. mucronata

In the oiled treatment, shoot length was significantly lower by 82% (Fig. 3.6A) while the lengths of the first and second internodes were lower by 76% and 100%, respectively, compared to their counterparts in the control (Fig. 3.5).

Number of leaves (Fig. 3.6B) leaf area (Fig. 3.6C) and chlorophyll content (Fig. 3.6D) were highest in the control and significantly lower in the oiled treatment by 100% respectively.

Seedlings in the oiled treatment did not produce leaves (Fig. 3.14) and propagules exhibited necrosis (black in colour) close to the base (Fig. 3.15). After two months there was 67% plant mortality in the oiled treatment.

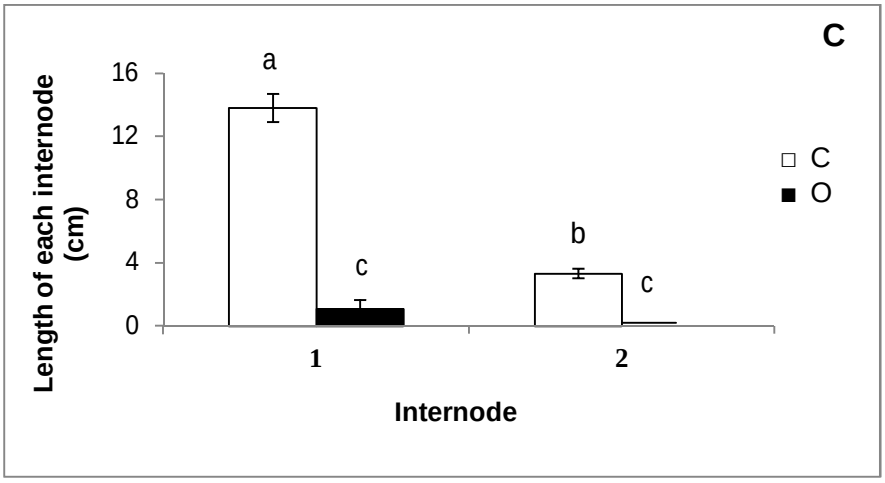

Fig. 3.5 Effects of oiling on length of each internode in *R. mucronata*. Measurements were taken after 245 days, C = control, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

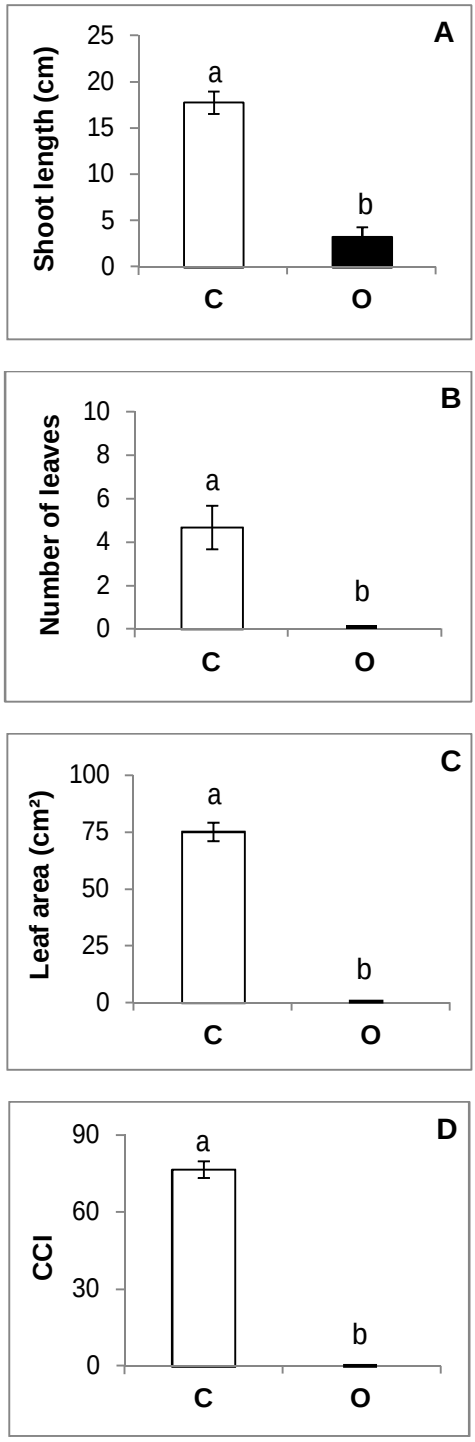

Fig. 3.6 Effects of oiling on shoot length (A), number of leaves (B), leaf area (C) and chlorophyll content index (CCI) (D) in *R. mucronata*. Measurements were taken after 245 days, C = control, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

3.3.1.2 Biomass allocation and root growth rate

A. marina

Oiling of propagules significantly reduced biomass by 42% compared to the control (Fig. 3.7A). In the ½ propagule oiled treatment, reductions in dry mass of leaves and roots were 59% and 44%, respectively, compared to the control (Table 3.1). There was no significant difference in stem dry mass between treatments.

In the ½ propagule oiled treatment, root growth rate was significantly lower by 50% compared to the control (Fig. 3.7C). There was no significant difference in root/shoot ratio between treatments (Fig. 3.7B).

B. gymnorrhiza

In the oiled treatment, biomass was significantly higher by 14% compared to the control (Fig. 3.8A). There were no significant differences in leaf, stem or propagule dry mass between treatments (Table 3.1).

In the oiled treatment, root dry mass was significantly higher by 17% compared to the control (Table 3.1). Root/shoot ratio (Fig. 3.8B) and root growth rate (Fig. 3.8C) were not significantly different between treatments.

R. mucronata

Oiling of propagules significantly reduced biomass by 20% compared to the control (Fig. 3.9A).

In the oiled treatment, reductions in dry mass of leaves, stems and roots were 100%, 94% and 85%, respectively, compared to the control (Table 3.1). There was no significant difference in propagule dry mass between treatments.

In the oiled treatment, root/shoot ratio was significantly higher by 88% compared to the control (Fig. 3.9B) while root growth rate was lower by 86% (Fig. 3.9C).

Table 3.1 Propagule, leaf, stem, root and total biomass accumulation in *A. marina*, *B. gymnorrhiza* and *R. mucronata*. Measurements were taken after 245 days, T = treatment, C = control, ½O = ½ propagule oiled, O = propagule oiled. Means ± SE are given, *A. marina* (n = 4), *B. gymnorrhiza* (n = 4 - control; 3 – propagule oiled) and *R. mucronata* (n = 3). Means with different letters within a column are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

	Dry Mass (g)				
T	Propagule	Leaf	Stem	Root	Total
A. marina					
C	0 ± 0	0.7 ± 0.05**a**	0.8 ± 0.05**a**	1.0 ± 0.06**a**	2.4 ± 0.33**a**
½O	0 ± 0	0.3 ± 0.03**b**	0.6 ± 0.04**a**	0.6 ± 0.08**b**	1.4 ± 0.25**b**
B. gymnorrhiza					
C	4.2 ± 0.11**a**	1.4 ± 0.09**a**	0.9 ± 0.01**a**	1.9 ± 0.04**a**	8.4 ± 0.22**a**
O	5.0 ± 0.16**a**	1.5 ± 0.07**a**	0.9 ± 0.08**a**	2.3 ± 0.02**b**	9.7 ± 0.88**b**
R. mucronata					
C	26.9 ± 1.38**a**	1.4 ± 0.13**a**	0.5 ± 0.01**a**	1.9 ± 0.05**a**	31 ± 2.01**a**
O	25.0 ± 0.95**a**	0.0 ± 0.00**a**	0.0 ± 0.00**b**	0.3 ± 0.07**b**	25 ± 0.75**b**

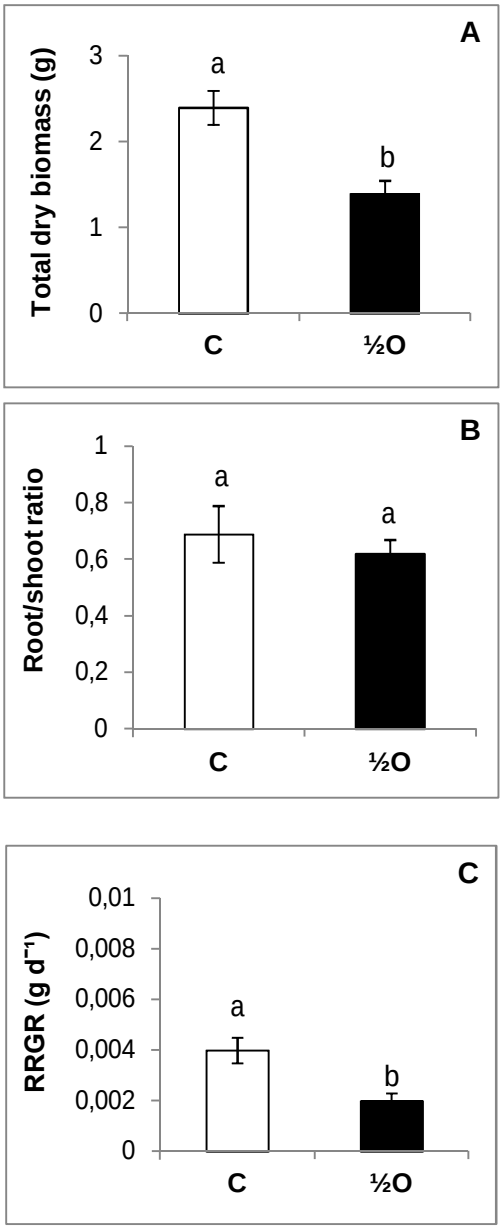

Fig. 3.7 Effects of oiling on dry biomass (A), root/shoot ratio (B) and relative root growth rate (RRGR) (C) in *A. marina.* Measurements were taken after 245 days, C = control, ½O = ½ propagule oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

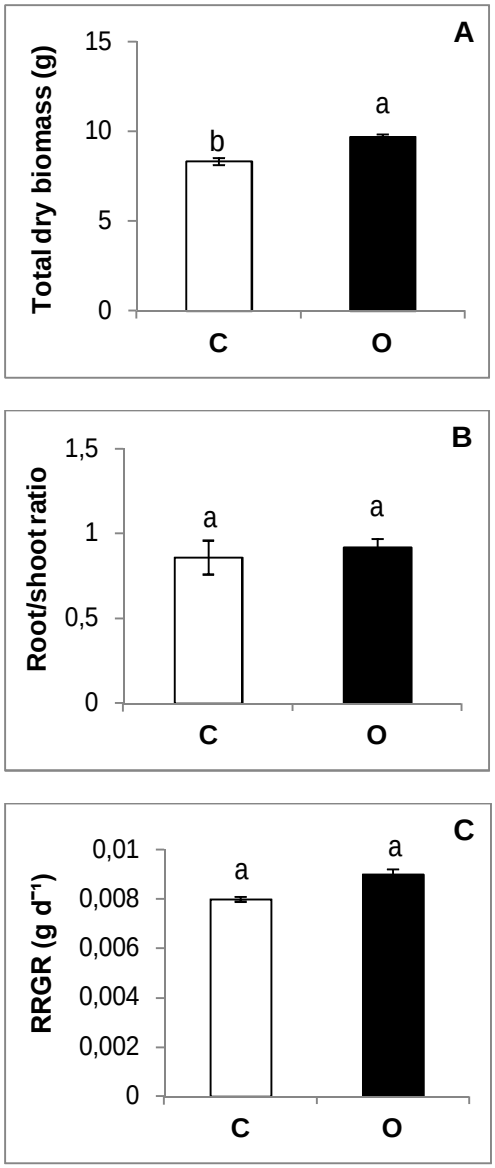

Fig. 3.8 Effects of oiling on dry biomass (A), root/shoot ratio (B) and relative root growth rate (RRGR) (C) in *B. gymnorrhiza.* Measurements were taken after 245 days, C = control, O = propagule oiled. Means ± standard error are given, n = 4 - control; 3 – propagule oiled. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

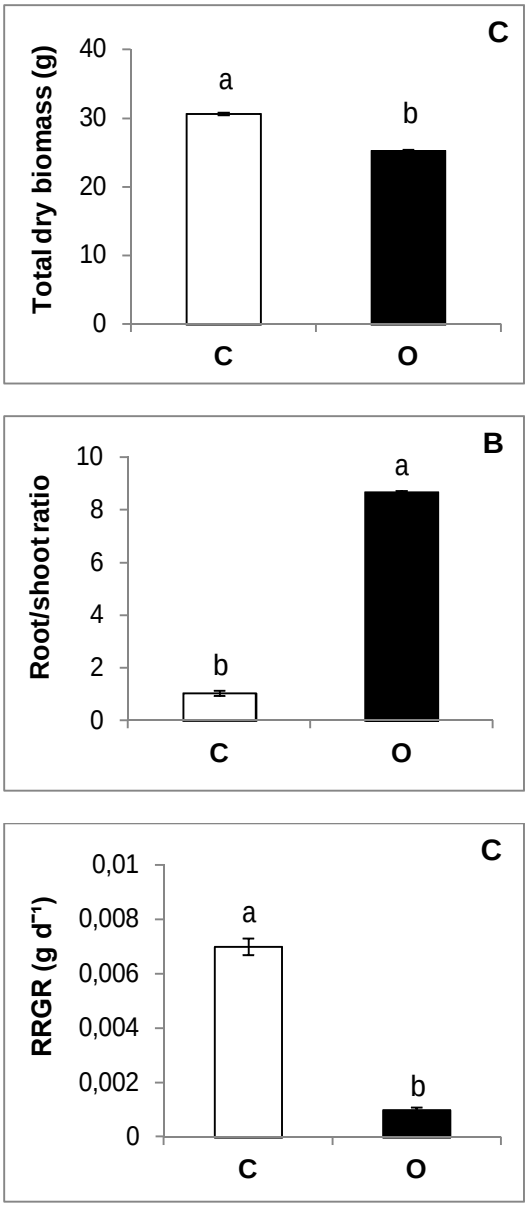

Fig. 3.9 Effects of oiling on dry biomass (A), root/shoot ratio (B) and relative root growth rate (RRGR) (C) in *R. mucronata.* Measurements were taken after 245 days, C = control, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

3.3.1.3 Root morphology and volume

A. marina

Root growth in seedlings commenced after one week in the control and four weeks in the ½ propagule oiled treatment (Fig. 3.10A). In the oiled treatment, root length and SRL were significantly lower by 54% and 19%, respectively (Table 3.2), while root length was lower in April, May, July, August, September, October, November and December compared to the control (Fig. 3.10A).

In the oiled treatment, root diameter and SRV were significantly higher by 31% and 21%, respectively, compared to the control (Table 3.2) while volume was lower by 30% (Fig. 3.11A).

B. gymnorrhiza

Root growth in seedlings commenced after two weeks in the control and four in the oiled treatment (Fig. 3.10B). In the oiled treatment, root length and SRL were significantly lower by 19% and 31%, respectively (Table 3.2), while root length was lower in April, May and December compared to the control (Fig. 3.10B).

In the oiled treatment, root diameter (Table 3.2), root volume (Fig. 3.11B) and SRV (Table 3.2) were significantly higher than the control by 27%, 26% and 14%, respectively.

R. mucronata

Root growth in seedlings commenced after two weeks in the control and four in the oiled treatment (Fig. 3.10C). Plants in oiled treatments produced numerous lateral roots (Fig. 3.14). In the oiled treatment, root length and SRL were significantly lower by 89% and 73%, respectively, than the control (Table 3.2). In the oiled treatment, root length was significantly lower than the control in all months (Fig. 3.10C).
In the oiled treatment, root diameter (Table 3.2) and volume (Fig. 3.11C) were significantly lower than the control by 51% and 79%, respectively, while SRV was higher by 31% (Table 3.2).

Table 3.2 Root length (RL), specific root length (SRL), root diameter (RD) and specific root volume (SRV) in *A. marina, B. gymnorrhiza* and *R. mucronata*. Measurements were taken after 245 days, T = treatment, C = control, ½O = ½ propagule oiled, O = propagule oiled. Means ± SE are given, *A. marina* (n = 4), *B. gymnorrhiza* (n = 4 - control; 3 – propagule oiled) and *R. mucronata* (n = 3). Means with different letters within a column are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

T	RL (cm)	SRL (cm g^{-1})	RD (mm)	SRV (cm^3 g^{-1})
A. marina				
C	147.55 ± 11.7**a**	149.41 ± 8.22**a**	2.14 ± 0.11**a**	4.33 ± 0.18**a**
½O	67.23 ± 6.61**b**	122.21 ± 10.9**b**	3.12 ± 0.13**b**	5.45 ± 0.31**b**
B. gymnorrhiza				
C	209.65 ± 8.41**a**	108.34 ± 3.41**a**	3.15 ± 0.09**a**	5.29 ± 0.23**a**
O	170.11 ± 6.65**b**	74.93 ± 4.61**b**	4.25 ± 0.15**b**	6.12 ± 0.11**b**
R. mucronata				
C	98.51 ± 6.91**a**	50.77 ± 2.32**a**	3.62 ± 0.12**a**	4.81 ± 0.33**a**
O	10.56 ± 4.02**b**	36.86 ± 2.45**b**	1.83 ± 0.03**b**	6.99 ± 0.41**b**

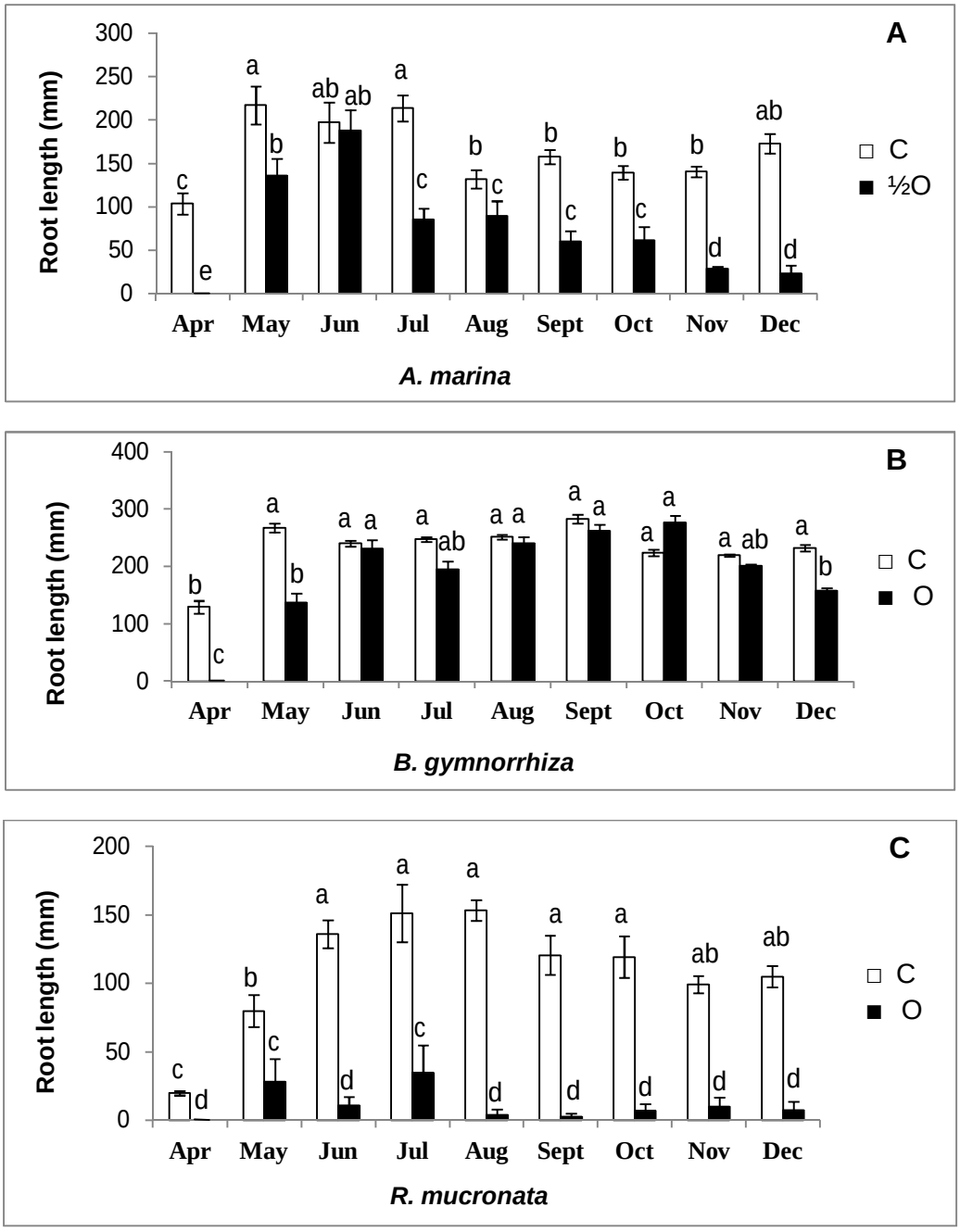

Fig. 3.10 Effects of oiling on root length in *A. marina* (A), *B. gymnorrhiza* (B) and *R. mucronata* (C). Measurements were recorded weekly for 245 days beginning eight days after treatment, C = control, ½O = ½ propagule oiled, O = propagule oiled. Means ± standard error are given, *A. marina* (n = 4), *B. gymnorrhiza* (n = 4 - control; 3 – propagule oiled) and *R. mucronata* (n = 3). Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

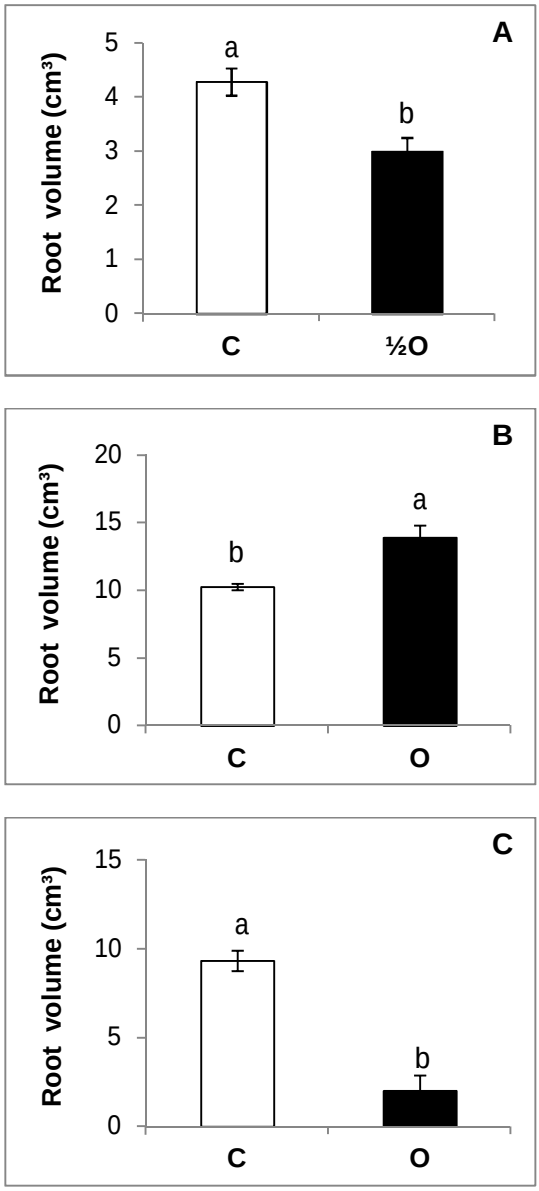

Fig. 3.11 Effects of oiling on root volume in *A. marina* (A), *B. gymnorrhiza* (B) and *R. mucronata* (C). Measurements were recorded after 245 days, C = control, ½O = ½ propagule oiled and O = propagule oiled. Means ± standard error are given, *A. marina* (n = 4), *B. gymnorrhiza* (n = 4 - control; 3 – propagule oiled) and *R. mucronata* (n = 3). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

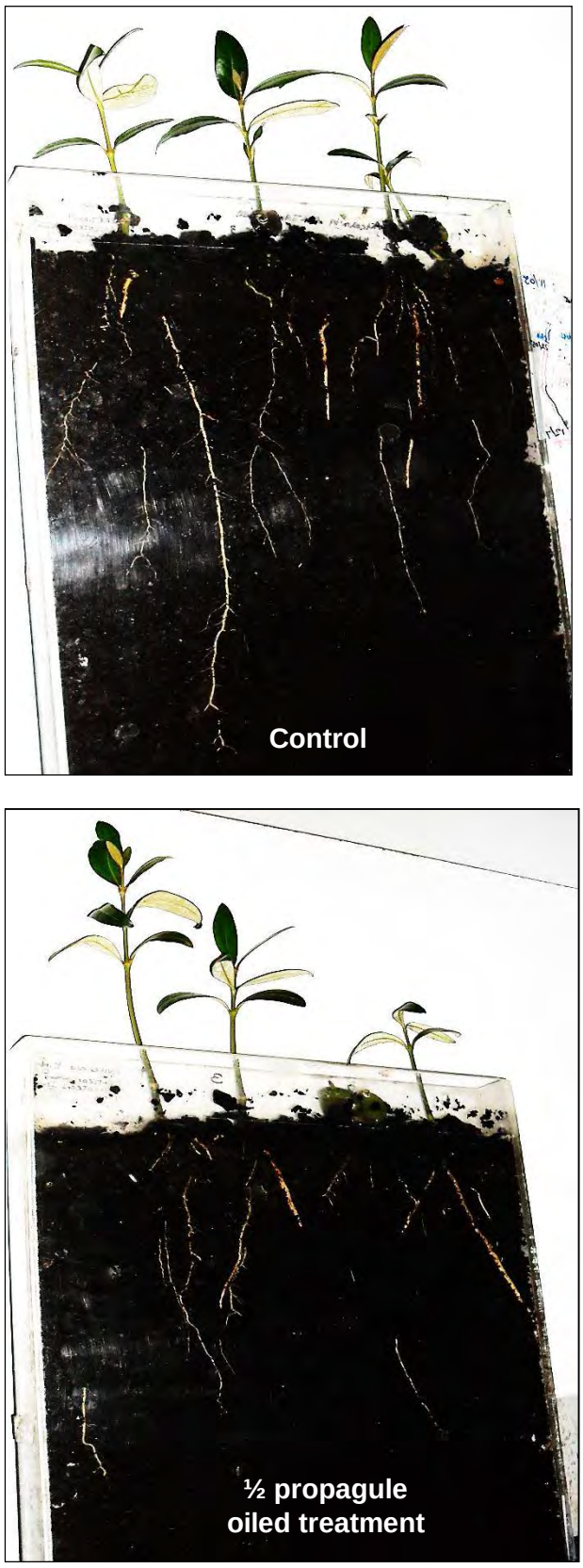

Fig. 3.12 Effects of oiling on root growth in *A. marina*, control (top) and ½ propagule oiled treatment (bottom) after four months.

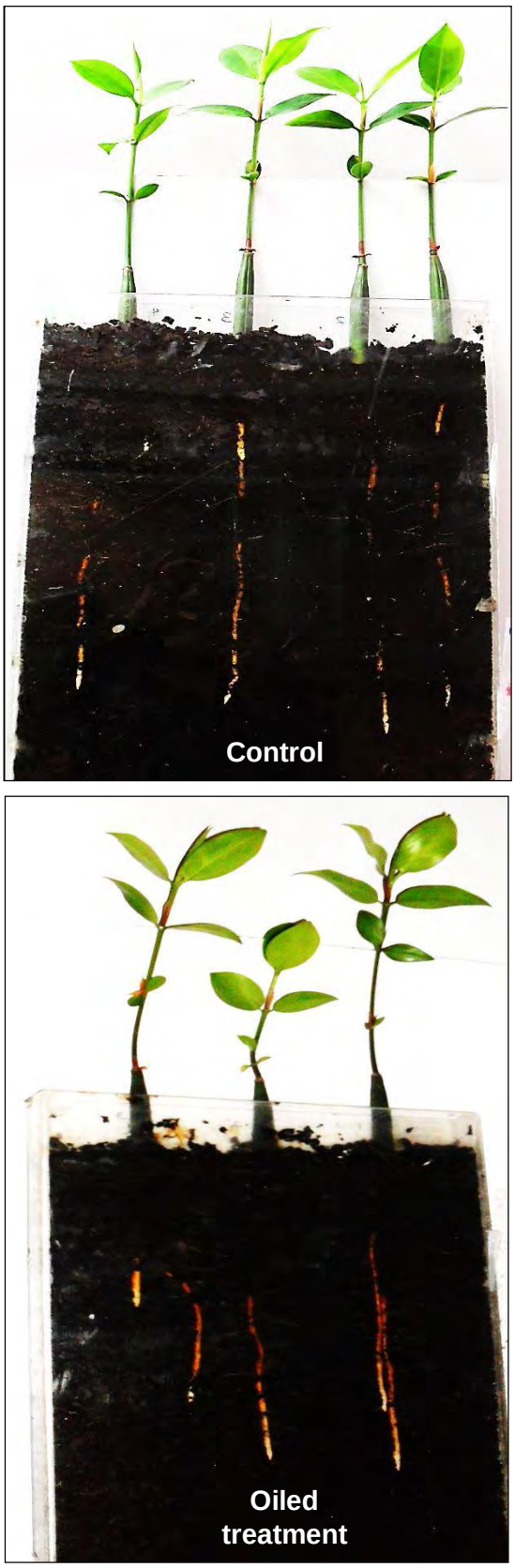

Fig. 3.13 Effects of oiling on root growth in *B. gymnorrhiza*, control (top) and oiled (bottom) treatment after four months.

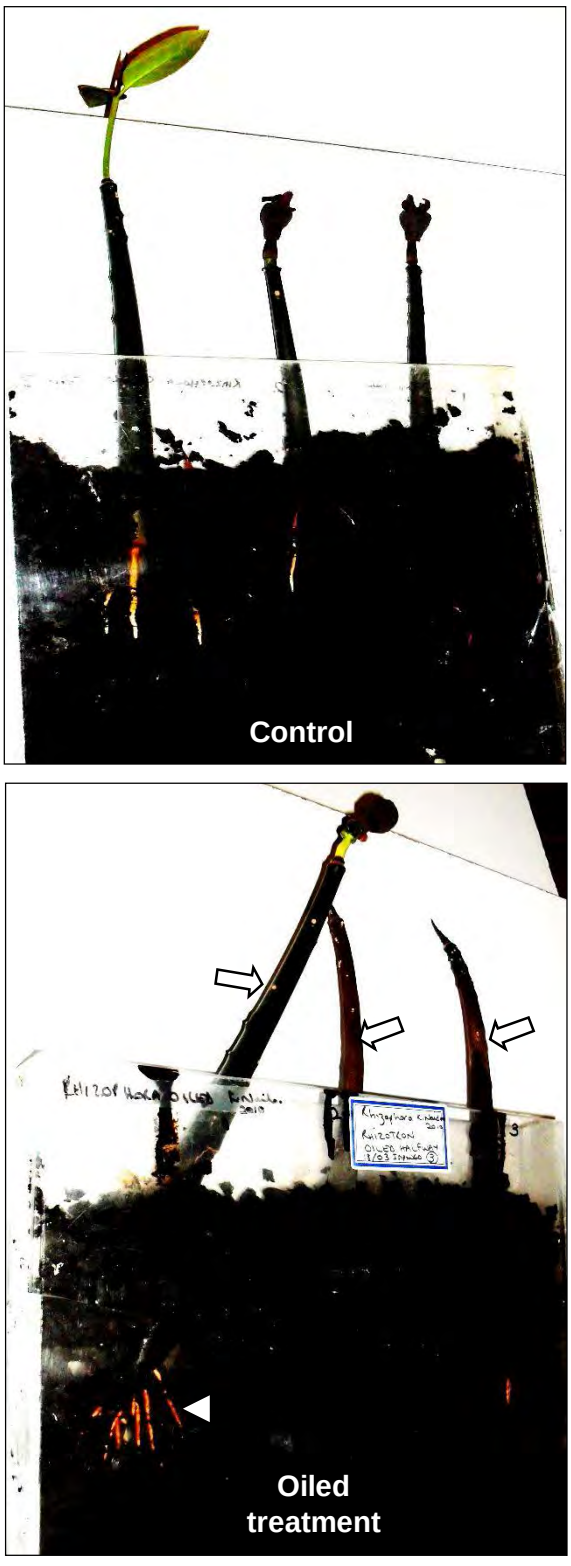

Fig. 3.14 Effects of oiling on root growth in *R. mucronata*, control (top) and oiled treatment (bottom) after two months. Note the propagule growing at a 45° angle (left arrow) and the two dead propagules (right arrows) in the oiled treatment. Note the numerous lateral roots beneath soil surface (arrowhead).

Fig. 3.15 Effects of oiling on root growth in *R. mucronata*, after two months. Note the necrosis on the propagule (arrow).

3.3.2 Second rhizotron study

3.3.2.1 Shoot growth and morphology

A. marina

Propagules in the control exhibited shoot growth within two weeks whilst those in the oiled treatments took twice as long.

Shoot length was highest in the control and significantly lower in the sediment and propagule oiled treatments by 80% and 82%, respectively (Fig. 3.16). There was no significant difference in shoot length between the oiled treatments (Fig. 3.16).

In the oiled treatments, the length of the first - fifth internode was significantly lower by 86% compared to the control (Fig. 3.18). There was no significant difference in the length of each internode between the oiled treatments (Fig. 3.18).

Number of leaves (Fig. 3.20), leaf area (Fig. 3.22) and chlorophyll content (Fig. 3.24) were highest in the control and significantly lower in the sediment oiled treatment by 80%, 81% and 79%, respectively. In the propagule oiled treatment, number of leaves, leaf area and chlorophyll content were significantly lower by 80%, 82% and 80%, respectively, compared to the control.

In both the sediment and propagule oiled treatments, there was 67% plant mortality after four weeks. Sixty seven percent of propagules in the oiled treatments produced roots, but shoots were absent (Figs. 3.37 and 3.38).

B. gymnorrhiza

Shoot length was highest in the control and significantly lower in the oiled treatment by 88% (Fig. 3.17A).

In the oiled treatment, the length of the first - fourth internode was significantly lower by 84% compared to the control and there were only four internodes in seedlings (Fig. 3.19A).

Number of leaves (Fig. 3.21A), leaf area (Fig. 3.23A) and chlorophyll content (Fig. 3.25A) were highest in the control and significantly lower in the oiled treatment by 93%, 94% and 81%, respectively.

After four weeks, there was 67% plant mortality in the oiled treatment.

R. mucronata

Shoot length was highest in the control and significantly lower in the oiled treatment by 43% (Fig. 3.17B).

In the oiled treatment, the length of the first – third internode was significantly lower by 65% compared to the control while the fourth was absent (Fig. 3.19B).

There was no significant difference in number of leaves between treatments (Fig. 3.21B). In the oiled treatment, leaf area (Fig. 3.23B) and chlorophyll content (Fig. 3.25B) were significantly lower than the control by 44% and 32%, respectively.

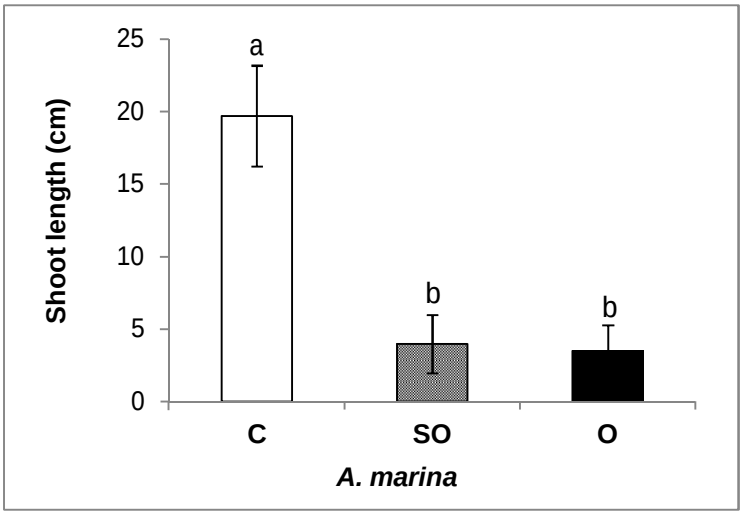

Fig. 3.16 Effects of oiling on shoot length in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

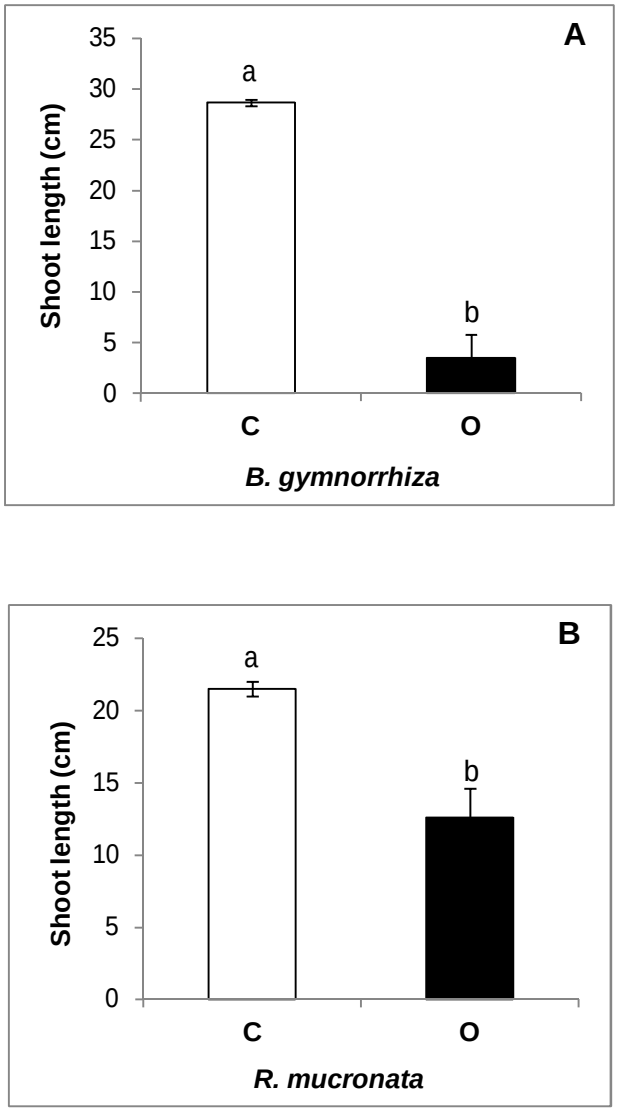

Fig. 3.17 Effects of oiling on shoot length in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

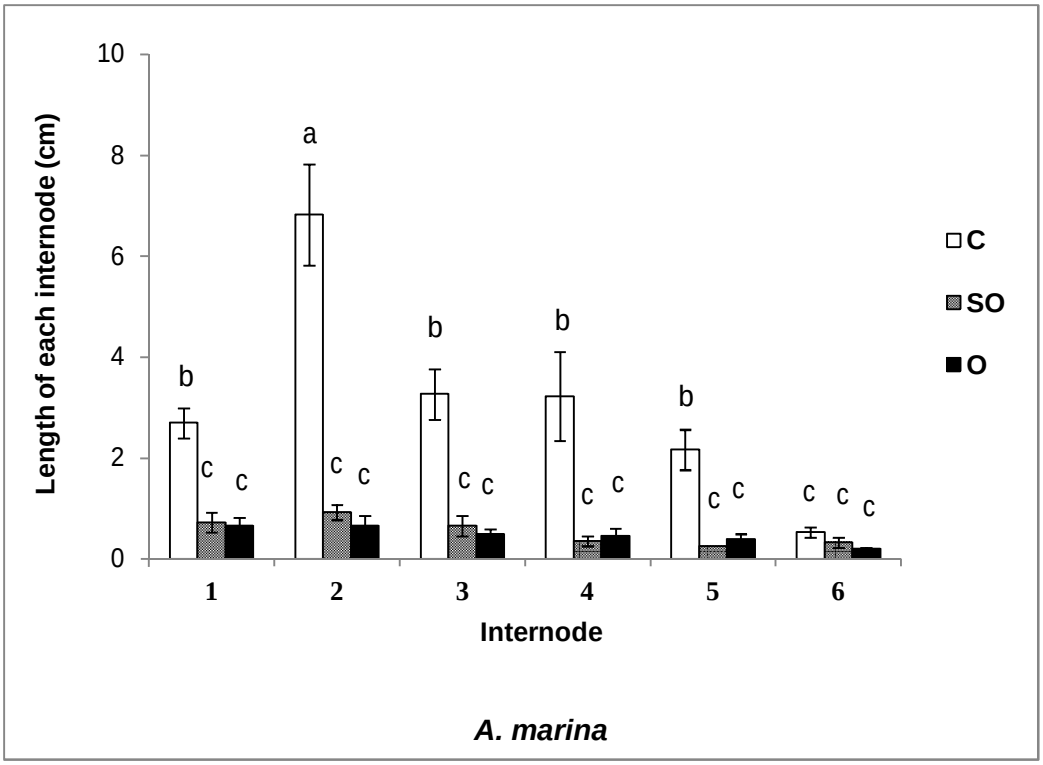

Fig. 3.18 Effects of oiling on length of each internode in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

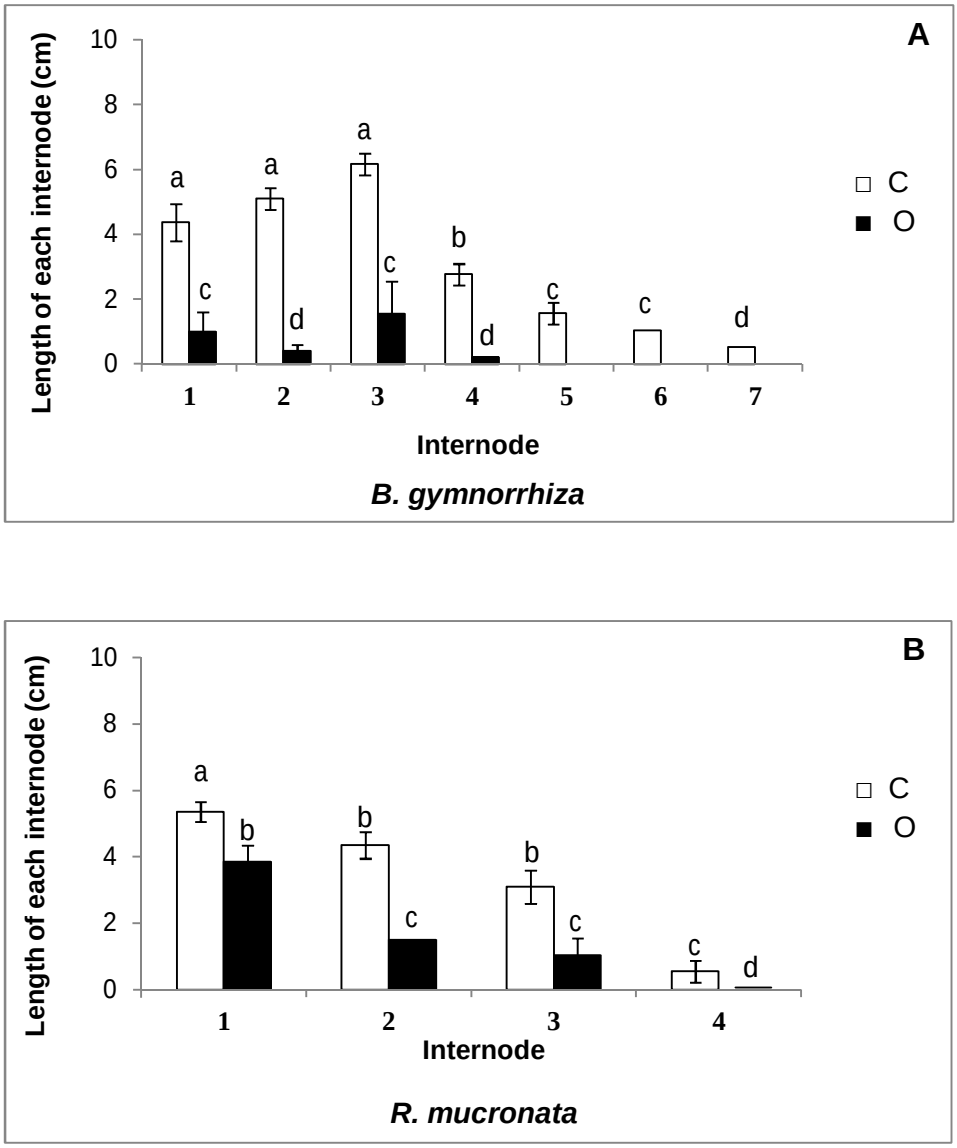

Fig. 3.19 Effects of oiling on length of each internode in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

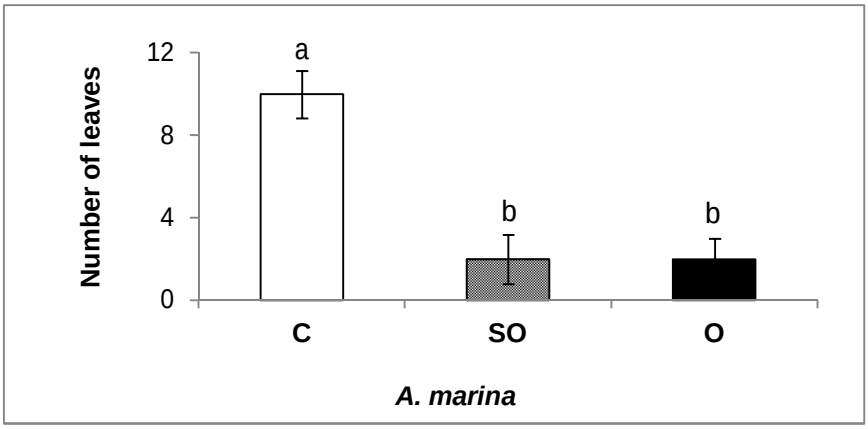

Fig. 3.20 Effects of oiling on number of leaves in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

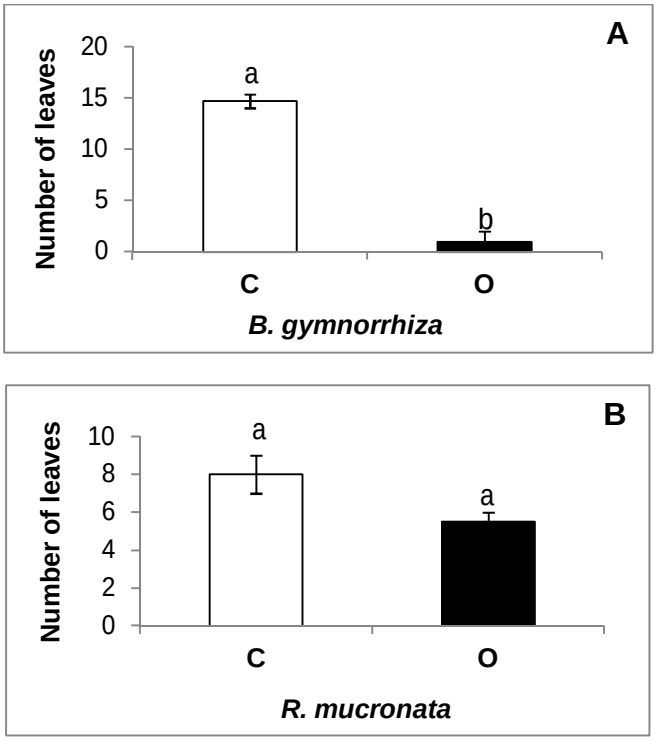

Fig. 3.21 Effects of oiling on number of leaves in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

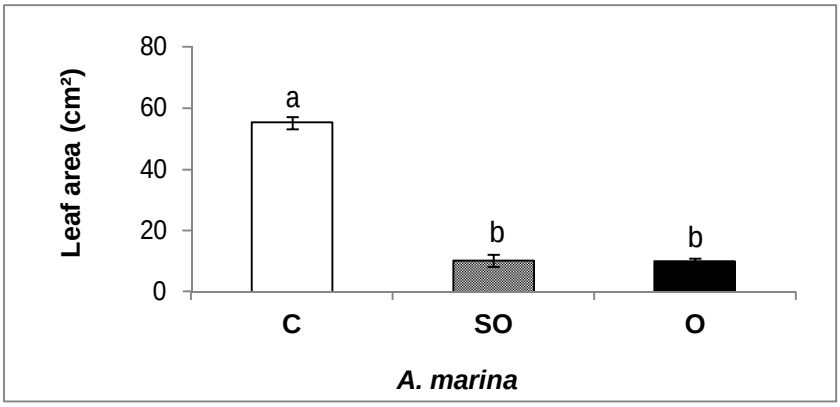

Fig. 3.22 Effects of oiling on leaf area in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

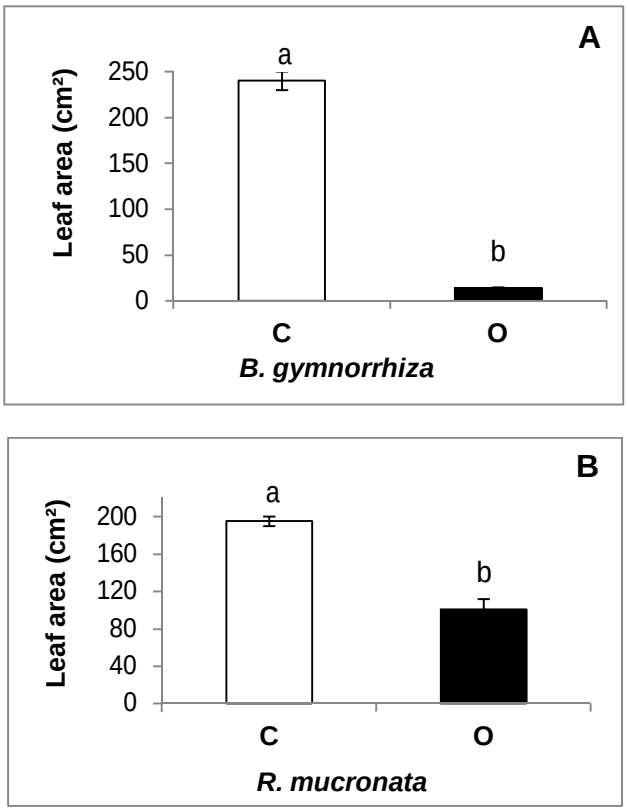

Fig. 3.23 Effects of oiling on leaf area in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

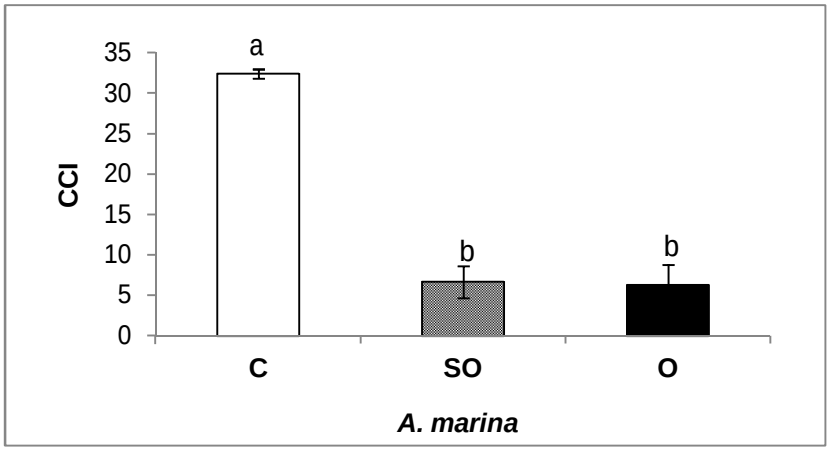

Fig. 3.24 Effects of oiling on chlorophyll content index (CCI) in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

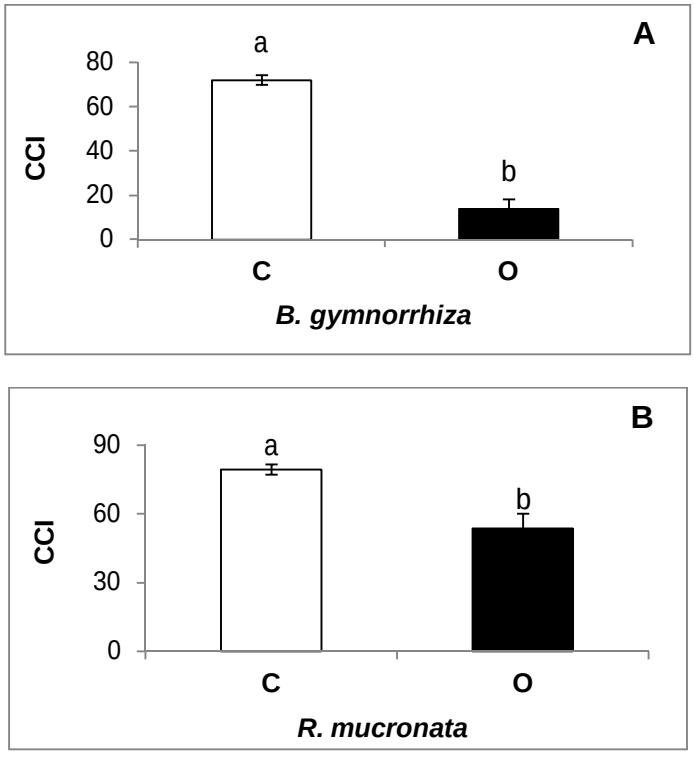

Fig. 3.25 Effects of oiling on chlorophyll content index (CCI) in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

3.3.2.2 Biomass allocation and root growth rate

A. marina

In the sediment and propagule oiled treatments, biomass was significantly reduced by 85% and 74%, respectively, compared to the control (Fig. 3.26). There was no significant difference in biomass between the oiled treatments (Fig. 3.26). In the oiled treatments, root/shoot ratio was significantly reduced by 14% and 37%, respectively, compared to the control (Fig. 3.28). Root/shoot ratio was significantly lower in the propagule than the sediment oiled treatment by 28% (Fig. 3.28).

In the sediment oiled treatment, reductions in dry mass of leaves, stems and roots were 76%, 92% and 86%, respectively, compared to the control (Table 3.3). In the propagule oiled treatment, dry mass of leaves, stems and roots were reduced by 56%, 80% and 81%, respectively, compared to the control (Table 3.3). Root growth rate was highest in the control and significantly lower in the sediment and propagule oiled treatments by 86% and 80% respectively (Fig. 3.30). There was no significant difference in root growth rate between the oiled treatments (Fig. 3.30).

B. gymnorrhiza

In the oiled treatment, biomass was significantly reduced by 31% compared to the control (Fig. 3.27A). In the oiled treatment, root/shoot ratio was significantly lower by 32% compared to the control (Fig. 3.29A).

In the oiled treatment, dry mass of leaves, stems and roots were significantly reduced by 99%, 89% and 97%, respectively, compared to the control while propagule dry mass was higher by 37% (Table 3.3). In the oiled treatment, root growth rate was significantly lower than the control by 97% (Fig. 3.31A).

R. mucronata

In the oiled treatment, biomass was significantly reduced by 35% (Fig. 3.27B) while root/shoot ratio was lower by 45% compared to the control (Fig. 3.29B). There were significant reductions in dry mass of leaves (52%) and roots (73%) in the oiled

treatment compared to the control (Table 3.3). In the oiled treatment, stem dry mass was significantly reduced by 38% compared to the control while there was no significant difference in propagule dry mass (Table 3.3). Root growth rate was highest in the control and significantly lower in the oiled treatment by 70% (Fig. 3.31B).

Table 3.3 Propagule, leaf, stem, root and total biomass accumulation in *A. marina*, *B. gymnorrhiza* and *R. mucronata*. Measurements were taken after 409 days, T = treatment, C = control, SO = sediment oiled, O = propagule oiled (*A. marina*); O = sediment oiled (*B. gymnorrhiza, R. mucronata*). Means ± SE are given, *A. marina,* n = 3; *B. gymnorrhiza,* n = 3; *R. mucronata*, n = 2. Means with different letters within a column are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

	Dry Mass (g)				
T	Propagule	Leaf	Stem	Root	Total
A. marina					
C	0 ± 0	0.5 ± 0.07**a**	0.5 ± 0.04**a**	1.1 ± 0.10**a**	2.2 ± 0.18**a**
SO	0 ± 0	0.1 ± 0.02**b**	0.1 ± 0.00**b**	0.2 ± 0.03**b**	0.4 ± 0.09**b**
O	0 ± 0	0.2 ± 0.05**b**	0.1 ± 0.01**b**	0.2 ± 0.02**b**	0.5 ± 0.08**b**
B. gymnorrhiza					
C	6.0 ± 0.06**a**	2.7 ± 0.12**a**	1.3 ± 0.04**a**	4.2 ± 0.10**a**	14 ± 0.18**a**
O	9.5 ± 0.54**b**	0.0 ± 0.00**b**	0.1 ± 0.02**b**	0.1 ± 0.03**b**	9.8 ± 0.75**b**
R. mucronata					
C	17.6 ± 0.55**a**	2.8 ± 0.17**a**	0.8 ± 0.05**a**	4.3 ± 0.16**a**	26 ± 0.81**a**
O	13.8 ± 0.21**a**	1.3 ± 0.15**b**	0.5 ± 0.07**b**	1.2 ± 0.06**b**	17 ± 0.10**b**

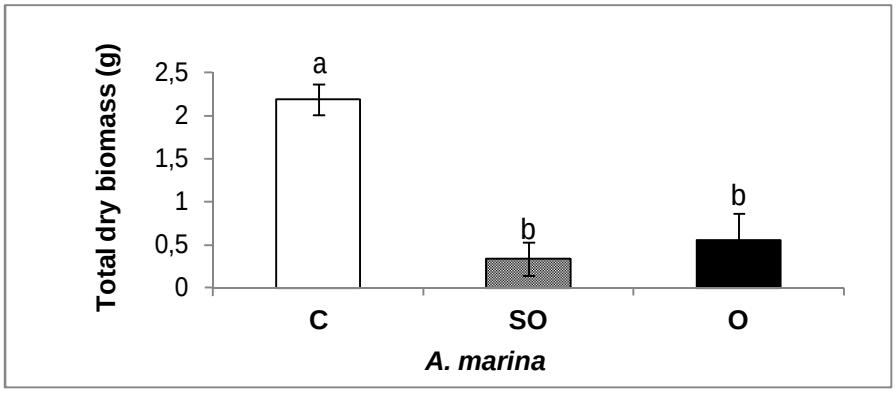

Fig. 3.26 Effects of oiling on dry biomass in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

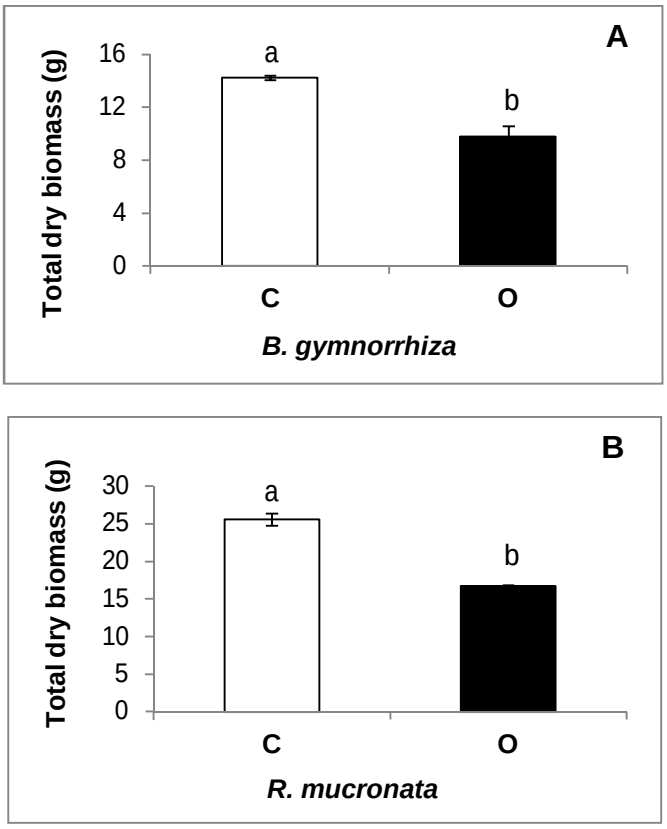

Fig. 3.27 Effects of oiling on dry biomass in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

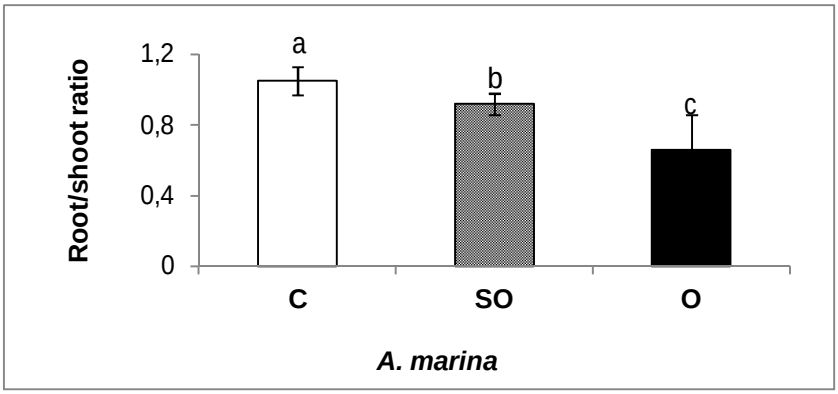

Fig. 3.28 Effects of oiling on root/shoot ratio in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

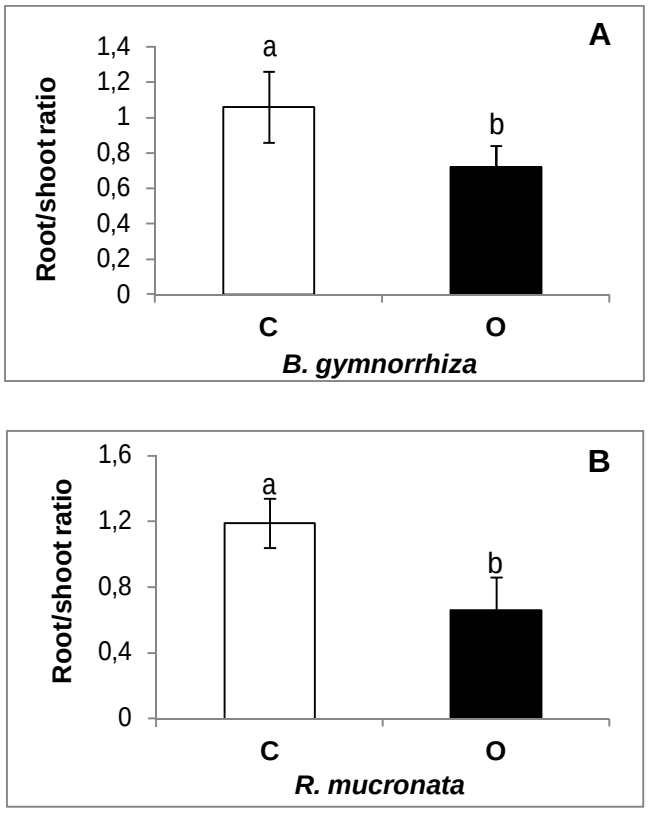

Fig. 3.29 Effects of oiling on root/shoot ratio in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

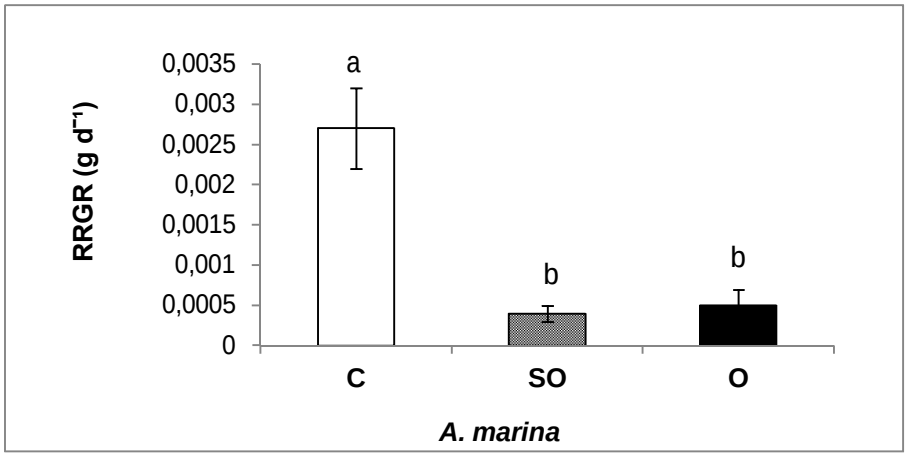

Fig. 3.30 Effects of oiling on relative root growth rate (RRGR) in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

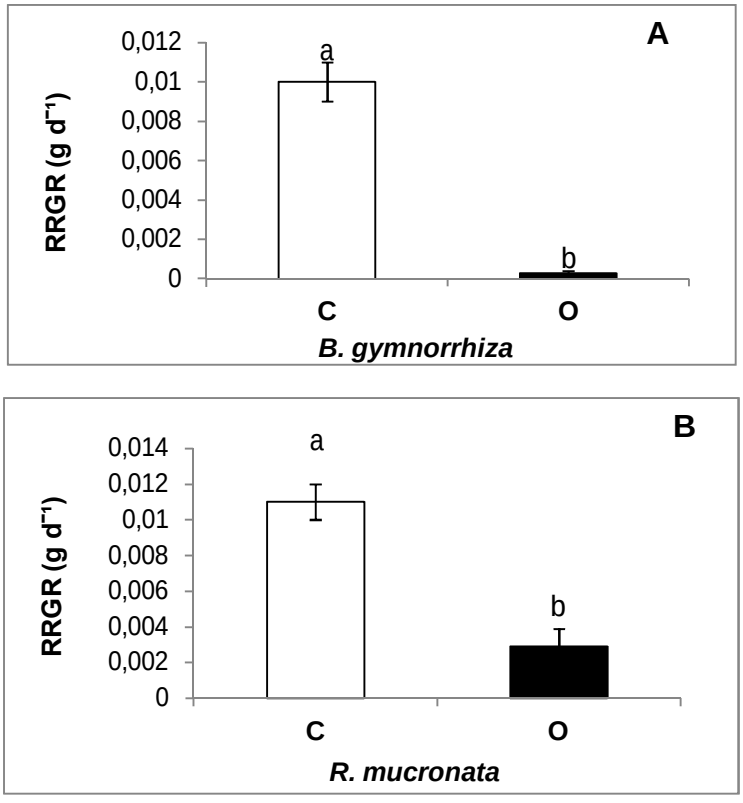

Fig. 3.31 Effects of oiling on relative root growth rate (RRGR) in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

3.3.2.3 Root morphology and volume

A. marina

Root length was highest in the control and significantly lower in the sediment and propagule oiled treatments in all months (Fig. 3.32). In the oiled treatments, root length was significantly lower by 96% and 91%, respectively, compared to the control (Table 3.4). Root length was significantly lower in the sediment than the propagule oiled treatment by 49% (Table 3.4). In the oiled treatments, SRL was significantly lower by 70% and 57%, respectively, compared to the control (Table 3.4). Specific root length was significantly lower in the sediment than the propagule oiled treatment by 30% (Table 3.4).

In the oiled treatments, root diameter was significantly lower by 61% and 72%, respectively, compared to the control (Table 3.4). There was no significant difference in root diameter between the oiled treatments (Table 3.4). In the oiled treatments, root volume was significantly lower by 78% and 84%, respectively (Fig. 3.35) while SRV was higher by 34% and 20%, respectively compared to the control (Table 3.4). Specific root volume was significantly lower in the propagule than the sediment oiled treatment by 18%. Roots in the oiled treatments were dark brown to black in colour and flaccid compared to the white turgid ones in the control (Figs. 3.37 and 3.38).

B. gymnorrhiza

Root length was highest in the control and significantly lower in the oiled treatment in all months (Fig. 3.33). In the oiled treatment, root length, diameter and SRL were significantly lower than the control by 99%, 83% and 58%, respectively (Table 3.4). In the oiled treatment, root volume was significantly lower by 97% (Fig. 3.36A) while SRV was higher by 28% compared to the control (Table 3.4). Roots in the oiled treatment were dark brown in colour and flaccid compared to the white turgid ones in the control (Fig. 3.41).

R. mucronata

Root length was highest in the control and significantly lower in the oiled treatment in all months (Fig. 3.34). In the oiled treatment, root length, diameter and SRL were

significantly lower than the control by 73%, 48% and 27%, respectively (Table 3.4). Root volume was highest in the control and significantly lower in the oiled treatment by 78% (Fig. 3.36B). In the oiled treatment, SRV was significantly higher than the control by 23% (Table 3.4). Roots in the oiled treatment were brown in colour, thinner and less healthy in appearance compared to the white, turgid, thicker ones in the control (Fig. 3.44).

Table 3.4 Root length (RL), specific root length (SRL), root diameter (RD) and specific root volume (SRV) in *A. marina, B. gymnorrhiza* and *R. mucronata*. Measurements were taken after 245 days, T = treatment, C = control, SO = sediment oiled, O = propagule oiled (*A. marina*); O = sediment oiled (*B. gymnorrhiza, R. mucronata*). Means ± SE are given, *A. marina,* n = 3; *B. gymnorrhiza,* n = 3; *R. mucronata*, n = 2. Means with different letters within a column are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

T	RL (cm)	SRL (cm g^{-1})	RD (mm)	SRV (cm^3 g^{-1})
A. marina				
C	239.97 ± 15.0**a**	214.30 ± 3.20**a**	3.27 ± 0.46**a**	3.10 ± 0.11**a**
SO	10.71 ± 7.02**b**	65.51 ± 1.91**b**	1.29 ± 0.12**b**	4.70 ± 0.33**b**
O	20.83 ± 9.20**c**	93.32 ± 2.82**c**	0.89 ± 0.06**b**	3.86 ± 0.40**c**
B. gymnorrhiza				
C	367.67 ± 11.7**a**	87.20 ± 2.22**a**	4.35 ± 0.55**a**	5.54 ± 0.34**a**
O	4.71 ± 2.00**b**	36.73 ± 1.10**b**	0.75 ± 0.07**b**	7.69 ± 0.56**b**
R. mucronata				
C	230.12 ± 8.75**a**	53.0 ± 0.50**a**	4.80 ± 0.63**a**	4.72 ± 0.23**a**
O	45.93 ± 5.19**b**	38.8 ± 0.89**b**	2.54 ± 0.33**b**	6.11 ± 0.07**b**

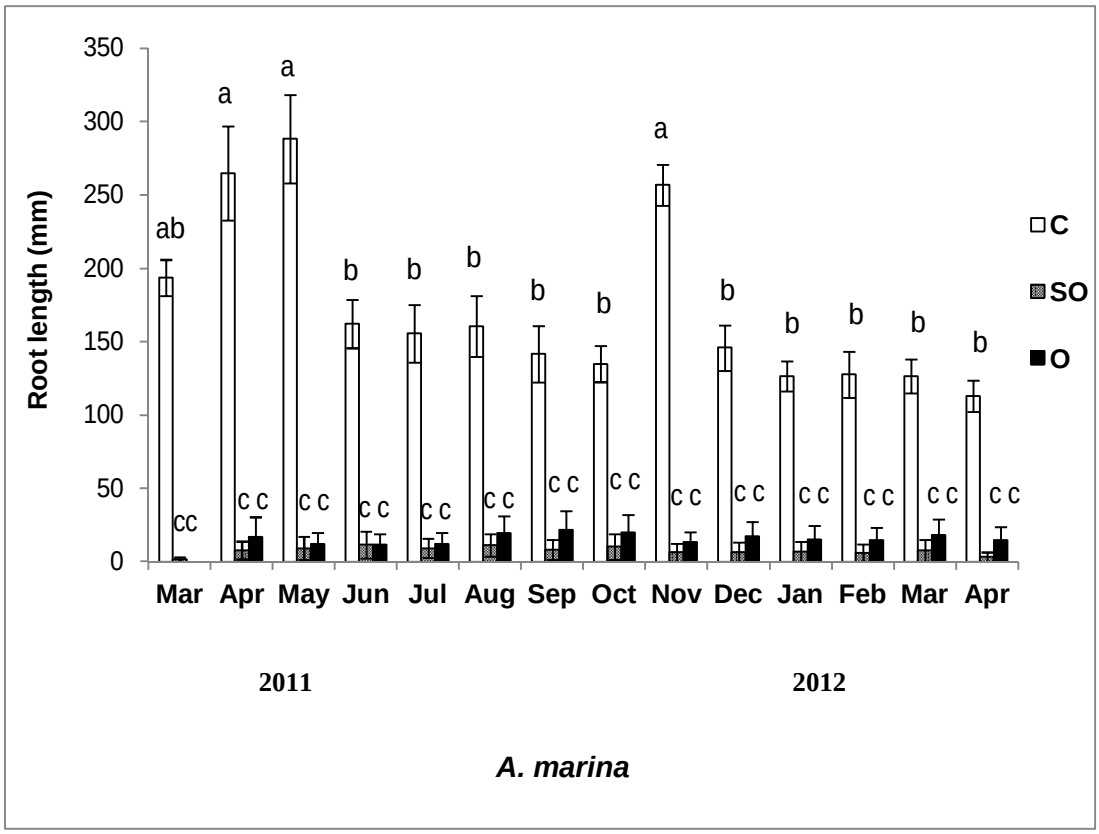

Fig. 3.32 Effects of oiling on root length in *A. marina*. Measurements were taken weekly for 409 days, beginning 17 days after treatment, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's comparisons test.

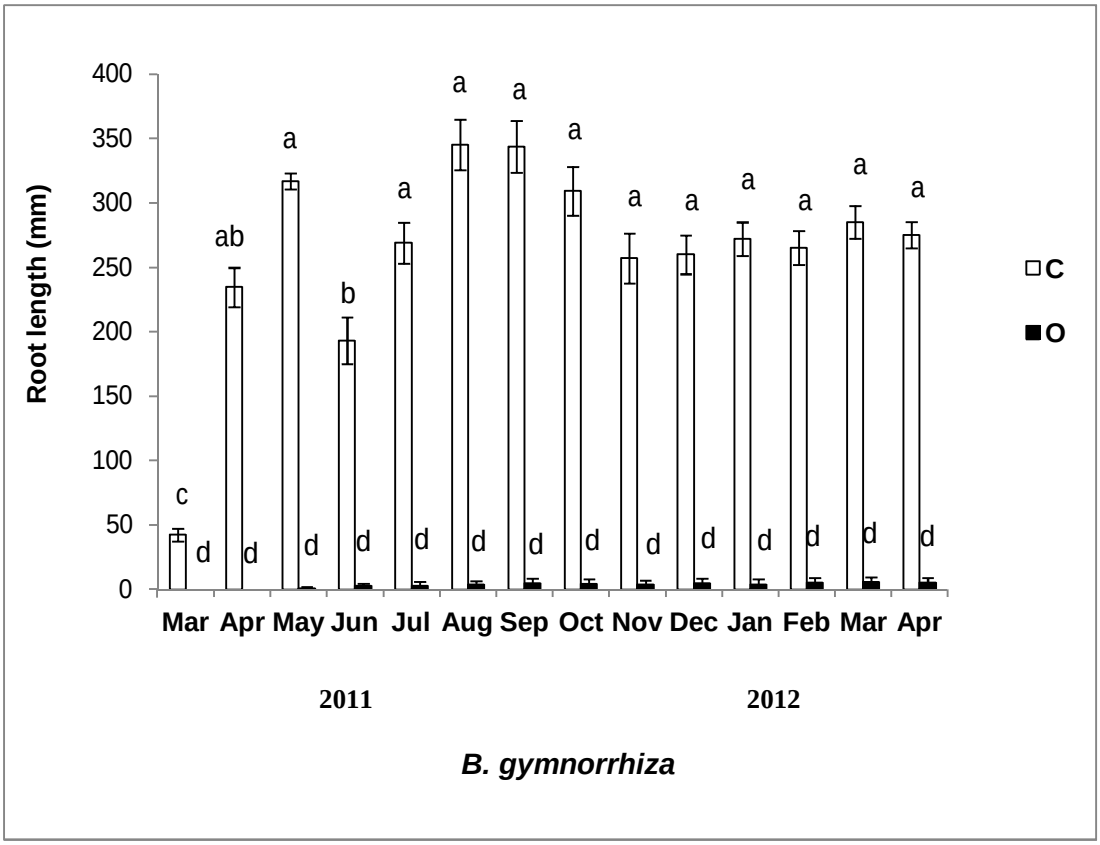

Fig. 3.33 Effects of oiling on root length in *B. gymnorrhiza*. Measurements were taken weekly for 409 days, beginning 17 days after treatment, C = control, O = sediment oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

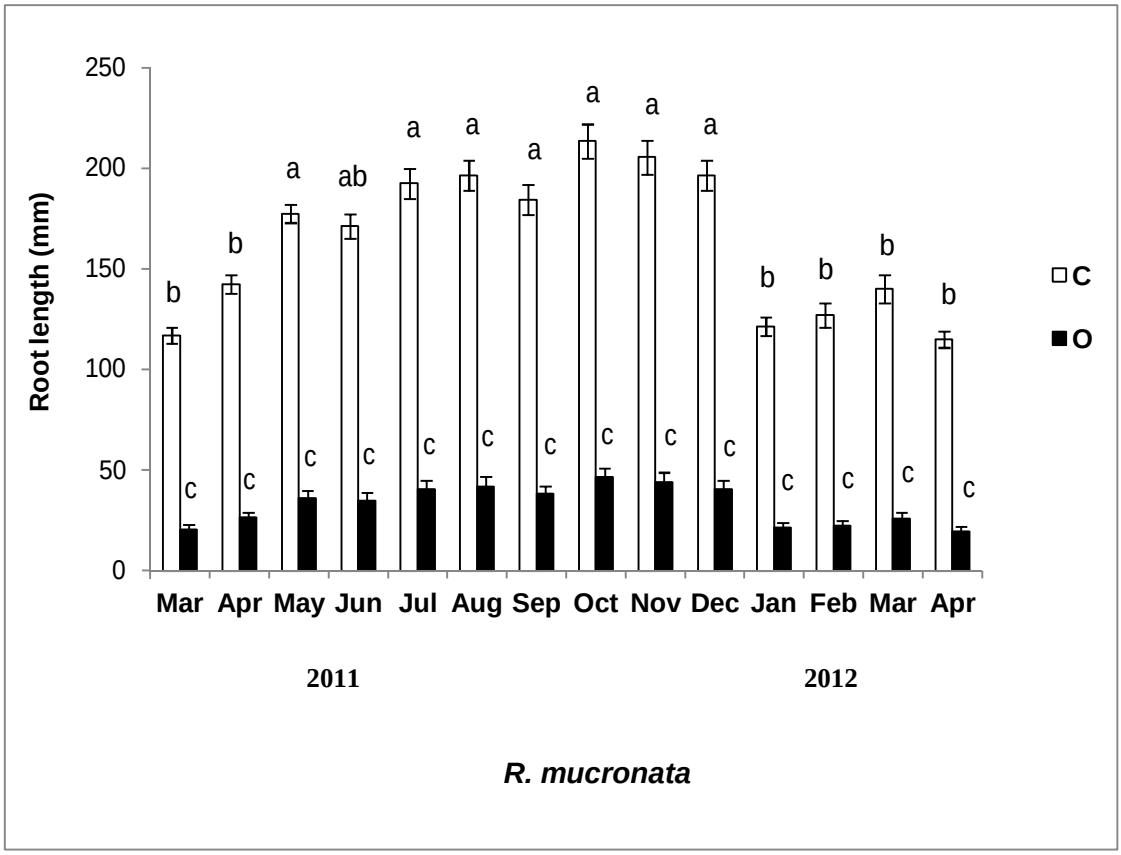

Fig. 3.34 Effects of oiling on root length in *R. mucronata*. Measurements were taken weekly for 409 days, beginning 17 days after treatment, C = control, O = sediment oiled. Means ± standard error are given, n = 2. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

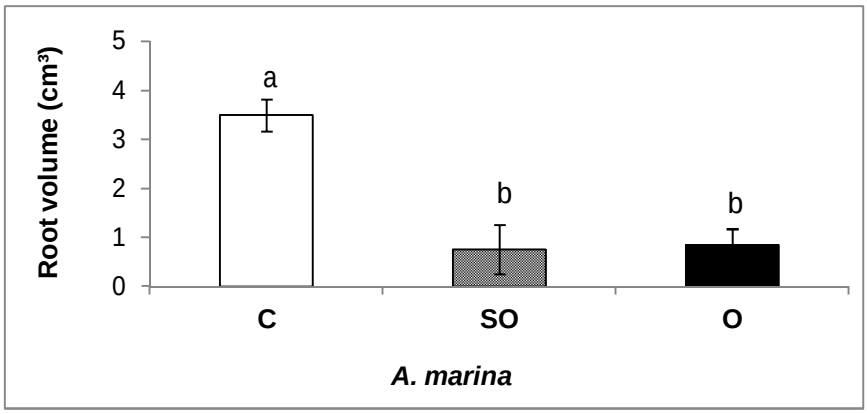

Fig. 3.35 Effects of oiling on root volume in *A. marina*. Measurements were taken after 409 days, C = control, SO = sediment oiled, O = propagule oiled. Means ± standard error are given, n = 3. Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

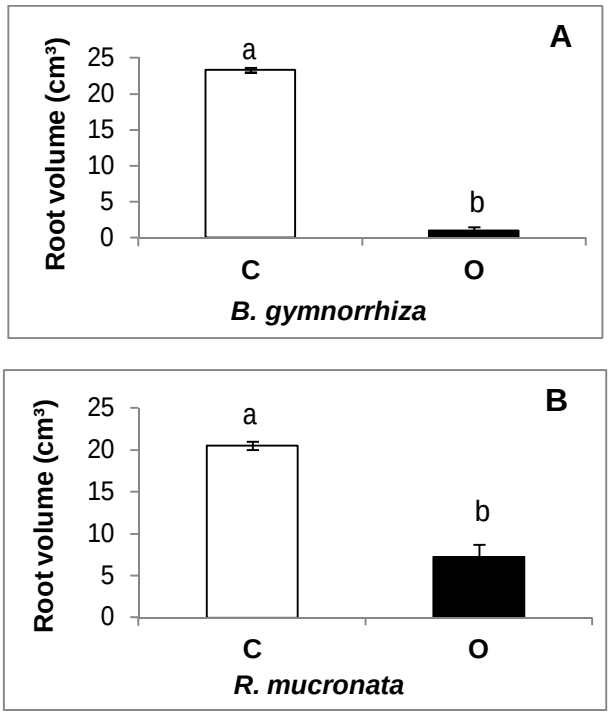

Fig. 3.36 Effects of oiling on root volume in *B. gymnorrhiza* (A) and *R. mucronata* (B). Measurements were taken after 409 days, C = control, O = sediment oiled. Means ± standard error are given, n = 3 (*B. gymnorrhiza*) and n = 2 (*R. mucronata*). Bars with different letters are significantly different at P ≤ 0.05 using Tukey-Kramer multiple comparisons test.

Fig. 3.37 Roots of *A. marina* in the sediment oiled (left) and control (right) treatments from the rhizotrons after 14 months. Note the dead propagule in the oiled treatment (arrow).

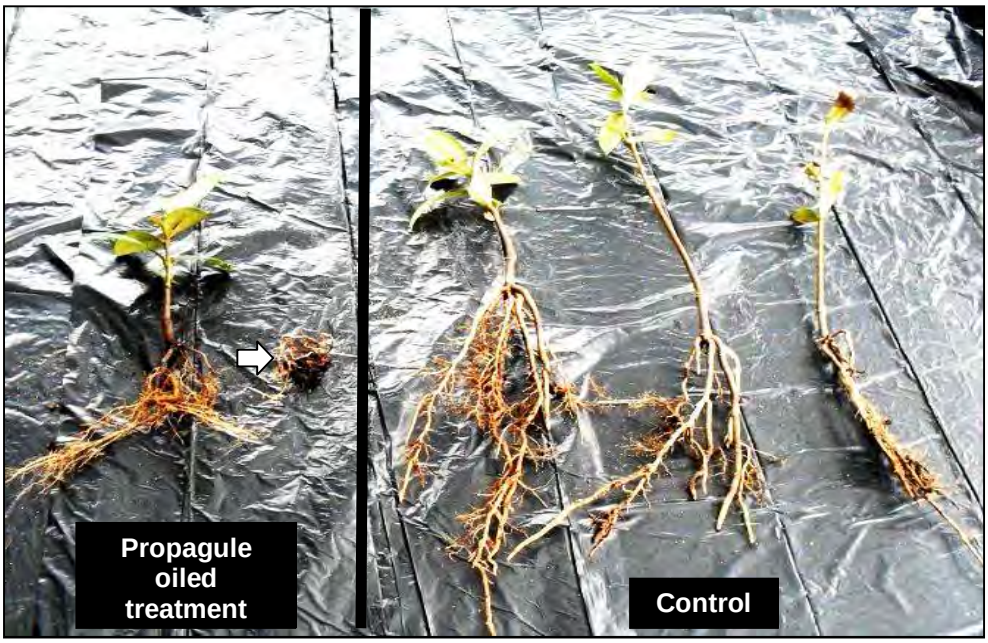

Fig. 3.38 Roots of *A. marina* in the propagule oiled (left) and control (right) treatments from the rhizotrons after 14 months. Note the dead propagule in the oiled treatment (arrow).

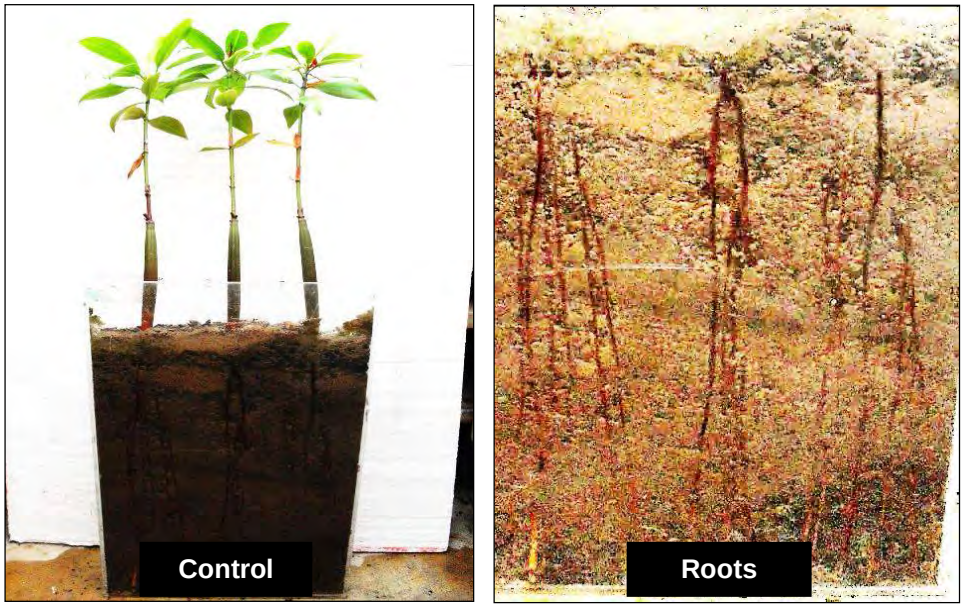

Fig. 3.39 Seedlings of *B. gymnorrhiza* in control rhizotron after 12 months. Note the magnified picture on the right showing root growth.

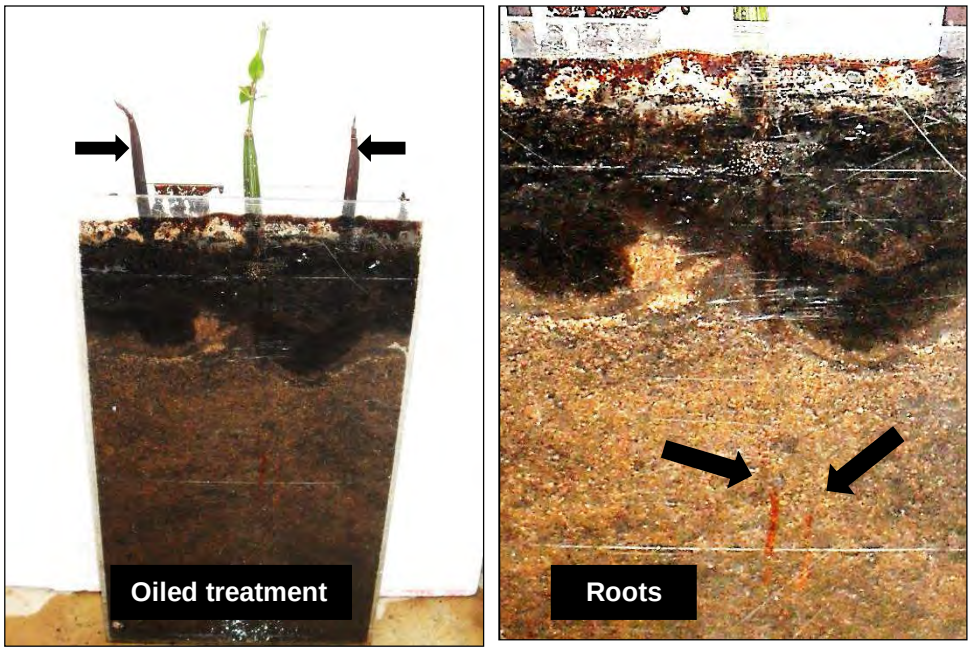

Fig. 3.40 Seedlings of *B. gymnorrhiza* in the oiled treatment in the rhizotron after 12 months. Note the two dead propagules on either side of the living plant on the left (arrows). Note the magnified picture on the right showing roots of the living plant (arrows).

Fig. 3.41 Roots of *B. gymnorrhiza* in the control (left) and oiled (right) treatments from the rhizotrons after 14 months. Note the long, thick healthy roots in the control.

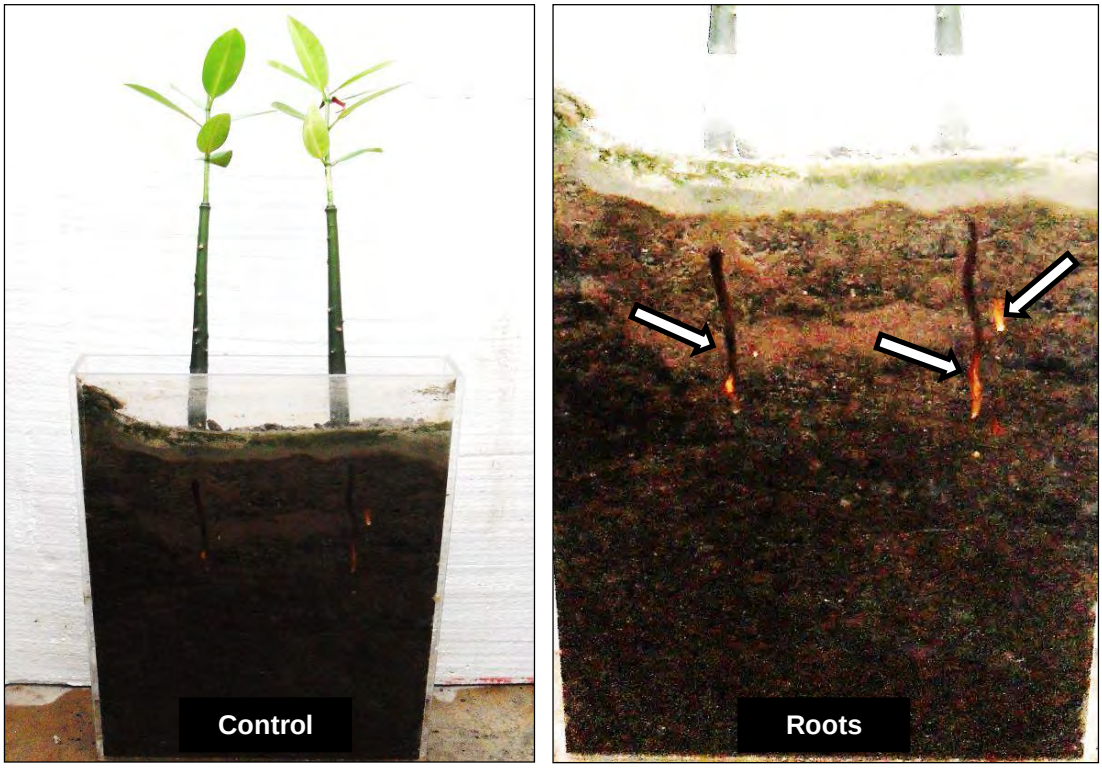

Fig. 3.42 Seedlings of *R. mucronata* in the control rhizotron after 12 months. Note the magnified picture on the right showing roots (arrows).

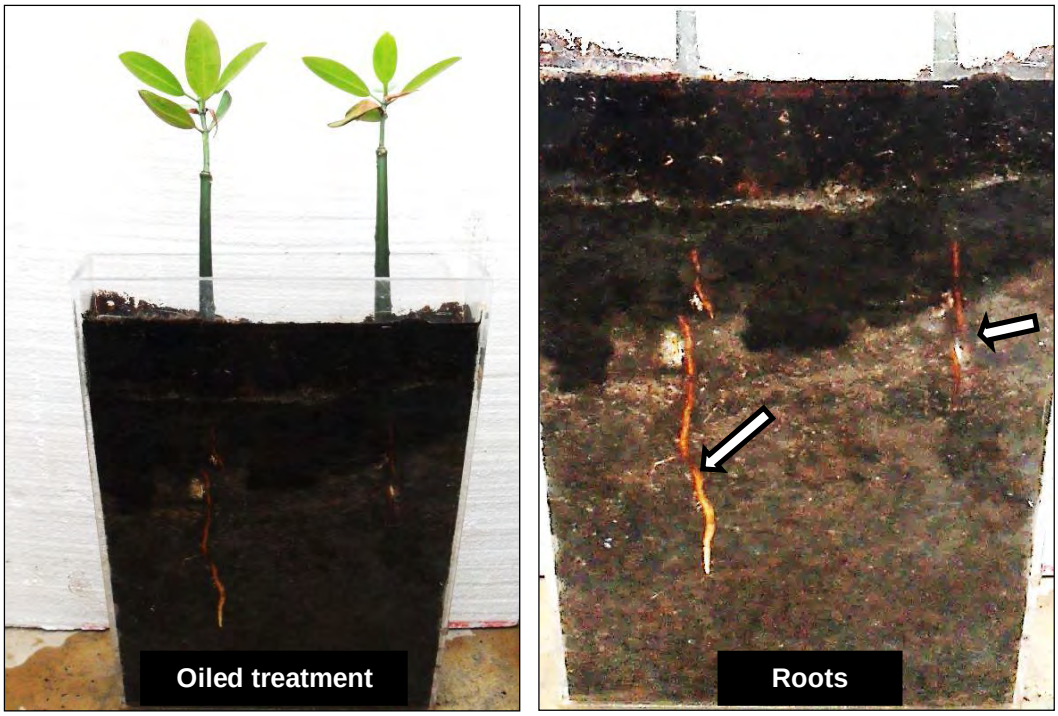

Fig. 3.43 Seedlings of *R. mucronata* in the oiled treatment in the rhizotron after 12 months. Note the magnified picture on the right showing roots (arrows).

Fig. 3.44 Roots of *R. mucronata* in the oiled (left) and control (right) treatments from the rhizotrons after 14 months. Note the thicker roots in the control.

3.4 Discussion

Studies on the toxic effects of PAHs on root growth in mangroves are few (Ye and Tam, 2007; Zhang *et al*., 2007a). Growth of mangrove propagules in rhizotrons provided a novel approach to determine the effects of oil on roots. In this study, oiling reduced root growth and altered morphology in *A. marina*, *B. gymnorrhiza* and *R. mucronata*.

In propagule oiled treatments, roots of *A. marina* and *B. gymnorrhiza* were broader in width and shorter in length than those in the control. Coarse roots had greater diameter and SRV and reduced length. This is the first study to show that increased root

diameter and reduced root length in mangroves is an adaptive response to sub-lethal oil contamination.

An increase in root diameter could be attributed to reduced soil permeability caused by PAHs, which was reported previously (Davies and Bacon, 2003). Oil has been reported to increase mechanical resistance of sediments (Bengough, 2003). Previous studies demonstrated that coarser roots exert greater force on sediments, for soil penetration (Fitter, 1996; Muthukumar *et al*., 2003).

Increased root diameter for greater soil penetration and nutrient absorption has been described previously (Fitter, 1994; Blouin *et al*., 2007). Previous studies showed that nutrient deficiency in soil, as a result of PAHs, can change root structure and produce roots with larger diameter (Zhang *et al*., 2003; Devinny *et al*., 2005). This study supports work by Merkl *et al*. (2005) on *Brachiaria brizantha* Hochst. ex A. Rich. Stapf (Poaceae) and *Cyperus aggregatus* (Willd.) Endl. (Cyperaceae) which reported increased root diameter and SRV but reduced root length in oil contaminated sediment.

In this study, sediment oiling reduced root length, volume, biomass, SRL and SRV in all species compared to the control. Oiling reduced root length in previous studies on grasses (Kechavarzi *et al*., 2007) and *Mimosa pilulifera* Benth. (Leguminosae) (Inckot *et al*., 2011). A decrease in root length reduces the surface area for absorption of nutrients and water (Eissenstat, 1991; Blouin *et al*., 2007), and leads to slower growth (Bengough, 2003; Reynoso-Cuevas *et al*., 2008). Reduced root length, volume, biomass, SRL and SRV in oiled treatments in all species suggests inefficient nutrient uptake (Merkl *et al*., 2005; Corrêa de Souza *et al*., 2013).

In all species, sediment oiling reduced the number and growth rate of roots as a result of mortality caused by PAHs (Getter *et al*., 1985; Tam *et al*., 2005). PAHs that penetrate root cell walls (Ke *et al*., 2011a); accumulate within cells (Gao and Zhu, 2004; Jiao *et al*., 2007) and damage tissues (Watts *et al*., 2006).

In oiled treatments, the roots of all species were flaccid and dark brown in colour. Dead and damaged roots appeared black in *A. marina* (Figs. 3.37 and 3.38), probably due to oil adherence and penetration (Smith *et al*., 2006). In the control, live roots were whitish or lighter in colour, turgid and structurally intact, as described before (McKee, 2001). Previous studies demonstrated that lubricating oil damaged fine roots of *A. marina* and

A. corniculatum (Ye and Tam, 2007). PAHs also disrupted cellular lipid membranes in the conducting tissue of roots (Zhang *et al*., 2007a).

In the propagule oiled treatment, *R. mucronata* produced numerous lateral roots about 5–7 cm beneath the soil surface (Fig. 3.14). These were similar in length and diameter but lacked the vertical extension and support of long, thick anchoring primary roots (Chapman, 1975; Blouin *et al*., 2007). Oiled seedlings did not grow upright as those in the control because they lacked primary root support (Figs. 3.14 and 3.15). Oil probably caused mortality of thicker primary roots, as described in previous studies (Ke *et al*., 2003; Tam *et al*., 2005), while the numerous lateral roots were probably oil-induced. Physiological stress can trigger lateral root growth in *Rhizophora* sp. (Sánchez, 2005) which is supported in this study.

Oiling increased the root/shoot ratio in seedlings of *R. mucronata* probably due to the production of numerous lateral roots to increase nutrient absorption. Previous studies found that PAHs decreased sediment permeability and reduced nutrient and water uptake (Suprayogi and Murray, 1999; Pereira *et al*., 2002). When nutrients are limited, plants allocate more biomass to roots (McKee, 1995; Sherman *et al*., 2003; Barton and Montagu, 2006). In addition, seedlings did not produce leaves and shoot growth was retarded contributing to increased root/shoot ratio, an adaptive response to oiling.

In the sediment oiled treatment, the root/shoot ratio and biomass were reduced in all species. Oil probably smothered roots and caused mortality. Previous studies reported reduced root biomass in sediment oiled treatments in grasses (Kechavarzi *et al*., 2007; Inckot *et al*., 2011).

Plant mortality occurred in completely oiled propagules of *A. marina*. Oil probably penetrated the propagule and developing embryo resulting in mortality. PAHs negatively affected the establishment of propagules in previous studies (Huang *et al*., 2005; Zhang *et al*., 2007a). Complete oil coating inhibited the establishment and growth of seedlings. Establishment and growth of seedlings are inhibited to a greater degree as oil coverage increases (Proffitt and Devlin, 1998). Oiling reduced shoot length and biomass of seedlings, the stage that is most sensitive to PAHs (Maila and Cloete, 2005).

Internodes of oiled seedlings were shorter and thicker than those of the control. In nature, internode growth follows a uniform pattern that is correlated with regular and annual seasonal changes (Duarte *et al*., 1999). However, oil adversely affected internode growth. Sediment oiling reduced the length of the first - third internode in all species, thereby altering growth patterns.

In the propagule oiled treatments, seedlings of *A. marina* and *B. gymnorrhiza* were taller in the first three months compared to those in the control. This shows that PAHs stimulated the growth of seedlings at low dose, similar to other studies (Lin and Mendelssohn, 1996; Lin *et al*., 2002). Suprayogi and Murray (1999) found that oil increased the concentration of nutrients in the leaves of mangroves thus serving as a fertiliser at low doses.

Sediment oiling reduced growth and caused mortality in seedlings of *A. marina* and *B. gymnorrhiza* similar to other studies (Proffitt *et al*., 1995; Ke *et al*., 2003). This was probably due to the higher oil dosage.

Sediment oiling reduced leaf area in all species. Leaf expansion is critical to plant growth as it determines the amount of photosynthetic material required for carbon fixation (Goodman *et al*., 2010). A reduction in leaf area is an indication of physiological stress in mangroves (Flores-de-Santiago *et al*., 2013) induced by PAHs (Zhang *et al*., 2007a; Ke *et al*., 2011a).

Oiling reduced number of leaves and chlorophyll content in all species. Decreases in leaf chlorophyll content (Blackburn, 2007; Liu *et al*., 2009) and photosynthesis (Naidoo *et al*., 2010) are indicative of oil induced plant stress. PAHs are known to disrupt the photosynthetic apparatus of chloroplasts (Naidoo *et al*., 2010; Yin *et al*., 2011) and break down chlorophyll (Huang *et al*., 1996).

In propagule oiled treatments, root growth rate, volume, biomass, leaf area, number of leaves and chlorophyll content were reduced in *A. marina* and *R. mucronata* but not in *B. gymnorrhiza*. In addition, plant mortality occurred in *A. marina* and *R. mucronata* in propagule oiled treatments. Seedlings of *A. marina* and *R. mucronata* were less tolerant of oil than *B. gymnorrhiza*. Oil appeared to have penetrated the propagules of *R. mucronata* and *A. marina* more easily than those of *B. gymnorrhiza*. The higher

tolerance of *B. gymnorrhiza* is probably due to the suberized root epidermal cells which bind PAHs, minimizing the amount of oil entering the plant (Ke *et al*., 2003).

Plant mortality occurred in the sediment oiled treatment in seedlings of *A. marina* and *B. gymnorrhiza* but not in *R. mucronata*. This study suggests that *A. marina* and *B. gymnorrhiza* are intolerant to sediment oiling at high doses. The propagules of *R. mucronata* had a greater dry mass than those of *B. gymnorrhiza*. Propagules of *R. mucronata* could be sites of accumulation thus minimizing the amount of oil entering the shoots, accounting for higher tolerance. The roots of *R. mucronata* secrete exudates (Holmer *et al*., 1999) which increases microbial activity around the roots thereby enabling PAHs to be degraded in the soil (Moreira *et al*., 2011) contributing to greater oil tolerance. Roots of *A. marina* have larger air spaces between aerenchyma cells and thinner epidermis and hypodermis compared to the other two species (Pi *et al*., 2009) allowing greater penetration of oil into cells and increased toxicity.

This study showed that oil altered patterns of growth and root morphology in the three mangroves. The data indicate that mangroves have the ability to survive sub-lethal or residual oil contamination.

Chapter 4

Effects of PAHs on cell ultrastructure in *A. marina*, *B. gymnorrhiza* and *R. mucronata*

Abstract

In this study, the effects of oil on cell ultrastructure in *A. marina*, *B. gymnorrhiza* and *R. mucronata* were investigated. Healthy one year old seedlings, grown under controlled conditions in a glasshouse, were subjected to control and sediment oiled treatments. Seedlings of all three species were subjected to oiling for three weeks. Roots and leaves were harvested and the concentrations of PAHs determined by gas chromatography / mass spectrometry. In the oiled treatment the total concentration of PAHs was higher in roots than in leaves of all species. In oiled treatments, the highest concentrations of PAHs were detected in roots of *A. marina* (44,045.9 µg/kg) followed by *B. gymnorrhiza* (10,280.4 µg/kg) and *R. mucronata* (6,979.1 µg/kg). In roots of all species in the oiled treatments, PAHs with two (naphthalene, acenaphthene and fluorene), three (phenanthrene and anthracene) and four (pyrene, benzo[a]anthracene and chrysene) rings were detected. Benzo[a]pyrene was the only PAH with five rings in roots of all species in the oiled treatments.

To test for living and dead cells and compare the effects of oil on cell ultrastructure in roots and leaves in the three species, seedlings were subjected to control and sediment oiled treatments for seven days. To test for living and dead cells in root tips, fresh sections were stained with fluorescein diacetate and propidium iodide. In control treatments, fluorescein diacetate exhibited green fluorescence in living root tip cells of all species. In oiled treatments, propidium iodide exhibited red fluorescence in most cells of the root tip in all species, which indicated cell death.

To compare the effects of oil on cell ultrastructure, root and leaf tissue were viewed under the transmission electron microscope. Transmission electron micrographs revealed that oil accumulated in root tips and leaf cells in all species. Anatomical changes induced by oil included, disorganization of cells in the root cap, epidermis, meristem and conducting tissue. Oil also induced loss of cell contents and fragmentation of organelles such as the nucleus and mitochondrion. In leaf cells of all species, oil caused dilation, distortion and disintegration of chloroplasts. This study

showed that oil accumulated in root tips and leaf tissue of all species and led to cell disorganisation.

Keywords:

Cell ultrastructure, chloroplasts, fluorescence, leaves, PAHs, roots

4.1 Introduction

Oil spills cause sediment and atmospheric contamination (Kechavarzi *et al*., 2007; Meudec *et al*., 2007). Fuel oil is a complex mixture of hydrocarbons, alkanes, cyclohexanes, asphaltenes, naphthenes, olefins and PAHs (Luz *et al*., 2010). PAHs contain two or more fused benzene rings (Haritash and Kaushik, 2009) and are highly toxic, persistent, organic pollutants (POPs) with carcinogenic and mutagenic properties (Luan *et al*., 2006; Li *et al*., 2010).

Each PAH compound has a different UV absorption spectrum making it easily identifiable (Orecchio, 2007). Currently, more than 200 types of PAHs have been described (Zhang *et al*., 2010). Sixteen PAHs have been identified as priority pollutants by the United States Environmental Protection Agency (Wilson and Jones, 1993; Binet *et al*., 2000).

PAHs are resistant to biodegradation due to their high insolubility and therefore persist in soils (Kottler and Alexander 2001; Taylor and Jones, 2001). The PAHs in fuel oil adsorb onto soil particles and accumulate in sediments (Wang *et al*., 2001; Tolosa *et al*., 2004). They are highly hydrophobic, tightly bound to soil particles and not readily bio-available (Pilon-Smits, 2005). PAHs with two to three rings volatize easily from soil into the atmosphere (Wetzel *et al*., 1997).

PAHs enter plant roots from contact with contaminated soil (Gao and Zhu, 2004; Meudec *et al*., 2006) and are drawn up through the root apoplastically with the transpiration stream via the xylem (Wild *et al*., 2005). They are translocated to the shoots symplastically via diffusion across cellular membranes and through plasmodesmata (Orcutt and Nielsen, 2000). Although PAHs constitute a small part of

oil components, they can be found in high concentrations within plant tissues (Lin *et al*., 2007; Reynoso-Cuevas *et al*., 2008).

Many studies have investigated PAH accumulation in vegetables (Tao *et al*., 2004; Parrish *et al*., 2006; Khan *et al*., 2008) and agricultural crops such as rice (Jiao *et al*., 2007) and maize (Lin *et al*., 2007). Other studies have concentrated on ryegrass (Binet *et al*., 2001) and the salt-marsh grass, *S. alterniflora* (Watts *et al*., 2006). Most studies on oil contamination in mangroves focus on the physical effects of oil that lead to reduced growth and plant mortality (Proffitt *et al*., 1995; Duke *et al*., 1997; Tam *et al*., 2005). Some studies have focussed on the harmful effects on physiological mechanisms like photosynthesis, respiration, transpiration and nutrient absorption (Youssef and Ghanem, 2002; Zhang *et al*., 2007a; Naidoo *et al*., 2010). There are limited studies on the uptake, accumulation, translocation and phytotoxicity of PAHs within plant tissues (Suprayogi and Murray 1999; Hong *et al*., 2009). The uptake of PAHs by roots is dependent on their chemical properties and the plant species (Zhu *et al*., 2007). Stress responses of plants to oil are dependent on both the chemical and physical effects of PAHs (Meudec *et al*., 2006).

Oil in tissues can disrupt cellular lipid membranes in the conducting tissue (Zhang *et al*., 2007a) and damage the xylem vessels of fine roots (Ye and Tam, 2007). However, information on the effects of PAHs on mangrove root and leaf ultrastructure is almost non-existent. Physiological damage is a result of the direct contact of organelles with PAHs (Watts *et al*., 2006; Kang *et al*., 2010). The accumulation of PAHs in plant tissues causes different degrees of ultrastructural damage, depending on the chemical structure and concentration of the compounds (Alkio *et al*., 2005; Liu *et al*., 2009).

4.2 Materials and methods

4.2.1 Growth conditions

Propagules of *A. marina*, *B. gymnorrhiza* and *R. mucronata* were collected and planted in March 2011 as described in Chapter 2.2.1. Pots were maintained in a glasshouse and seedlings allowed to grow for 12 months. The temperature in the glasshouse was the same as in Chapter 2.2.1.

Healthy one year old seedlings of *A. marina*, *B. gymnorrhiza* and *R. mucronata* were subjected to control and oiled treatments for three weeks. In the oiled treatments, 200 ml of oil were carefully poured onto the soil surface. There were 13 replications for the control and 10 per species for the oiled treatment.

4.2.2 Determination of PAHs

After three weeks, the leaves and roots of four replicates were harvested and fresh mass determined by weighing on a Toledo scale. Leaves and roots from replicates where pooled to obtain the weight requirement (10 g) for determination of PAHs. The properties of the bunker fuel oil used in this study are indicated in Table 2.1, Chapter 2.

PAHs were extracted from 10 g of leaf and root material that were freeze dried and ground into a powder. The powder was combined with acetonitrile and buffered with magnesium sulphate, sodium chloride and citrate salts (pH 5 - 5.5) and then centrifuged. Formic acid was added to the mixture. The resulting extracts were analysed by gas chromatography on an HP-5890 gas chromatograph (Hewlett Packard, USA) equipped with an HP-DB 5MS column (60 m x 0.25 mm x 0.25 µm thick film). The column was coupled to a mass spectrometer detector (HP 5972) operating in a selected ion monitoring (SIM) mode. Deuterated pyrene and benzo(a)pyrene were used as internal standards. The temperature was set at 300°C. Fifteen PAHs were identified based on the EPA list of priority PAHs (United States Environmental Protection Agency, 2008). Benzo[k]fluoranthene and benzo[b]fluoranthene were grouped as one compound - benzo[k+b]fluoranthene.

4.2.3 Staining with fluorescein diacetate (FDA) and propidium iodide (PI)

After seven days, fresh roots from the control and oiled treatments were harvested. Fresh root tips were placed between small pieces of polystyrene and sectioned with a Vibratome 1000 (Lancer, U.S.A). Sections were approximately 300 µm thick.

The plant cell viability assay kit containing the stains, PI and FDA, was used for the staining of root cells (Sigma, USA). The kit components were thawed in a 30 - 37°C water bath. After thawing, a precipitate formed in the PI solution which was re-dissolved by vortexing vigorously. Each stain (1 µL) was diluted in 99 µL of deionised

molecular water (pH 7.4) in Eppendorf tubes using a 10 µL pipette. The solutions were vortexed to mix. Sections were stained for 5 min with PI and for 20 min with FDA on microscope glass slides.

Slides were examined with a Nikon Eclipse 80i, utilizing Nikon filter sets, FITC (fluorescein isothiocyanate) (510 to 560 nm) and TRITC (tetramethylrhodamine isothiocyanate) (475 to 490 nm). Each section was individually stained. Sections stained with FDA were viewed with the FITC band pass filter, and those stained with PI were viewed with the TRITC band pass filter. Photomicrography was performed with the Nikon imaging software (NIS-D) with a Nikon sight DS-Fli digital camera.

4.2.4 Preparation of material for light microscopy

After seven days, the root tips of three replicates of all species were observed with the compound light microscope to view the structural organisation of cells. Fresh root tips of all species were harvested (about 5 mm in length) by cutting with a sterilised blade. Samples were fixed in 10% formalin acetic acid (FAA) (Jensen, 1962) within 15 min of procurement. Tissues were fixed for at least 24 hours followed by storage in 70% ethanol. Tissues were processed through a tertiary butanol series in which each sample was in a butanol solution for at least 24 hours. Tissues were placed in two changes of paraffin, embedded in paraffin wax and sectioned (5 to 7 µm) using a LKB Bromma 2218 Historange microtome (LKB, Bromma, Sweden). Sections were placed on microscope glass slides and stained with safranin and methylene *blue*. Photomicrography was performed with the Nikon imaging software (NIS-D) with a Nikon sight DS-Fli digital camera.

4.2.5 Preparation of material for the transmission electron microscope (TEM)

After seven days, fresh leaves and roots of three replicates from all species were harvested. Small segments (1 mm^2) of material were taken from root tips and flag leaves i.e. the youngest, fully expanded leaves. The material was fixed under vacuum for 24 hours in 2.5% glutaraldehyde (4°C) and then washed three times for 5 min each in phosphate buffer (pH 7.2). Samples were post fixed in 0.5% osmium tetroxide (OsO_4) for 1 hour in a dark cupboard at room temperature and washed three times for 5 min each in phosphate buffer (pH 7.2). The material was then dehydrated through a

graded series of acetone (i.e. two times for 5 min each in 30%, 50%, 75% and two changes of 10 min each in 100% acetone). The samples were left for 4 hours in equal parts of Spurr's (1969) low viscosity resin and 100% acetone. The material was then embedded in 100% resin for 24 hours. The specimens were orientated in a mould with fresh 100% resin and placed in an oven to polymerize for 8 hours at 70°C.

Sectioning was carried out using a Reichert Ultracut E microtome (Leica, Germany). Ultrastructural analysis was undertaken using ultrathin sections (60 to 100 nm) collected on copper grids. Sections were initially post stained with 2% aqueous uranyl acetate for 10 min and thereafter with Reynolds' (1963) lead citrate for 10 min. Grids were placed in a petri dish lined with filter paper and surrounded by sodium hydroxide (NaOH) pellets for 5 min. Sections were viewed with a Jeol 1010 transmission electron microscope operating at 100kV and images captured using the Megaview 3 soft imaging system (SIS).

4.3 Results

4.3.1 Determination of PAHs

Fifteen PAH compounds detected in root and leaf samples of *A. marina*, *B. gymnorrhiza* and *R. mucronata* are shown in Tables 4.1 – 4.3. In oiled treatments of all species, the total concentration of PAHs was higher in roots (6,979.1 – 44,045.9 µg/kg) than in leaves (64.5 – 1,642.3 µg/kg).

In oiled treatments, the concentrations of all 15 PAHs in roots and leaves were higher in *A. marina* than in *B. gymnorrhiza* or *R. mucronata* (Tables 4.1 – 4.3).

Concentrations of PAHs in roots

In the roots of oiled treatments, the total concentration of PAHs was highest in *A. marina* (44045.9 µg/kg) followed by *B. gymnorrhiza* (10280.4 µg/kg) and *R. mucronata* (6979.1 µg/kg) (Table 4.4).

In the roots of all oiled treatments, the more volatile PAHs with two rings, naphthalene, acenaphthene and fluorene were detected but acenaphthylene was undetected (Tables 4.1 – 4.3). Fluorene concentrations in oiled treatments of all species were higher than those of naphthalene and acenaphthene in *A. marina* (3,938.8 µg/kg), *B. gymnorrhiza* (1,183 µg/kg) and *R. mucronata* (725 µg/kg).

In roots of all oiled treatments, volatile PAHs with three rings, phenanthrene and anthracene, but not fluoranthene, were detected (Tables 4.1 – 4.3). The concentration of phenanthrene in oiled treatments of all species was higher than all other PAHs. Phenanthrene concentrations in oiled treatments were 27,044.8 µg/kg in *A. marina* (Table 4.1), 5,632 µg/kg in *B. gymnorrhiza* (Table 4.2) and 3,485 µg/kg in *R. mucronata* (Table 4.3).

In roots of all oiled treatments, the PAHs with four rings (pyrene, benzo[a]anthracene and chrysene) were detected. In *A. marina*, the concentration of chrysene was 2,278.1 µg/kg compared to pyrene (1,508.3 µg/kg) or benzo[a]anthracene (867.6 µg/kg) (Table 4.1). The concentrations of pyrene were higher than other PAHs with four rings, 607 µg/kg in *B. gymnorrhiza* (Table 4.2) and 500 µg/kg in *R. mucronata* (Table 4.3).

In roots of all oiled treatments, benzo[a]pyrene was the only PAH with five rings. Benzo[a]pyrene concentrations were 562.7 µg/kg in *A. marina* (Table 4.1), 78 µg/kg in *B. gymnorrhiza* (Table 4.2) and 315 µg/kg in *R. mucronata* (Table 4.3). In oiled treatments, roots of all species lacked PAHs with six rings (Tables 4.1 – 4.3).

Control roots of all species had very low concentrations of PAHs with two (naphthalene and acenaphthene) and three (phenanthrene) rings (Tables 4.1 – 4.3). In the control, PAHs with four (chrysene) and five (benzo[a]pyrene and benzo[k+b]fluoranthene) rings were detected in roots of *A. marina* in very low concentrations but undetected in *B. gymnorrhiza* and *R. mucronata*.

Concentrations of PAHs in leaves

In the leaves of oiled treatments, the total concentration of PAHs was highest in *A. marina* (1,642.3 µg/kg) followed by *R. mucronata* (71 µg/kg) and *B. gymnorrhiza* (64.5 µg/kg) (Table 4.4).

In the leaves of all oiled treatments, PAHs with two rings (naphthalene, acenaphthene and fluorene) were detected. In *A. marina* the concentration of acenaphthene (136.2 µg/kg) was considerably higher than naphthalene (26.3 µg/kg) or fluorene (88.3 µg/kg) (Table 4.1). In *B. gymnorrhiza*, the concentration of acenaphthene (22.4 µg/kg) was higher than naphthalene (21.4 µg/kg) or fluorene (11.8 µg/kg). In *R. mucronata* the concentration of naphthalene (16.5 µg/kg) was higher than acenaphthene (3.5 µg/kg) or fluorene (5.1 µg/kg). In oiled treatments, the concentration of acenaphthylene (two rings) was 7.5 µg/kg in *A. marina* but undetected in *B. gymnorrhiza* and *R. mucronata.*

In leaves of oiled treatments, the concentration of phenanthrene (three rings) was higher in *A. marina* (451.3 µg/kg) and *R. mucronata* (36.5 µg/kg) than all other PAHs. Anthracene (three rings) was detected in leaves of *A. marina* (173.8 µg/kg) but undetected in *B. gymnorrhiza* or *R. mucronata*. In oiled treatments, the concentrations of fluoranthene (three rings) in leaves of *A. marina* and *R. mucronata* were 58.5 µg/kg and 7.7 µg/kg respectively but undetected in *B. gymnorrhiza*.

In oiled treatments, the concentration of pyrene (four rings) in leaves was 112.7 µg/kg in *A. marina*, and 1.8 µg/kg in *R. mucronata* but undetected in *B. gymnorrhiza* (Tables 4.1 – 4.3). In oiled treatments, benzo[a]anthracene (122.4 µg/kg) and chrysene (193.5 µg/kg) (four rings) were detected in leaves of *A. marina* but undetected in the other two species.

In leaves of oiled treatments, the PAHs with five rings, benzo[k+b]fluoranthene (180.4 µg/kg) and benzo[a]pyrene (91.4 µg/kg) were detected in *A. marina* but undetected in *B. gymnorrhiza* and *R. mucronata* (Table 4.1) while PAHs with six rings were undetected.

Control leaves of all species had negligible concentrations of PAHs with two (naphthalene and acenaphthene) and three (phenanthrene) rings (Tables 4.1 – 4.3).

Table 4.1 Concentrations of 15 PAHs (µg/kg) in leaves and roots of *A. marina* in control (C) and oiled (O) treatments. *asterisk indicates undetected in sample.

		Leaves		Roots	
# rings	PAH	C	O	C	O
2	Naphthalene	*	26.3	35.7	238.2
2	Acenaphthene	21.1	136.2	228.4	2,933.7
2	Acenaphthylene	*	7.5	5.4	*
2	Fluorene	21.1	88.3	191.1	3,938.8
3	Phenanthrene	50.3	451.3	434.9	27,044.8
3	Anthracene	*	173.8	82.9	4,673.8
3	Fluoranthene	5.2	58.5	*	*
4	Pyrene	*	112.7	*	1,508.3
4	Benzo[a]anthracene	*	122.4	*	867.6
4	Chrysene	*	193.5	6.1	2,278.1
5	Benzo[k+b]fluoranthene	*	180.4	39.3	*
5	Benzo[a]pyrene	*	91.4	21.5	562.7
5	Dibenz[a,h]anthracene	*	*	*	*
6	Benzo[g,h,i]perylene	*	*	*	*
6	Indeno[1,2,3-cd]pyrene	*	*	*	*

Table 4.2 Concentrations of 15 PAHs (µg/kg) in leaves and roots of *B. gymnorrhiza* in control (C) and oiled (O) treatments. *asterisk indicates undetected in sample.

		Leaves		Roots	
# rings	PAH	C	O	C	O
2	Naphthalene	3.3	21.4	4.5	79
2	Acenaphthene	*	22.4	6.6	1,306
2	Acenaphthylene	*	*	*	*
2	Fluorene	*	11.8	*	1,183
3	Phenanthrene	2.1	8.9	7.3	5,632
3	Anthracene	*	*	*	839
3	Fluoranthene	*	*	*	*
4	Pyrene	*	*	*	607
4	Benzo[a]anthracene	*	*	*	123
4	Chrysene	*	*	*	433
5	Benzo[k+b]fluoranthene	*	*	*	*
5	Benzo[a]pyrene	*	*	*	78
5	Dibenz[a,h]anthracene	*	*	*	*
6	Benzo[g,h,i]perylene	*	*	*	*
6	Indeno[1,2,3-cd]pyrene	*	*	*	*

Table 4.3 Concentrations of 15 PAHs (µg/kg) in leaves and roots of *R. mucronata* in control (C) and oiled (O) treatments. *asterisk indicates undetected in sample.

		Leaves		Roots	
# rings	PAH	C	O	C	O
2	Naphthalene	17.8	16.5	3.0	156
2	Acenaphthene	6.3	3.5	6.0	717
2	Acenaphthylene	*	*	*	*
2	Fluorene	*	5.1	*	725
3	Phenanthrene	27.7	36.5	5.4	3,485
3	Anthracene	*	*	*	460
3	Fluoranthene	6.9	7.7	*	*
4	Pyrene	*	1.8	*	500
4	Benzo[a]anthracene	*	*	*	138
4	Chrysene	*	*	*	483
5	Benzo[k+b]fluoranthene	*	*	*	*
5	Benzo[a]pyrene	*	*	*	315
5	Dibenz[a,h]anthracene	*	*	*	*
6	Benzo[g,h,i]perylene	*	*	*	*
6	Indeno[1,2,3-cd]pyrene	*	*	*	*

Table 4.4 Total concentration of 15 PAHs (µg/kg) in roots and leaves of *A. marina, B. gymnorrhiza* and *R. mucronata* in control (C) and oiled (O) treatments.

	Leaves		Roots	
	C	O	C	O
A. marina	97.6	1,642.3	1,125.2	44,045.9
B. gymnorrhiza	5.4	64.5	18.4	10,280.4
R. mucronata	58.7	71	14.4	6,979.1

4.3.2 Fluorescence of living and dead cells

In the control, fresh sections of root tips stained with FDA, exhibited green fluorescence in living cells of the meristematic and conducting tissue in *A. marina* (Fig. 4.1), *B. gymnorrhiza* (Fig. 4.3A and B) and *R. mucronata* (Fig. 4.5A and B).

In the oiled treatment, fresh sections of root tips stained with PI, exhibited red fluorescence in dead cells of the meristematic and conducting tissue in *A. marina* (Fig. 4.2A and B), *B. gymnorrhiza* (Fig. 4.4) and *R. mucronata* (Fig.4.6A and B).

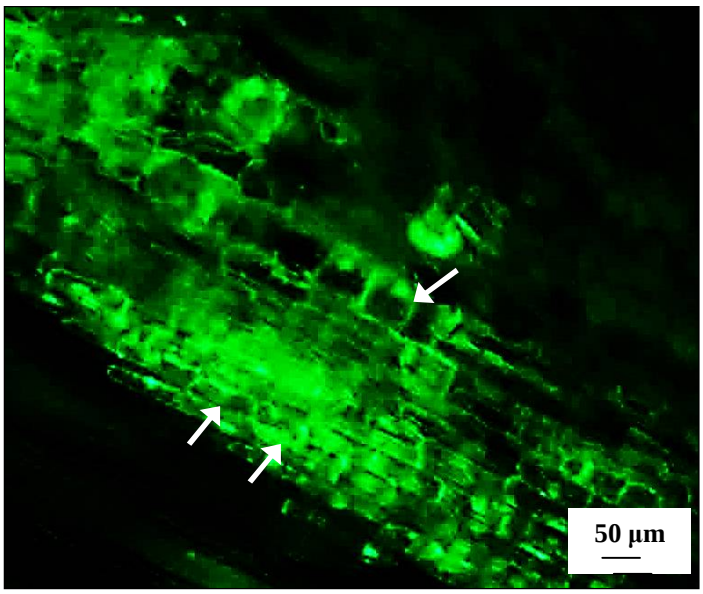

Fig. 4.1 Fluorescence light micrographs of root tips in longitudinal section of *A. marina* from the control. After staining, fluorescein diacetate (indicated by bright green fluorescence) penetrated the cell walls and plasma membranes in cells of meristematic tissue (arrows). Note: green = living.

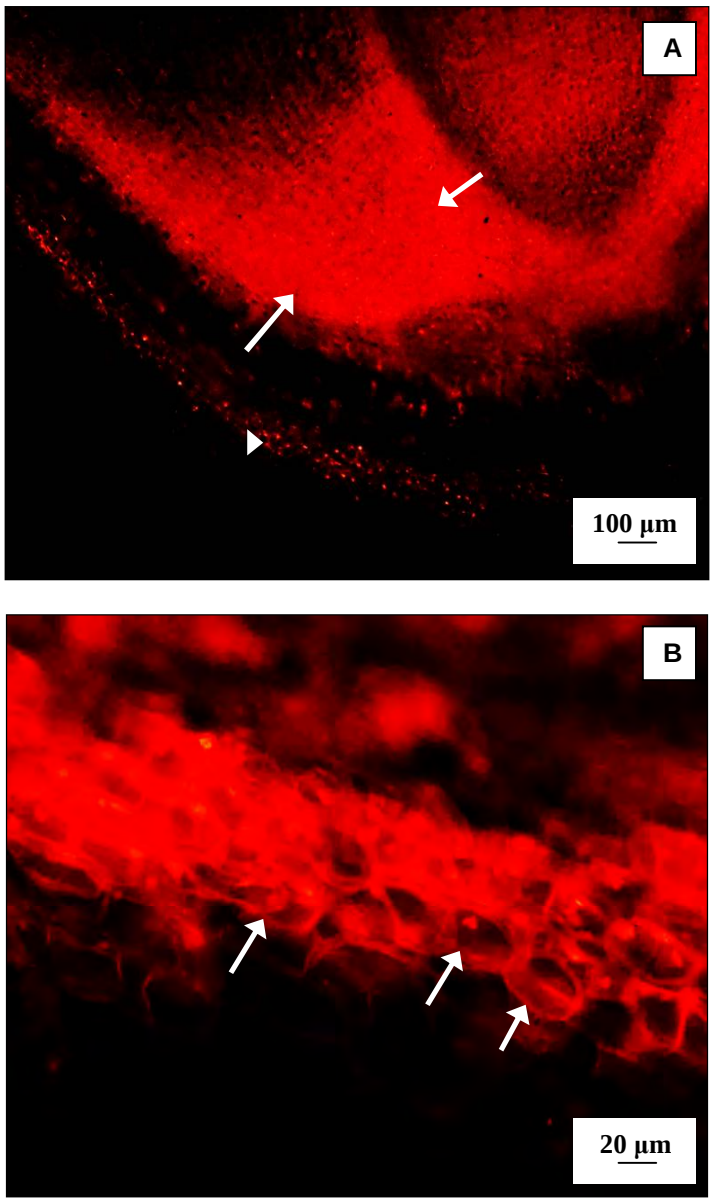

Fig. 4.2 Fluorescence light micrographs of root tips in longitudinal section of *A. marina* from the oiled treatment. (A and B) After staining, propidium iodide (indicated by bright red fluorescence) penetrated the cell walls and plasma membranes in cells of meristematic tissue (arrows). Note: red = dead.

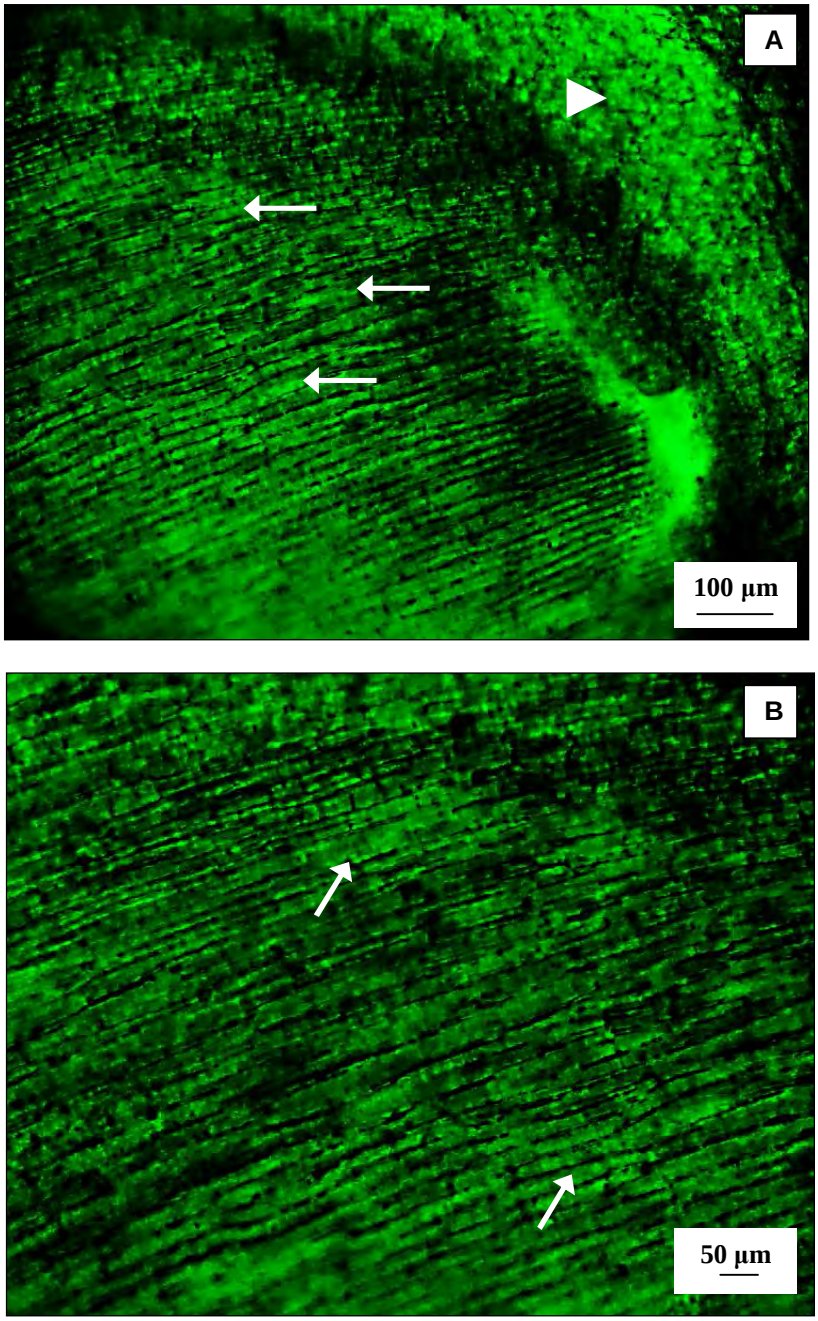

Fig. 4.3 Fluorescence light micrographs of root tips in longitudinal section of *B. gymnorrhiza* in the control. (A) After staining, fluorescein diacetate (indicated by bright green fluorescence) penetrated the cell walls and plasma membranes in cells of root cap (arrowhead) and meristematic tissue (arrow). (B) Cells of meristematic tissue exhibiting bright green fluorescence (arrows). Note: green = living.

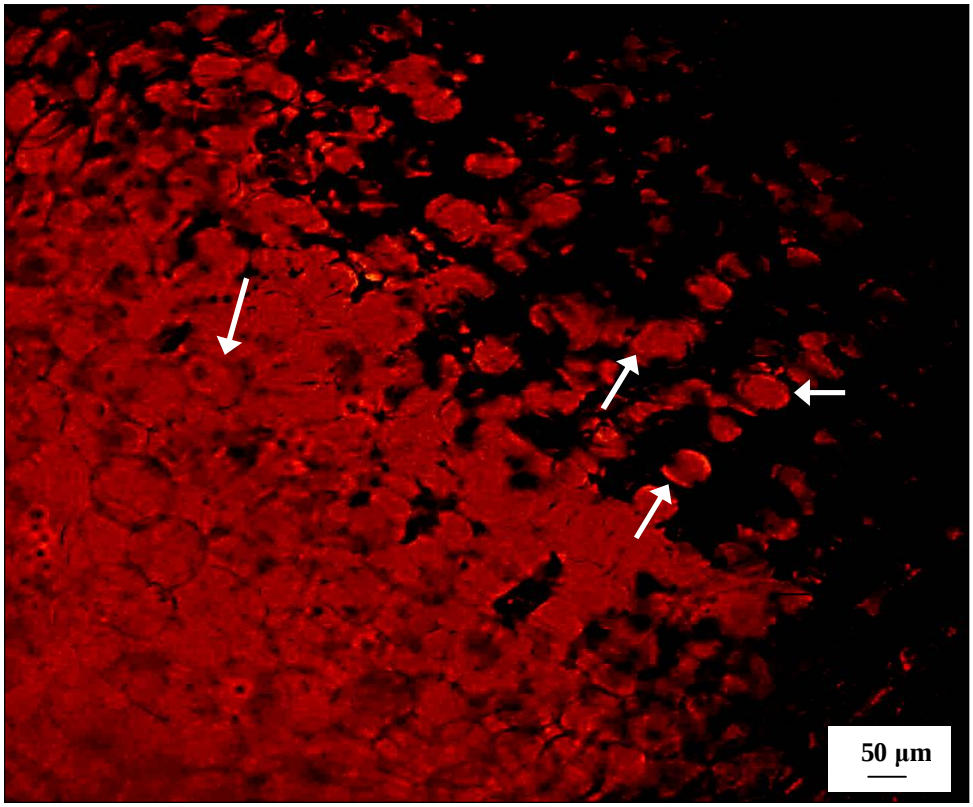

Fig. 4.4 Fluorescence light micrographs of root tips in longitudinal section of *B. gymnorrhiza* in the oiled treatment. After staining, propidium iodide (indicated by bright red fluorescence) penetrated the cell walls and plasma membranes in cells of meristematic tissue (arrows). Note: red = dead.

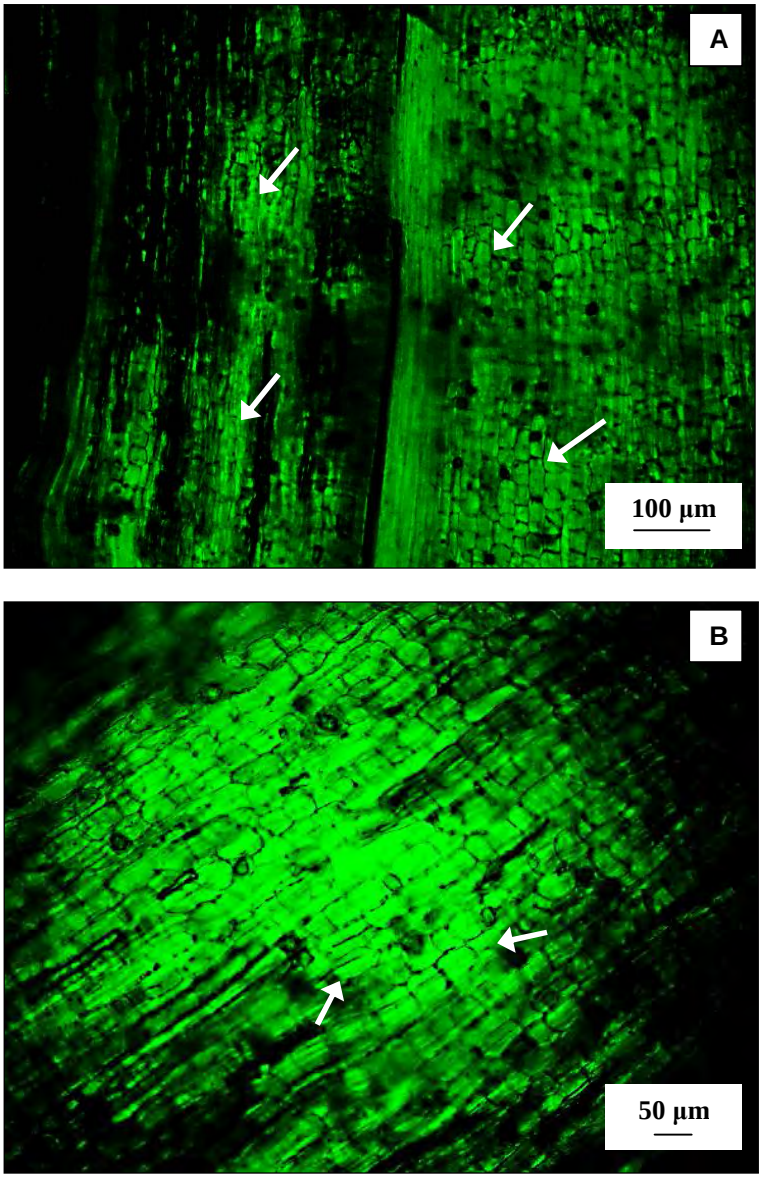

Fig. 4.5 Fluorescence light micrographs of root tips in longitudinal section of *R. mucronata* in the control. (A and B) After staining, fluorescein diacetate (indicated by bright green fluorescence) penetrated the cell walls and plasma membranes in cells of meristematic tissue (arrows). Note: green = living.

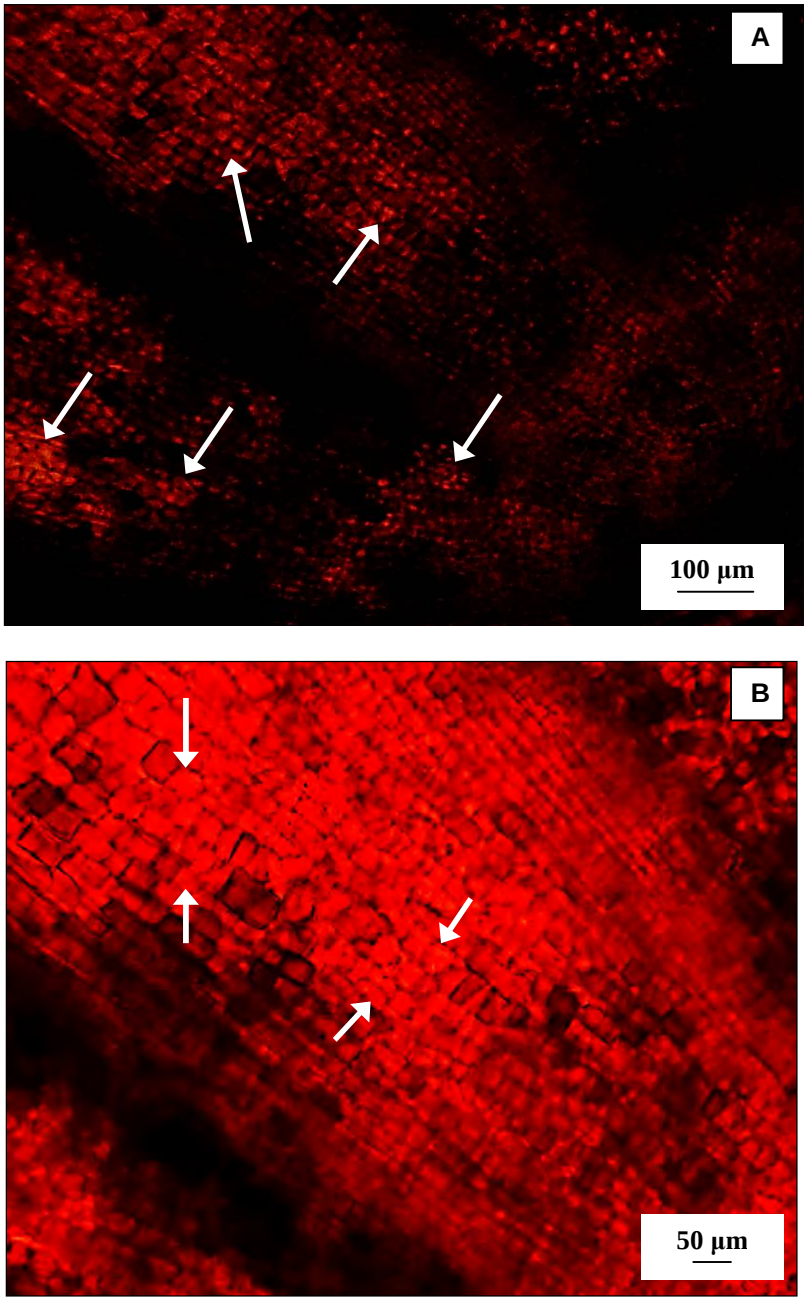

Fig. 4.6 Fluorescence light micrographs of root tips in longitudinal section of *R. mucronata* in the oiled treatment. (A and B) After staining, propidium iodide (indicated by bright red fluorescence) penetrated the cell walls and plasma membranes in cells of meristematic tissue (arrows). Note: red = dead.

4.3.3 Light micrographs of control root tips in longitudinal section

In the control, light micrographs of root tips in longitudinal section of *A. marina* (Figs. 4.7 and 4.8), *B. gymnorrhiza* (Figs. 4.9 and 4.10) and *R. mucronata* (Figs. 4.11 and 4.12), showed the typical cell organization of the meristematic region. The structural organization of the root tip included cells of the root cap and epidermis; then meristem, cortex, xylem and phloem tissue.

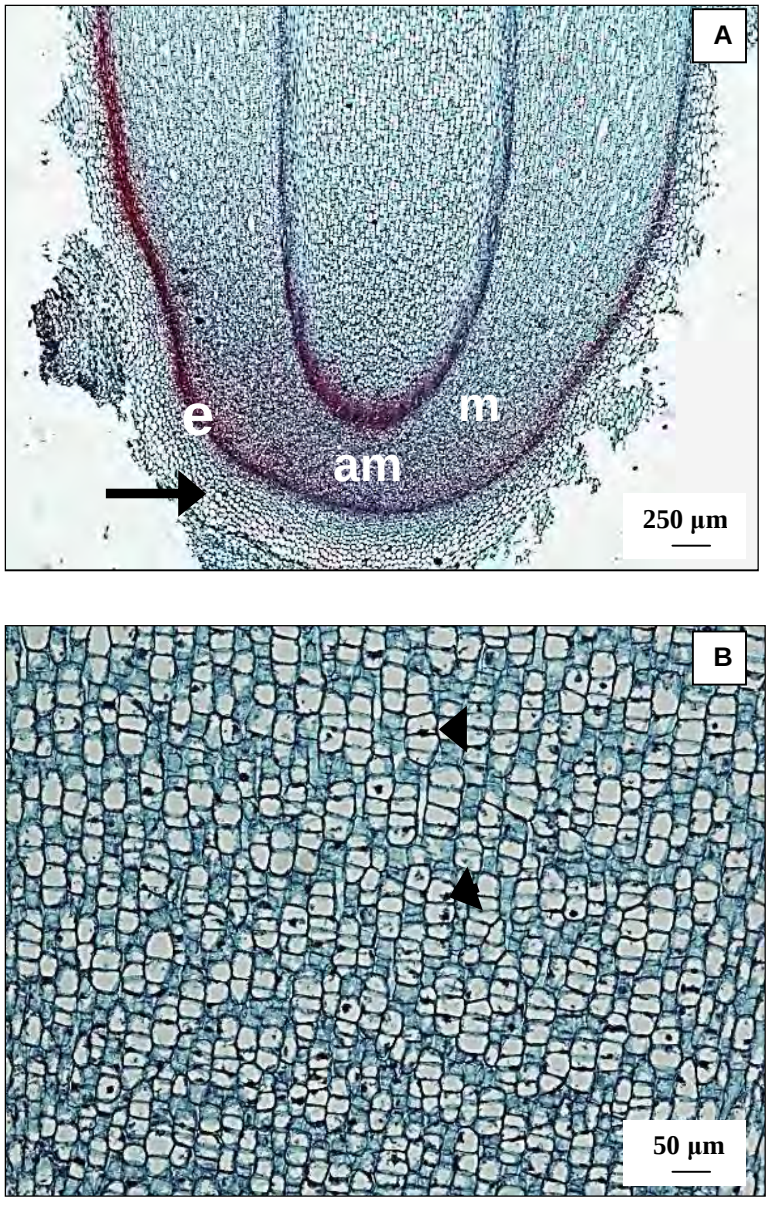

Fig. 4.7 Light micrographs of root tip of *A. marina* in the control treatment. (A) Root tip showing root cap (arrow), epidermis (e), apical meristem (am) and meristematic tissue (m). (B) Meristematic cells exhibiting cell division (arrowheads).

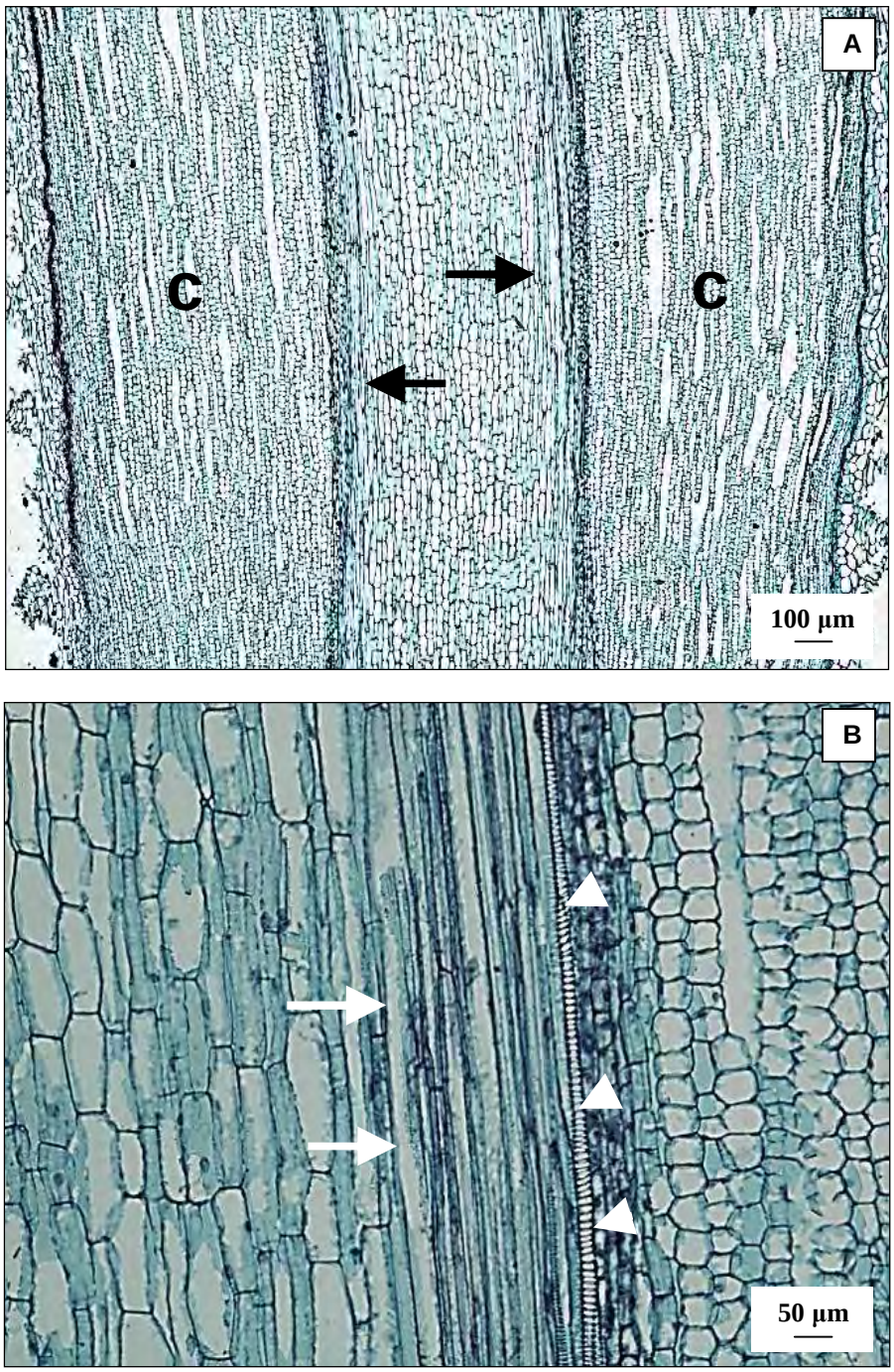

Fig. 4.8 Light micrographs of root tip of *A. marina* in the control treatment. (A) Centre of root primordium showing cortex (c) and conducting tissue (arrows). (B) Conducting tissue showing xylem tracheid (arrowheads) and sieve tube of phloem (arrows).

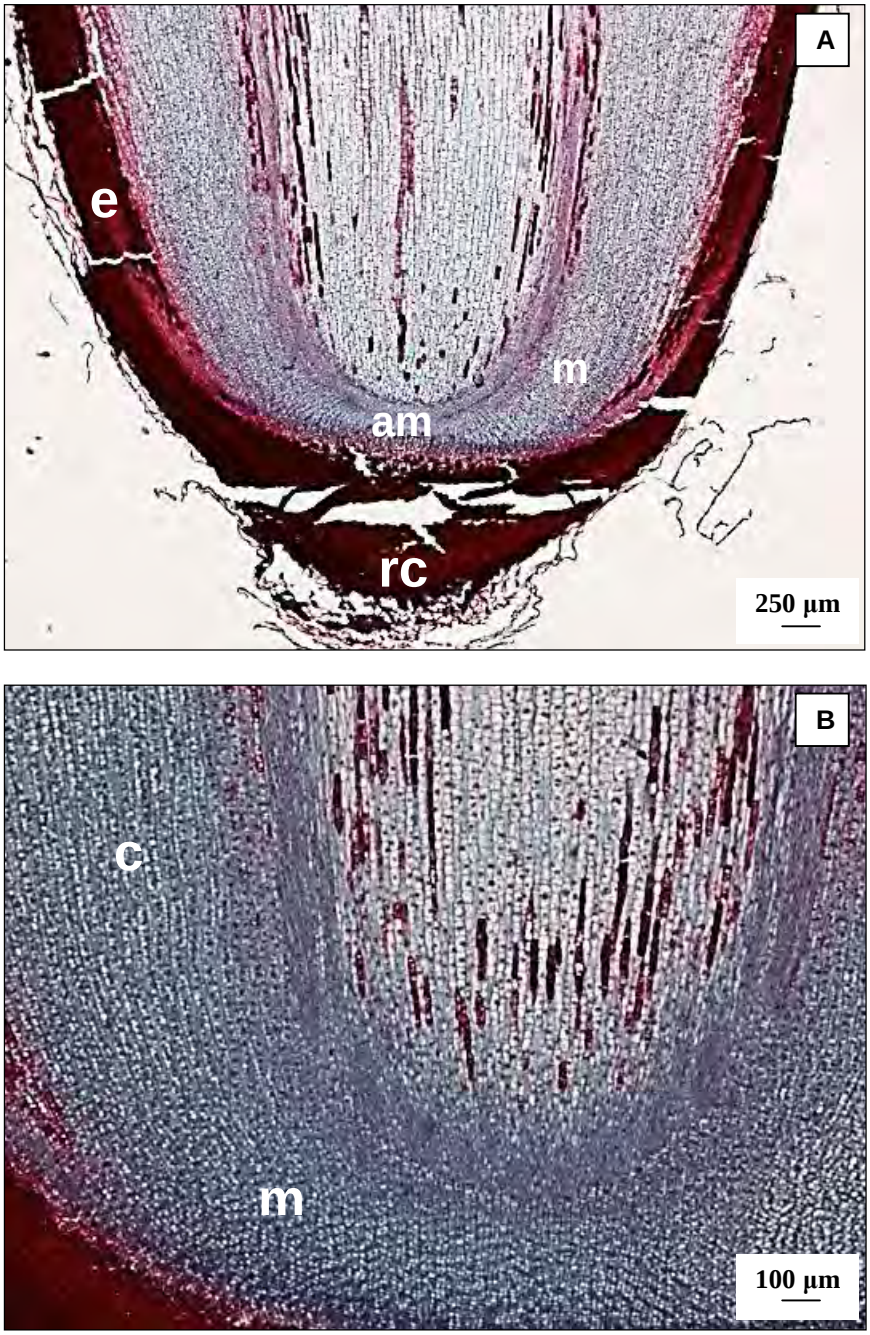

Fig. 4.9 Light micrographs of root tip of *B. gymnorrhiza* in the control treatment. (A) Root tip showing root cap (rc), epidermis (e), apical meristem (am) and meristematic tissue (m). (B) Centre of root primordium showing cortex (c) and meristematic tissue (m).

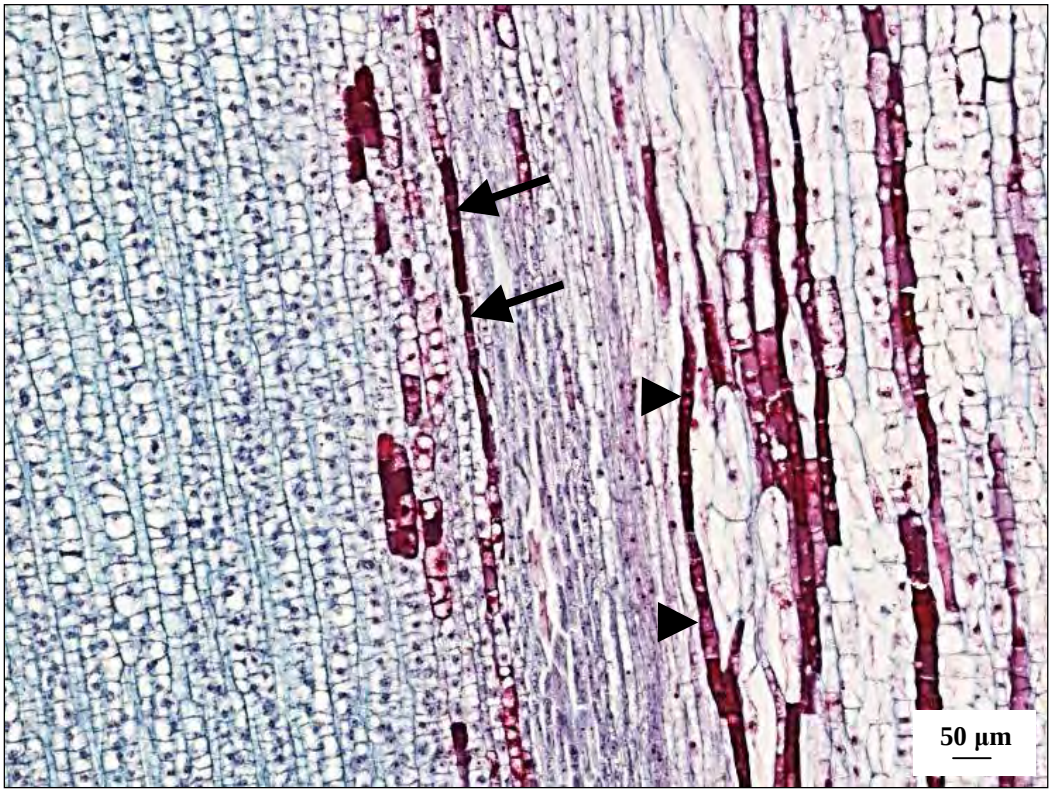

Fig. 4.10 Light micrograph of root tip of *B. gymnorrhiza* in the control treatment showing xylem vessels (arrowheads) and sieve tubes of phloem (arrows).

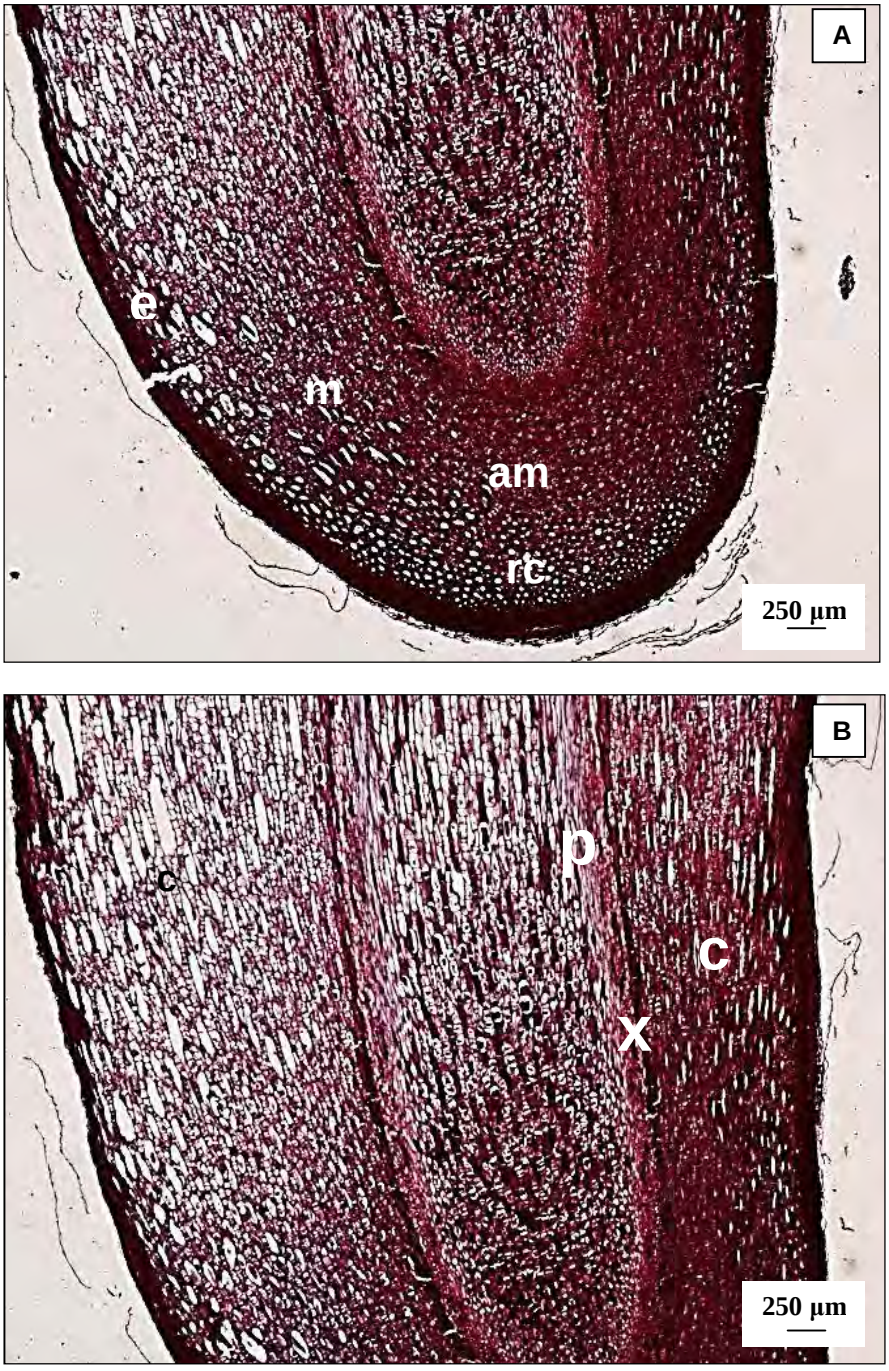

Fig. 4.11 Light micrographs of root tip of *R. mucronata* in the control treatment. (A) Root tip showing root cap (rc), epidermis (e), apical meristem (am) and meristematic tissue (m). (B) Centre of root primordium showing cortex (c) and immature xylem (x) and phloem tissue (p).

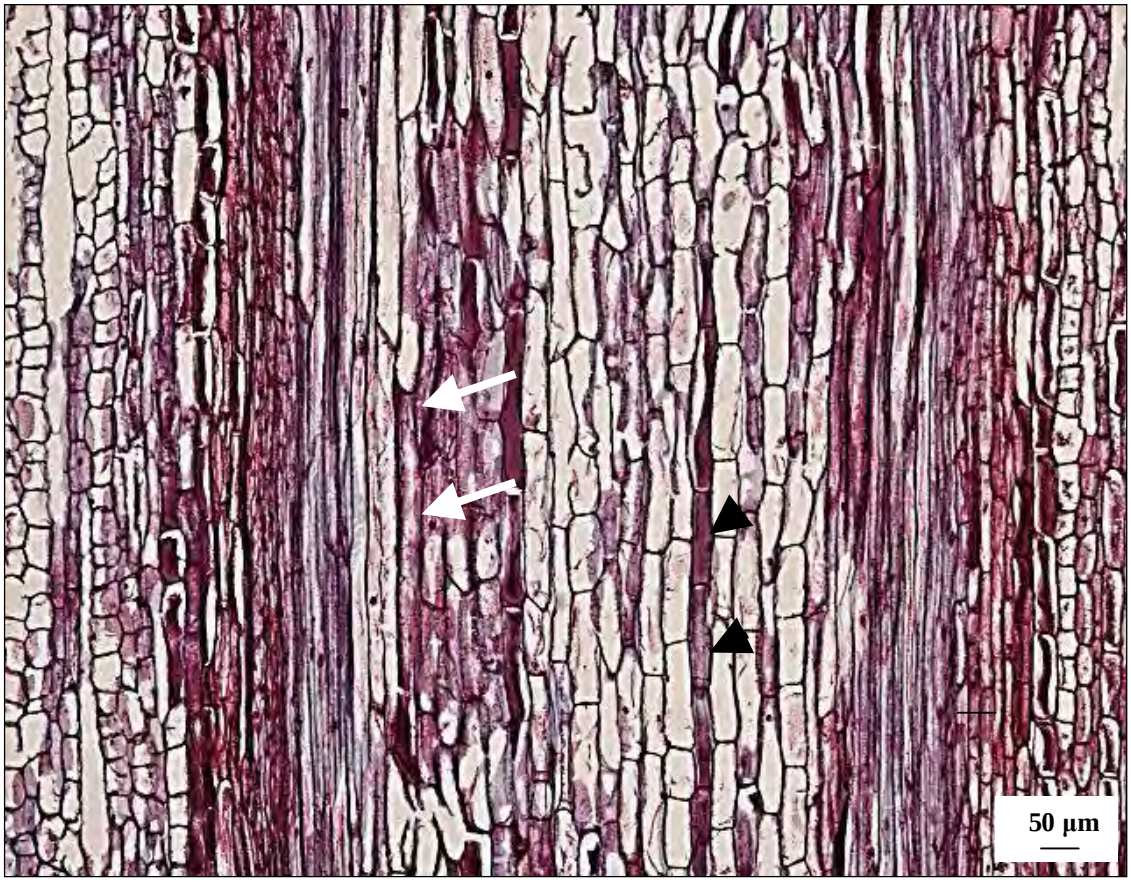

Fig. 4.12 Light micrograph of root tip of *R. mucronata* in the control treatment showing xylem vessel (arrowheads) and phloem sieve tube (arrows).

4.3.4 Transmission electron micrographs of root tips

Ultrastructure of root tip cells in the control treatment

In the control, transmission electron micrographs of root tips of *A. marina* (Figs. 4.13 – 4.16), *B. gymnorrhiza* (Figs. 4.17 - 4.20) and *R. mucronata* (Figs. 4.21 - 4.23), showed the typical ultrastructure of normal, healthy cells.

Cells of the meristematic tissue were robust and exhibited cell division in *A. marina* (Fig. 4.13B), *B. gymnorrhiza* (Fig. 4.18A) and *R. mucronata* (Fig. 4.22A). Meristematic cells were turgid with uniform cell walls and intact plasma membranes in *A. marina* (Fig. 4.14B), *B. gymnorrhiza* (Fig. 4.18B) and *R. mucronata* (Fig. 4.22B). In the meristematic cells of *R. mucronata*, plasmodesmata were clearly observed (Fig. 4.22B). Cells of the cortex were healthy and structurally intact in *A. marina* (Fig. 4.15A).

In xylem tissue, vessel members were uniform and had intact scalariform thickenings in *A. marina* (Figs. 4.16A and B). In phloem tissue, parenchyma cells were structurally intact and healthy in *A. marina* (Fig. 4.15B), *B. gymnorrhiza* (Figs. 4.19A and 4.20) and *R. mucronata* (Fig. 4.23A). Sieve tubes of phloem tissue in *R. mucronata* were also healthy with intact cell walls (Fig. 4.23B).

All cells had well-developed nuclei with prominent nucleoli in *A. marina* (Figs. 4.13B and 4.14A), *B. gymnorrhiza* (Figs. 4.17B and 4.19B) and *R. mucronata* (Figs. 4.22A and 4.23A). In control root tip cells, vacuoles were well-distributed throughout the cell in *A. marina* (Fig. 4.14A), *B. gymnorrhiza* (Fig. 4.17B) and *R. mucronata* (Fig. 4.22A). Cellular organelles including mitochondria and endoplasmic reticula were well-defined in *A. marina* (Fig. 4.14A) and *B. gymnorrhiza* (Fig. 4.17B).

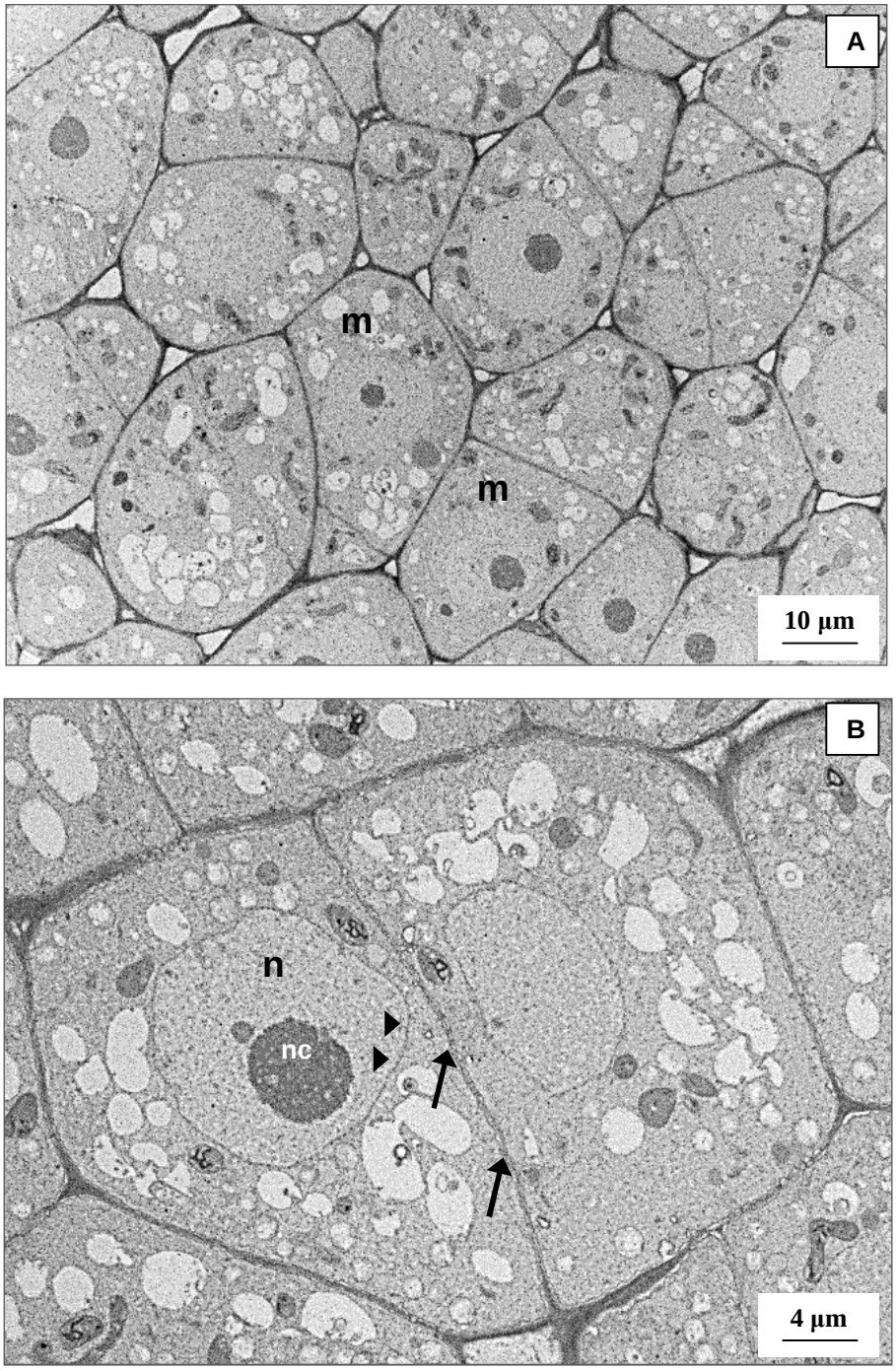

Fig. 4.13 Transmission electron micrographs of root tip cells of *A. marina* in the control treatment. (A) Meristematic tissue showing densely cytoplasmic intact cells (m). (B) Cells of meristematic tissue exhibiting cell division (arrows). Note the intact nucleus (n) with prominent nucleolus (nc) and well-defined nuclear membrane (arrowheads).

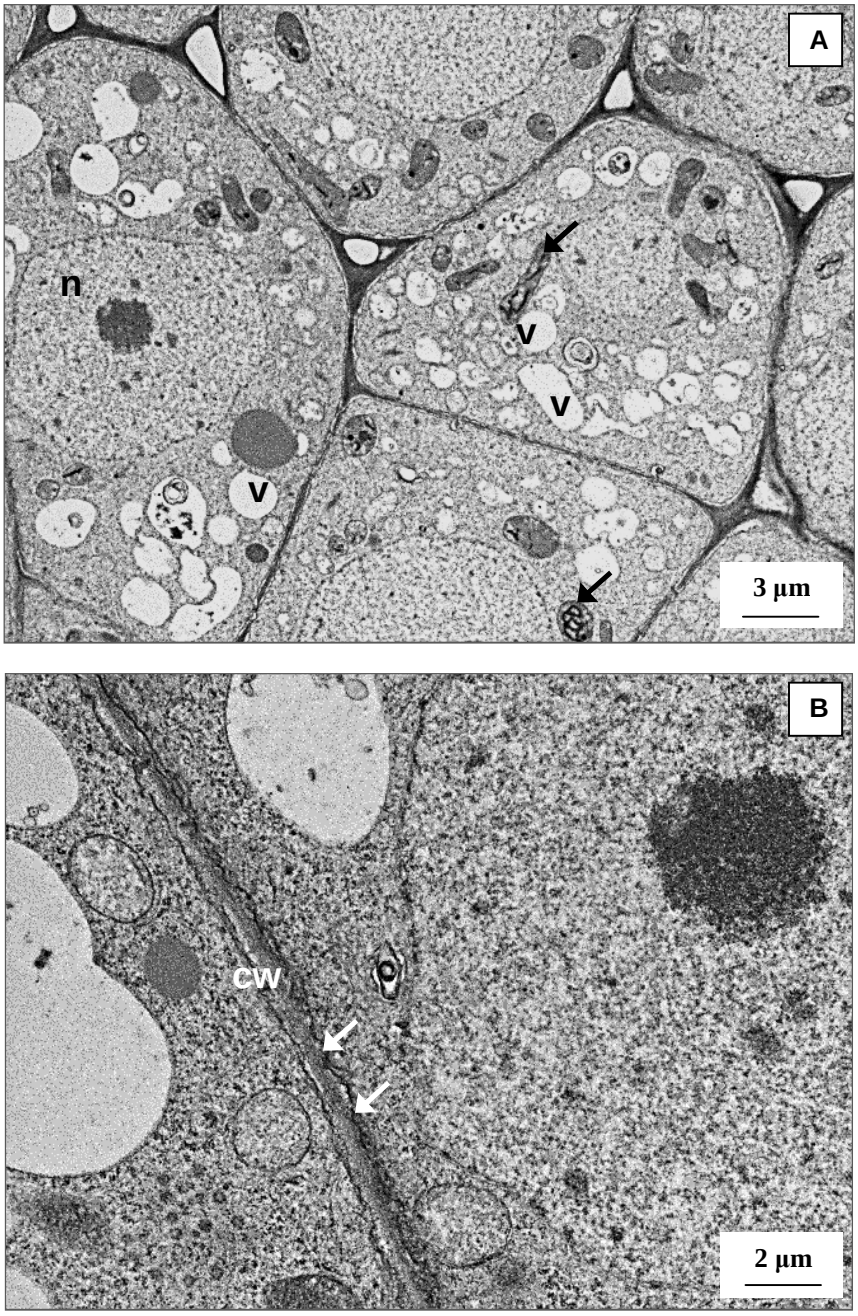

Fig. 4.14 Transmission electron micrographs of root tip cells of *A. marina* in the control treatment. (A) Cells of meristematic tissue showing mitochondria (arrows) and nucleus (n) with dense chromatin network. Note vacuoles (v) distributed throughout cells. (B) Cell of meristematic tissue showing intact and uniform cell wall (cw) and plasma membrane (arrows).

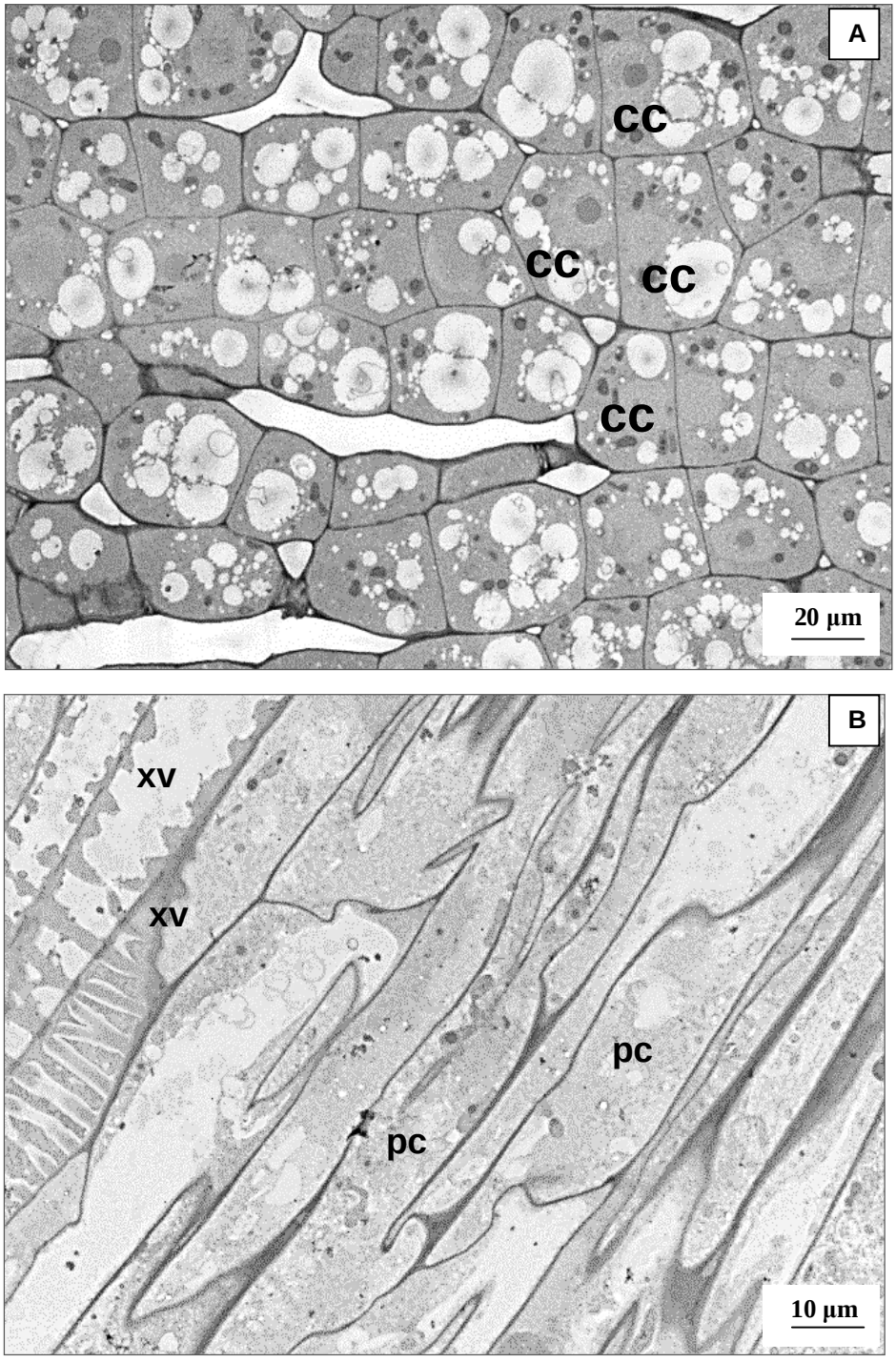

Fig. 4.15 Transmission electron micrographs of root tip cells of *A. marina* in the control treatment. (A) Cortex region of root tip showing intact cells (cc). (B) Centre of the root tip showing xylem vessel members (xv) and phloem parenchyma cells (pc).

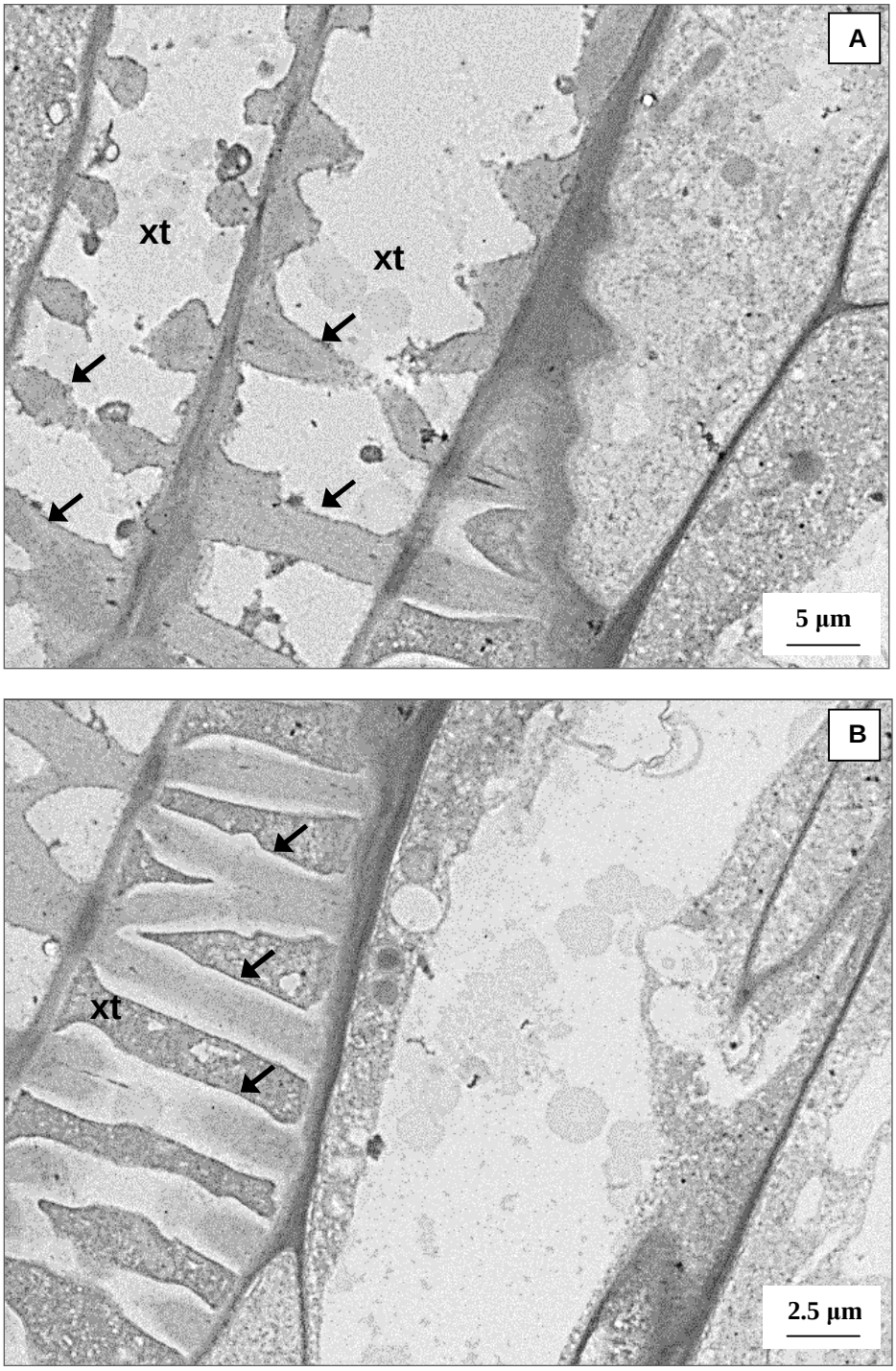

Fig. 4.16 Transmission electron micrographs of root tip cells of *A. marina* in the control treatment. (A) Xylem tracheid (xt) showing scalariform thickening of cell walls (arrows). (B) Xylem tracheid (xt) showing intact scalariform thickening (arrows).

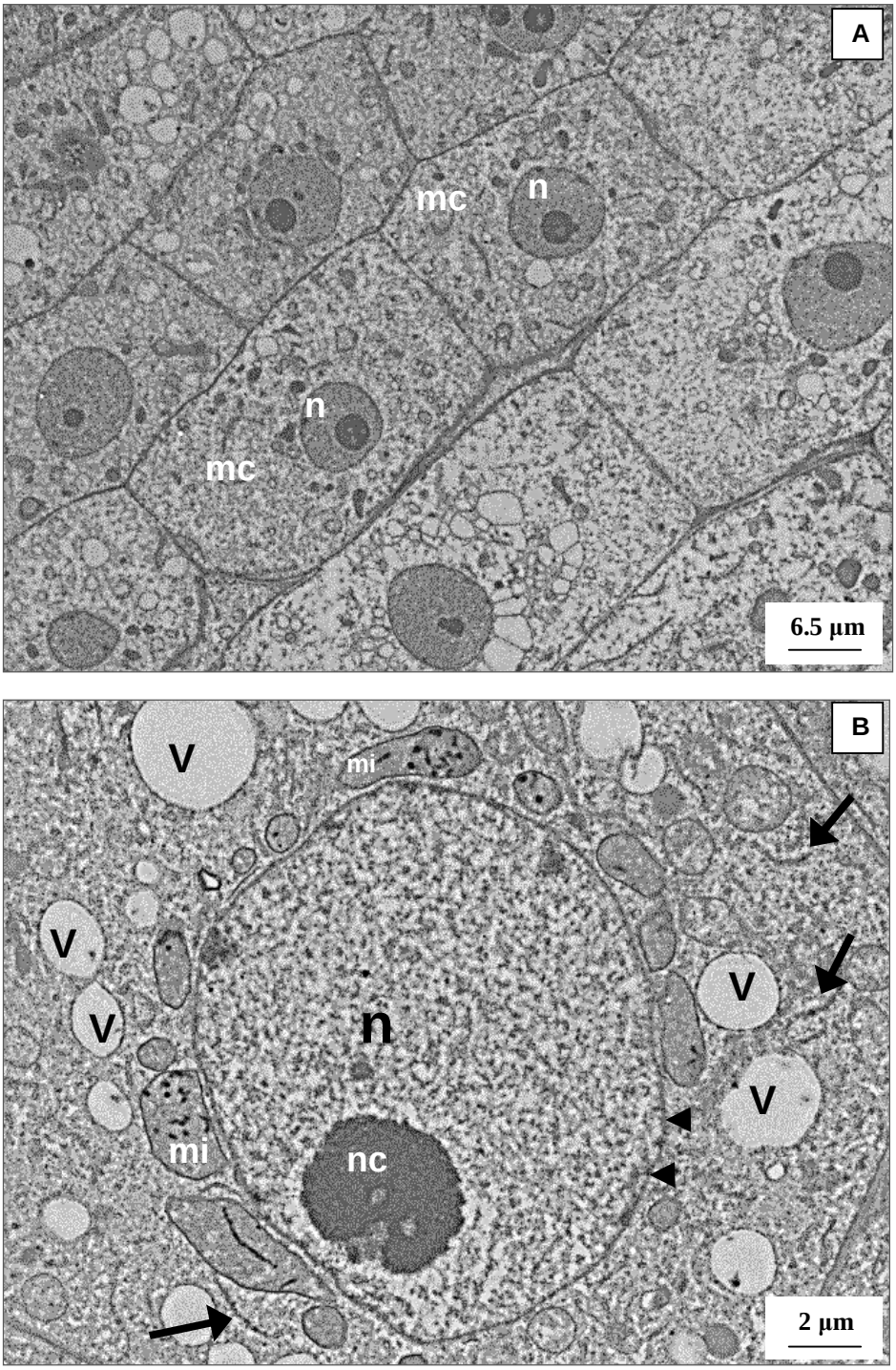

Fig. 4.17 Transmission electron micrographs of root tip cells of *B. gymnorrhiza* in the control treatment. (A) Meristematic tissue showing healthy, intact and uniform cells (mc). Note intact nuclei (n). (B) Meristematic cells showing nucleus (n) with dense chromatin network and uniform intact nuclear membrane (arrowheads) and prominent nucleolus (nc). Note mitochondria (mi) and cisternae of endoplasmic reticulum (arrows). Note vacuoles well-distributed within the cell (v).

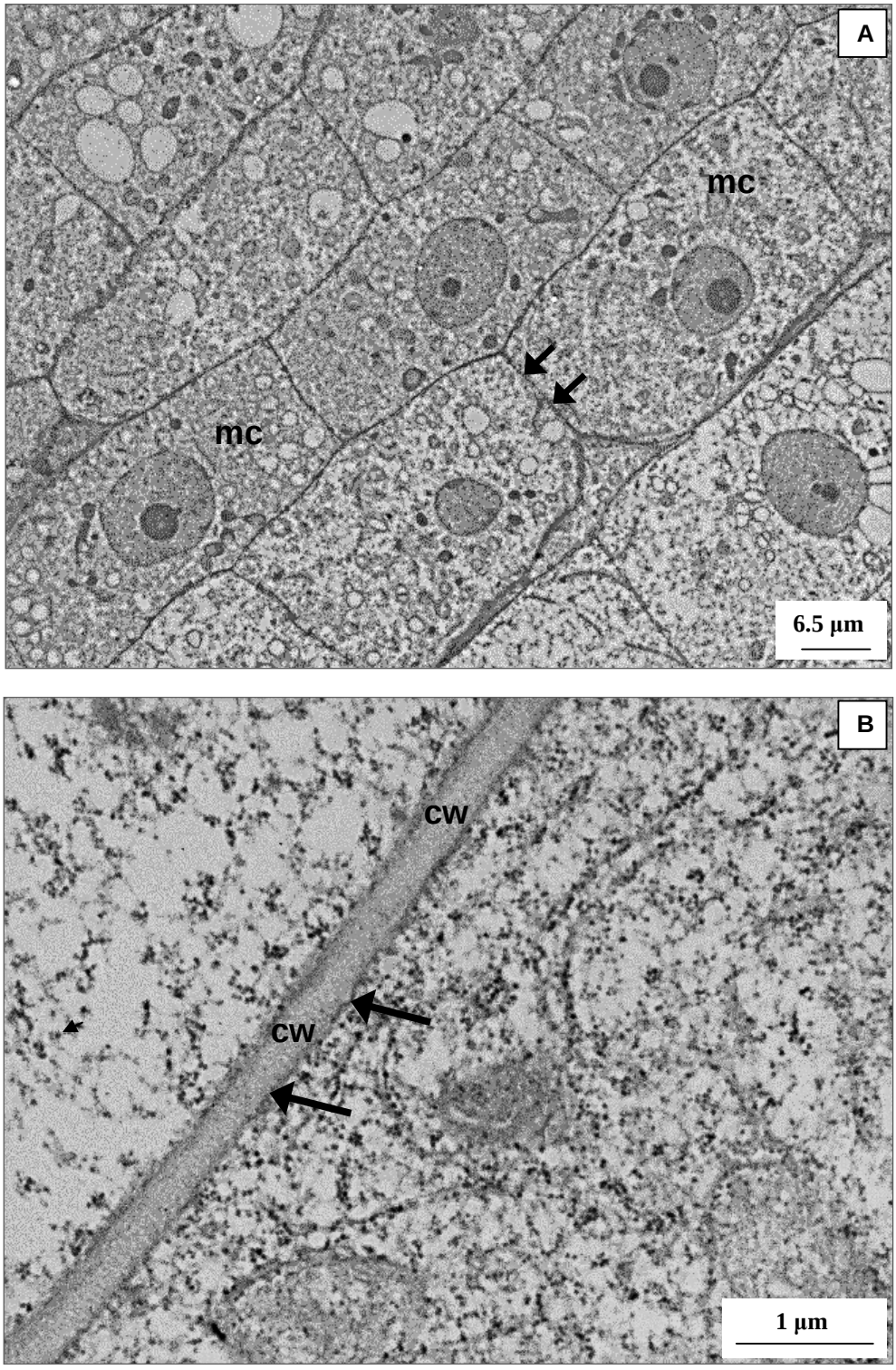

Fig. 4.18 Transmission electron micrographs of root tip cells of *B. gymnorrhiza* in the control treatment. (A) Cells of meristematic tissue (mc) exhibiting cell division (arrows). (B) Meristematic cell showing intact uniform cell wall (cw) and plasma membrane (arrows).

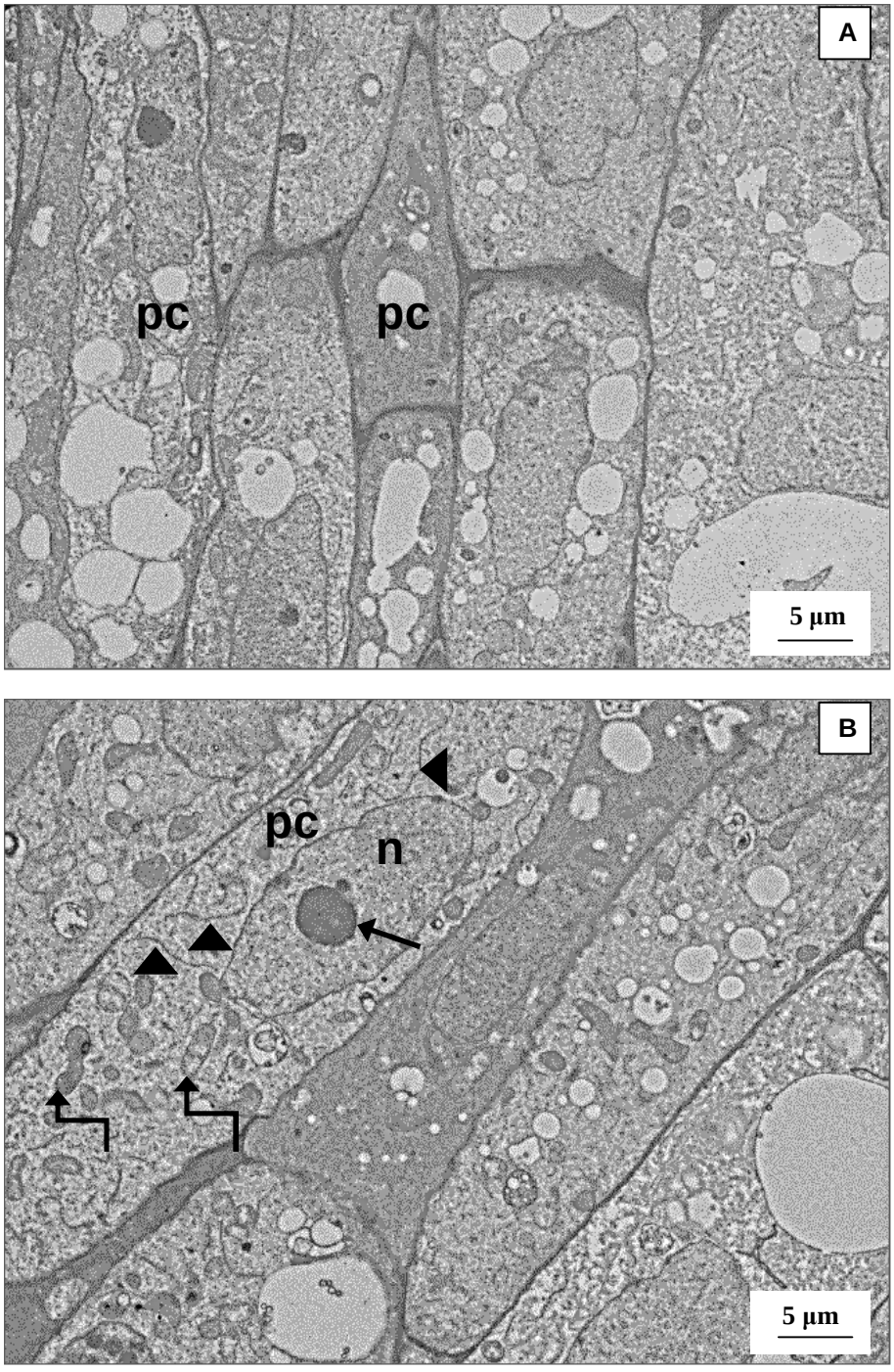

Fig. 4.19 Transmission electron micrographs of root tip cells of *B. gymnorrhiza* in the control treatment. (A) Phloem tissue showing intact organized parenchyma cells (pc). (B) Phloem parenchyma cell (pc) showing nucleus (n) with prominent nucleolus (straight arrow). Note cell organelles, mitochondria (elbow arrows) and cisternae of endoplasmic reticulum (arrowheads).

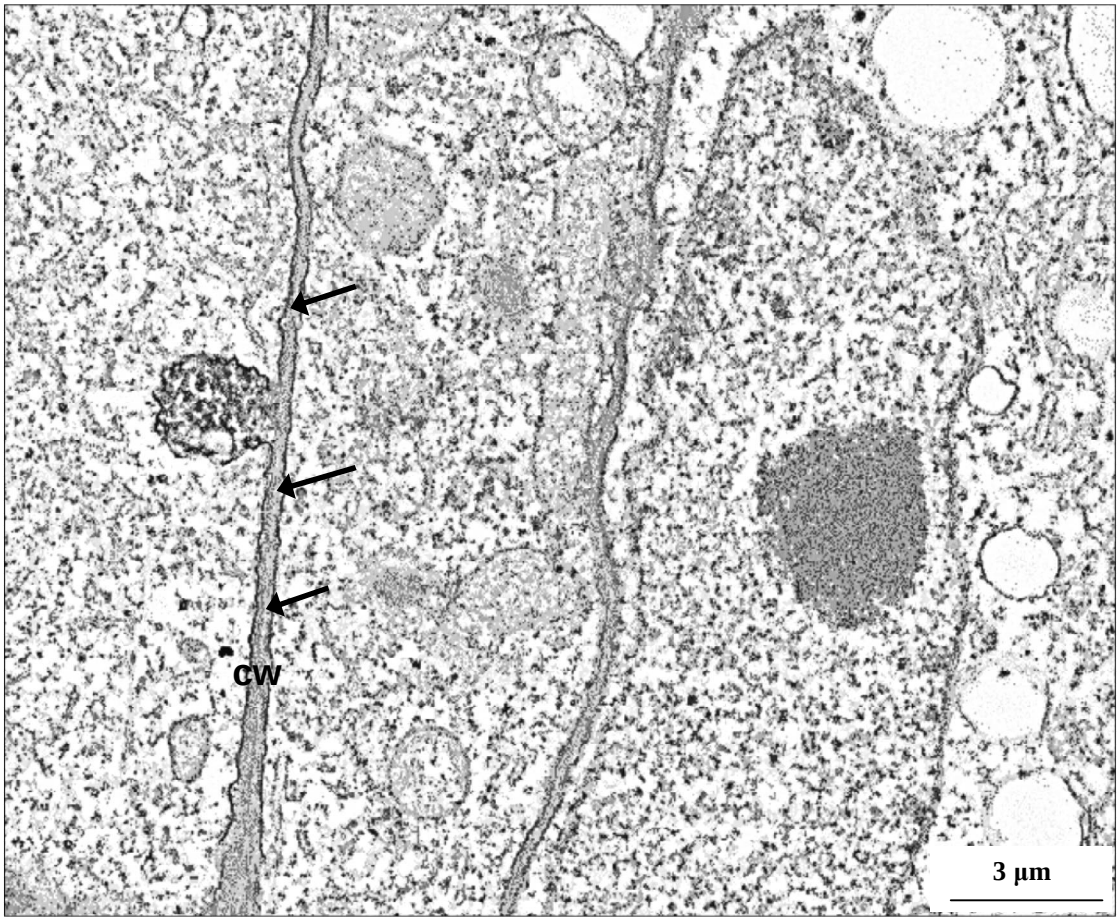

Fig. 4.20 Transmission electron micrograph of phloem parenchyma cell in root tip of *B. gymnorrhiza* in the control treatment showing uniform cell wall (cw) and plasma membrane (arrows).

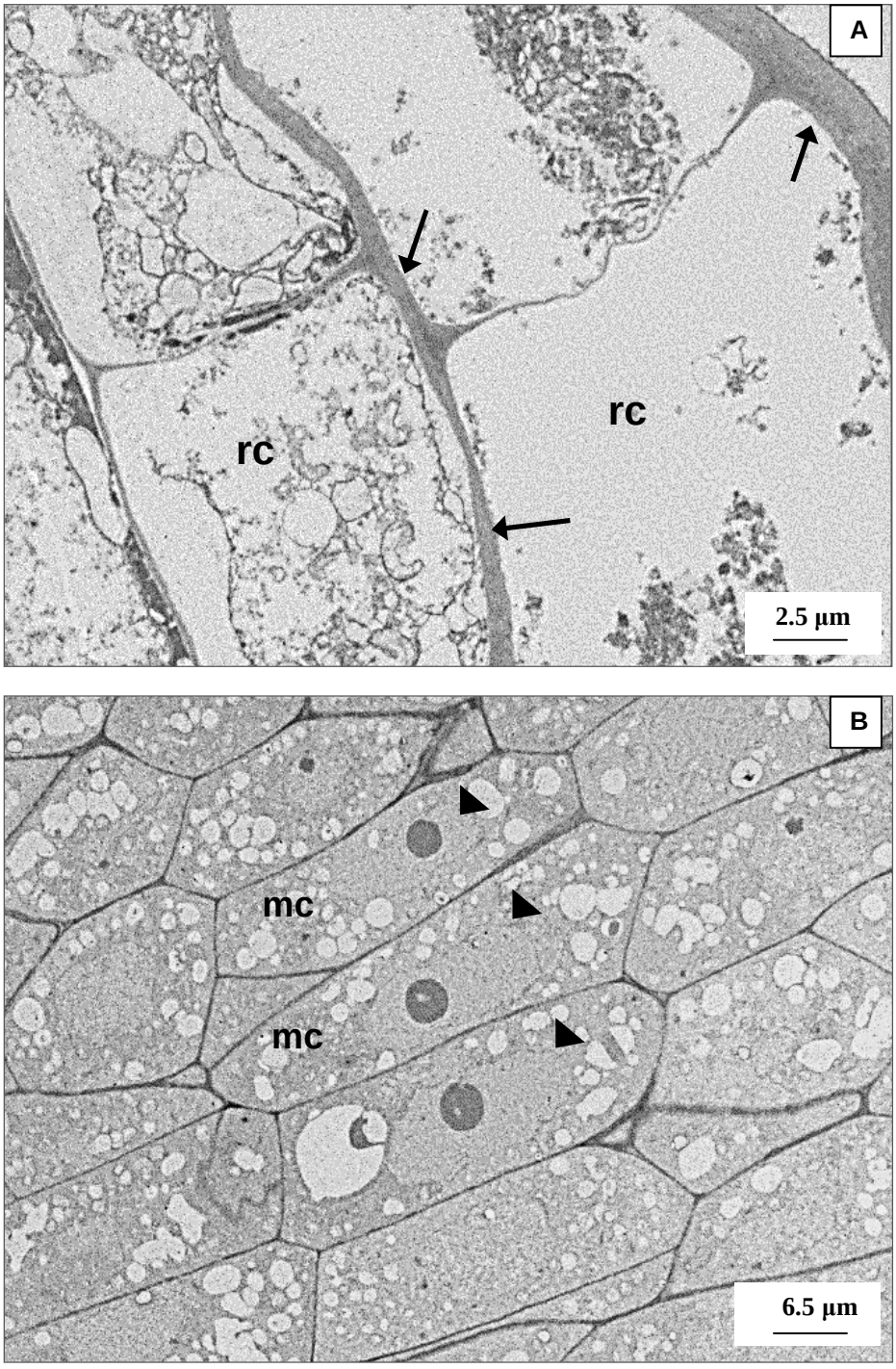

Fig. 4.21 Transmission electron micrographs of root tip cells of *R. mucronata* in the control treatment. (A) Cells of the root cap (rc) showing intact uniform cell walls (arrows). (B) Meristematic tissue showing healthy cells (mc) with nuclei (arrowheads).

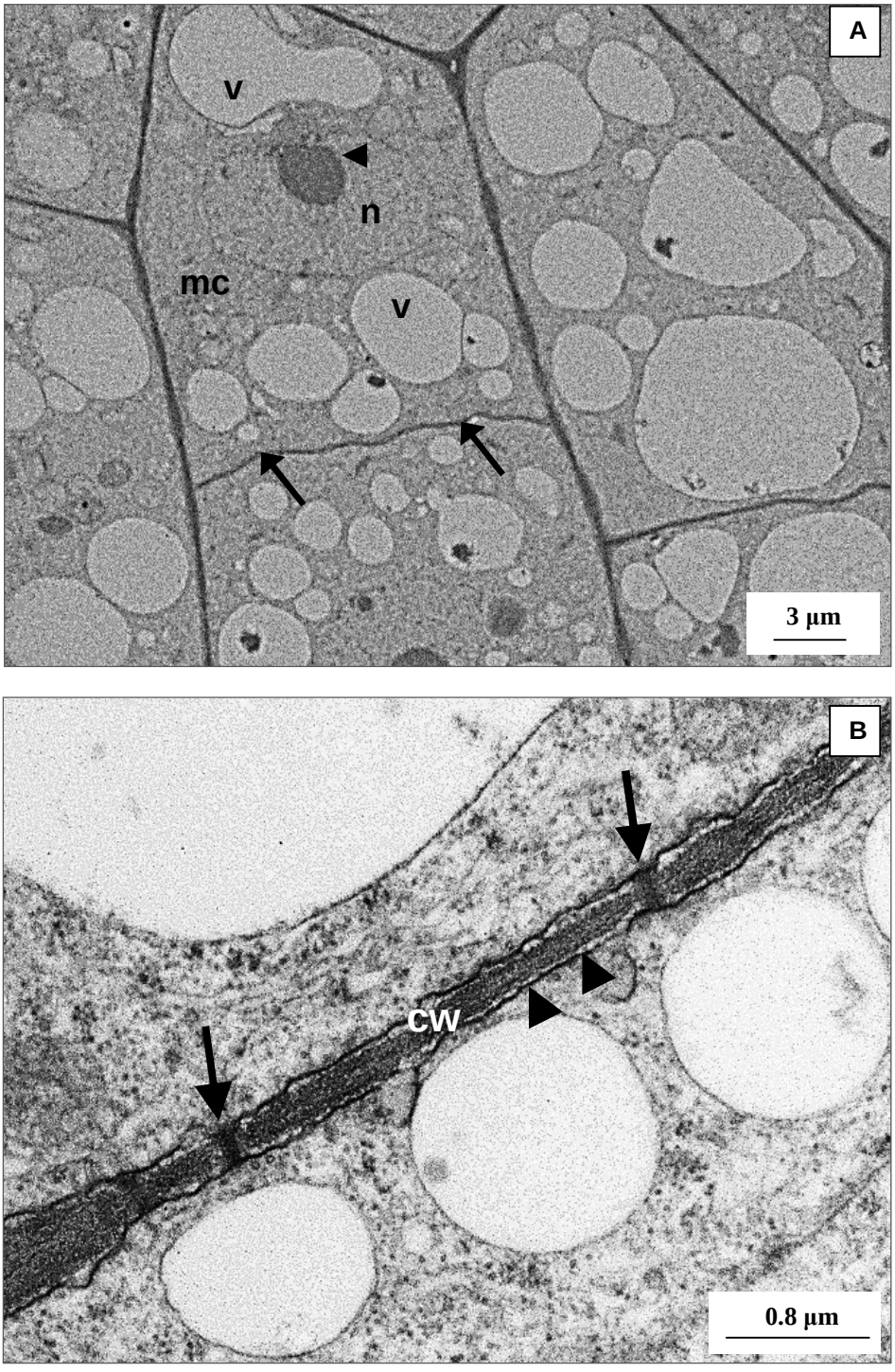

Fig. 4.22 Transmission electron micrographs of root tip cells of *R. mucronata* in the control treatment. (A) Cells of meristematic tissue (mc) exhibiting cell division (arrows). Note vacuoles (v) distributed throughout the cell. Note the nucleus (n) with prominent nucleolus (arrowhead). (B) Cell of meristematic tissue showing intact uniform cell wall (cw) and plasma membrane (arrowheads). Note the distinct plasmodesmata (arrows).

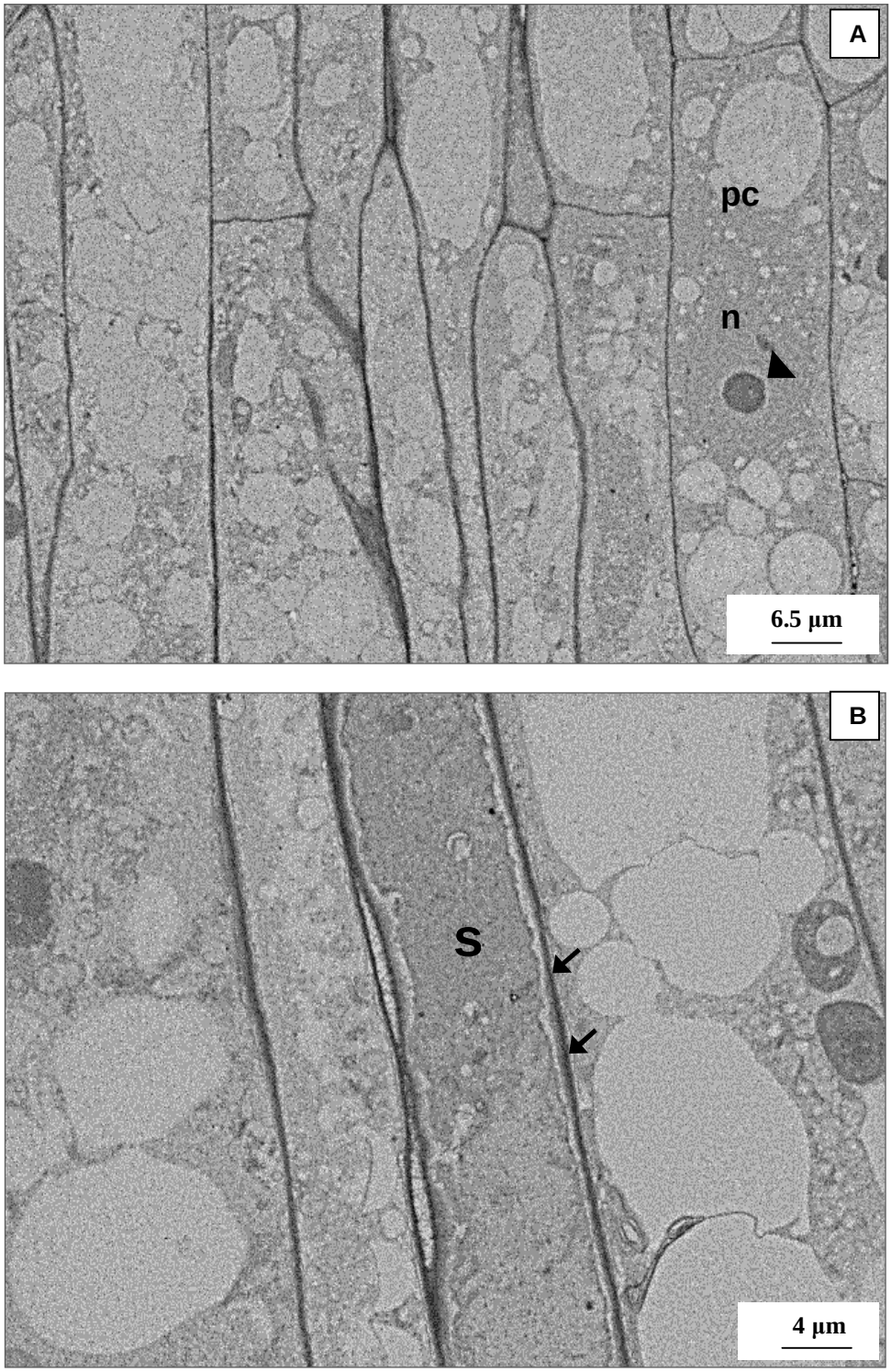

Fig. 4.23 Transmission electron micrographs of root tip cells of *R. mucronata* in the control treatment. (A) Parenchyma cells (pc) of phloem tissue showing nucleus (n) with prominent nucleolus (arrowhead). (B) Phloem tissue showing sieve tube (s) with intact cell walls (arrows).

Ultrastructure of root tip cells in the oiled treatment

The effects of oil were deleterious on fine structure of root tip cells in *A. marina* (Figs. 4.24 – 4.29), *B. gymnorrhiza* (Figs. 4.30 – 4.33) and *R. mucronata* (Figs.4.34 – 4.37). Oil accumulated in root tip cells and resulted in their complete disorganisation.

Effects of oil on cell ultrastructure were similar in all three species. In the root cap region in *R. mucronata*, oil deposits were observed in the interior of cells (Fig. 4.34A) and around root hairs (Fig. 4.34B).

In the epidermal region in *R. mucronata*, cell contents and organelles such as the nucleus, mitochondrion and endoplasmic reticulum were unrecognizable as oil deposits occurred throughout the cells (Fig. 4.35A).

In the meristematic tissue, cells were deformed in *B. gymnorrhiza* (Fig. 4.30A) and distorted in *R. mucronata* (Fig. 4.35B). Cell walls and plasma membranes were irregular and invaginated (with numerous inward folds) in meristematic cells of *A. marina* (4.24B) and *B. gymnorrhiza* (Fig. 4.30B). Cell walls appeared darkened and large oil deposits were observed in *A. marina* (Fig. 4.25A), *B. gymnorrhiza* (Fig. 4.30A) and *R. mucronata* (Fig. 4.35B). Oil appeared to infiltrate adjacent cells via deteriorated cell walls in *B. gymnorrhiza* (Fig. 4.30A).

Cell contents of meristematic tissue appeared disorganized in *A. marina* (Fig. 4.24A). Vacuoles appeared to make up the bulk of the cell volume in *A. marina* (Fig. 4.24B) and *B. gymnorrhiza* (Fig. 4.31A). In meristematic cells of *B. gymnorrhiza*, oil accumulated within large vacuoles (Fig. 4.31A) while in *R. mucronata*, oil filled entire cells (Fig. 4.35B). Oil deposits were observed close to the nucleus in meristematic cells of *A. marina* (Fig. 4.24B) while in *B. gymnorrhiza*, the nuclear membrane was perforated (Fig. 4.31B).

Cell organelles such as mitochondria and endoplasmic reticula were fragmented and unrecognizable in meristematic cells of *A. marina* (Figs. 4.24A and B), *B. gymnorrhiza* (Figs. 4.31A and B) and *R. mucronata* (Fig. 4.35B).

In the cortex region of the root tip in *A. marina*, cells were distorted and filled with oil deposits and large vacuoles made up the bulk of the cell volume (Fig. 4.26A and B). Oil induced loss of cell contents in the cortex region of the root tip of *A. marina*, and cell organelles such as mitochondria and endoplasmic reticula were fragmented and unrecognizable (Fig. 4.26A and B).

In the phloem tissue, in *A. marina* (Fig. 4.27A), *B. gymnorrhiza* (Fig. 4.32B) and *R. mucronata* (Fig. 4.37A), parenchyma cells contained large oil deposits. The cell walls of parenchyma cells were invaginated and darkened in *A. marina* (Fig. 4.27A), *B. gymnorrhiza* (Figs. 4.32B and 4.33) and *R. mucronata* (Fig. 4.37A and B). Cell contents leaked out through ruptured cell walls in *R. mucronata* (Fig. 4.36A).

In phloem parenchyma cells in *A. marina*, large oil deposits leaked from cell walls into the cytoplasm (Fig. 4.28) while large vacuoles were observed (Fig. 4.27A). In *B. gymnorrhiza*, oil deposits were closely appressed to parenchyma cell walls (Figs. 4.32A and 4.33).

In *R. mucronata*, phloem cells were distorted, indistinguishable from each other and were filled with oil (Fig. 4.36A). Sieve tubes of phloem tissue in *R. mucronata* were also filled with oil (Fig. 4.36B). In *A. marina*, xylem vessels with intact scalariform thickenings exhibited minimal damage compared to phloem cells (Fig. 4.29A and B).

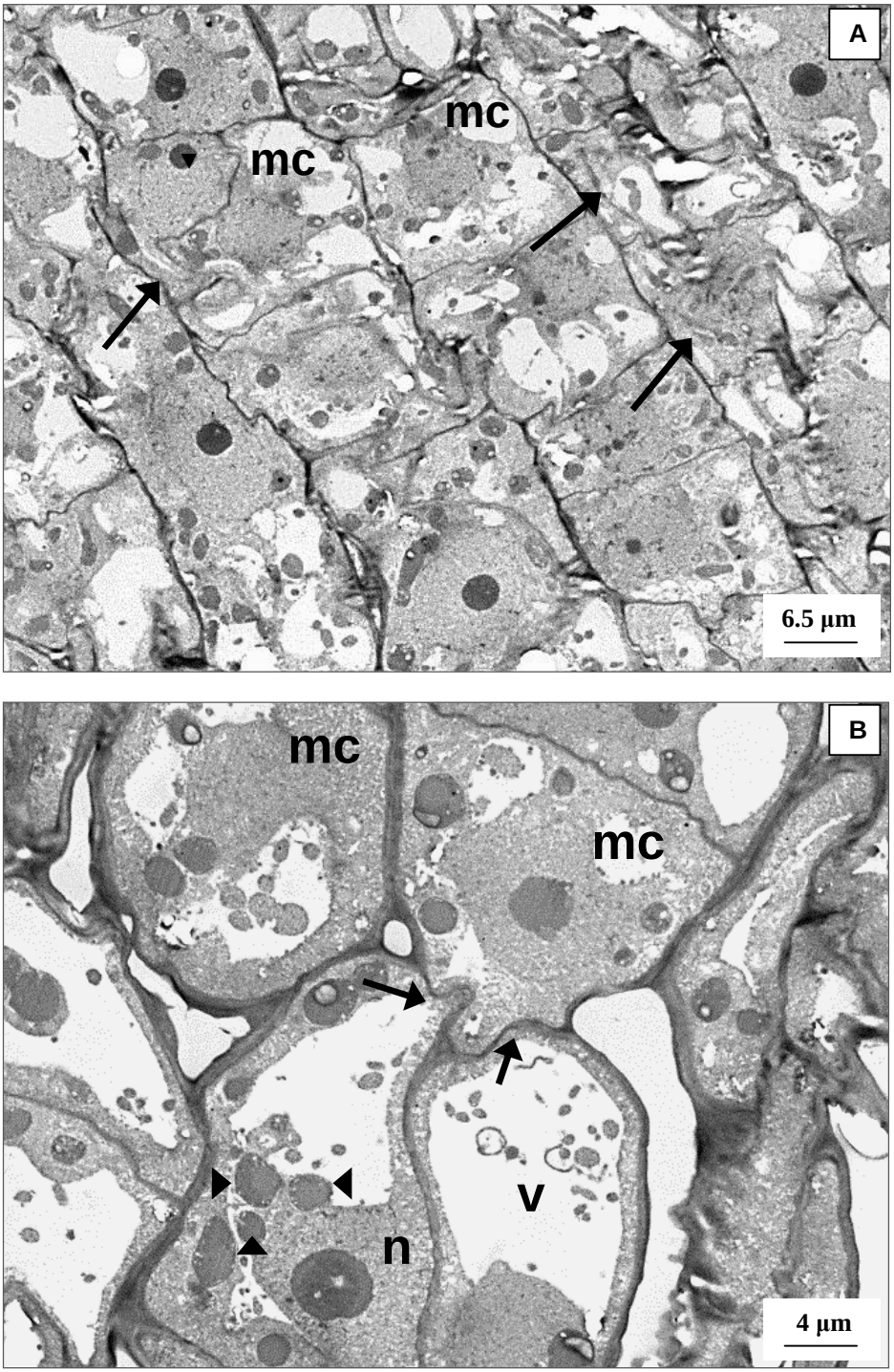

Fig. 4.24 Transmission electron micrographs of root tip cells of *A. marina* in the oiled treatment. (A) Meristematic cells (mc) showing disorganization of cell contents (arrows). (B) Meristematic cells showing irregular invaginated cell walls (arrows). Note oil deposits (arrowheads) appeared to enter nucleus (n). Note also the large vacuole (v) making up the bulk of the cell volume.

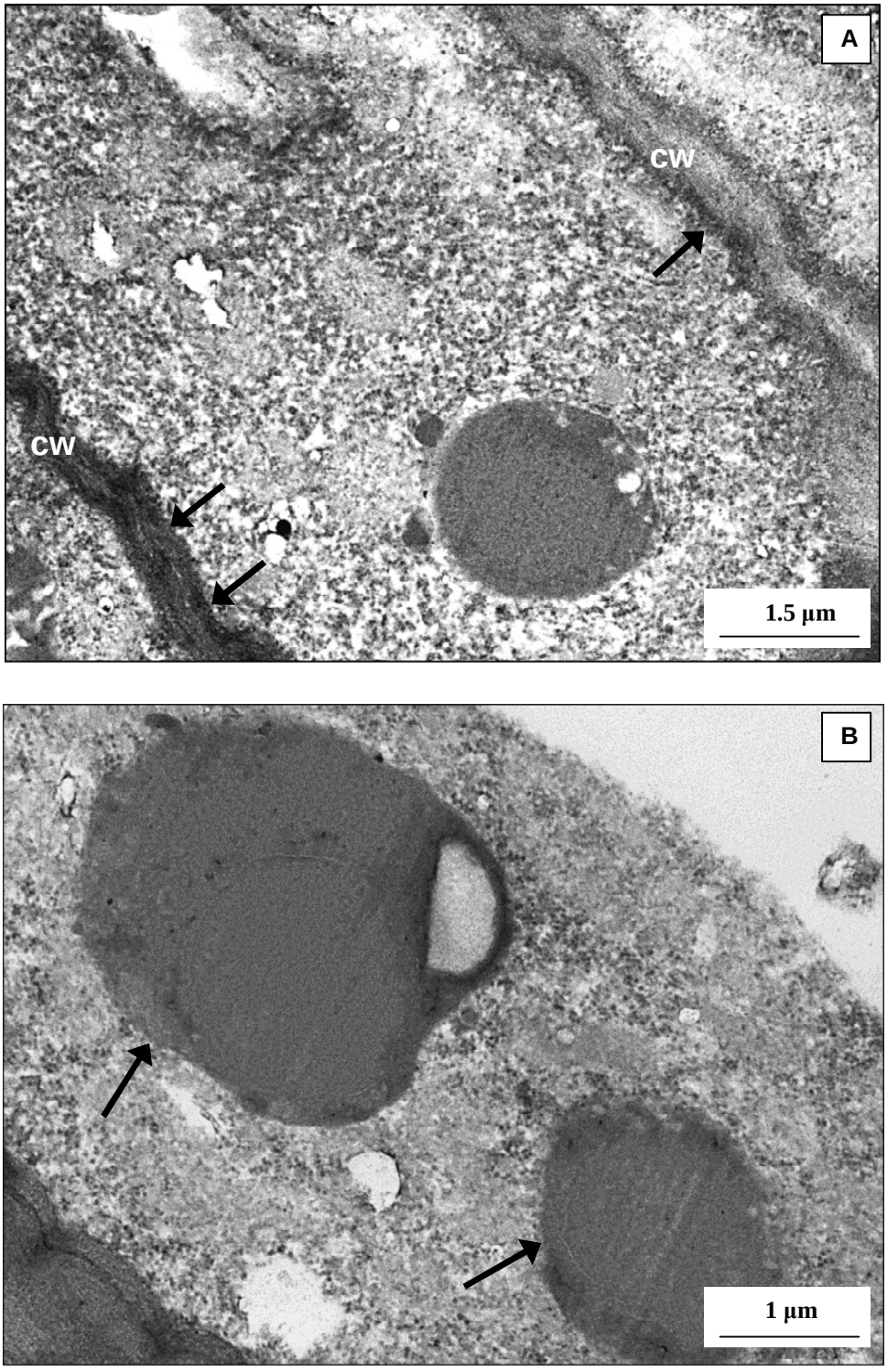

Fig. 4.25 Transmission electron micrographs of root tip cells of *A. marina* in the oiled treatment. (A) Cell of meristematic tissue showing cell walls (cw) that appeared to be darkened by oil (arrows). (B) Cytoplasm of meristematic cell showing higher magnification of oil deposits (arrows).

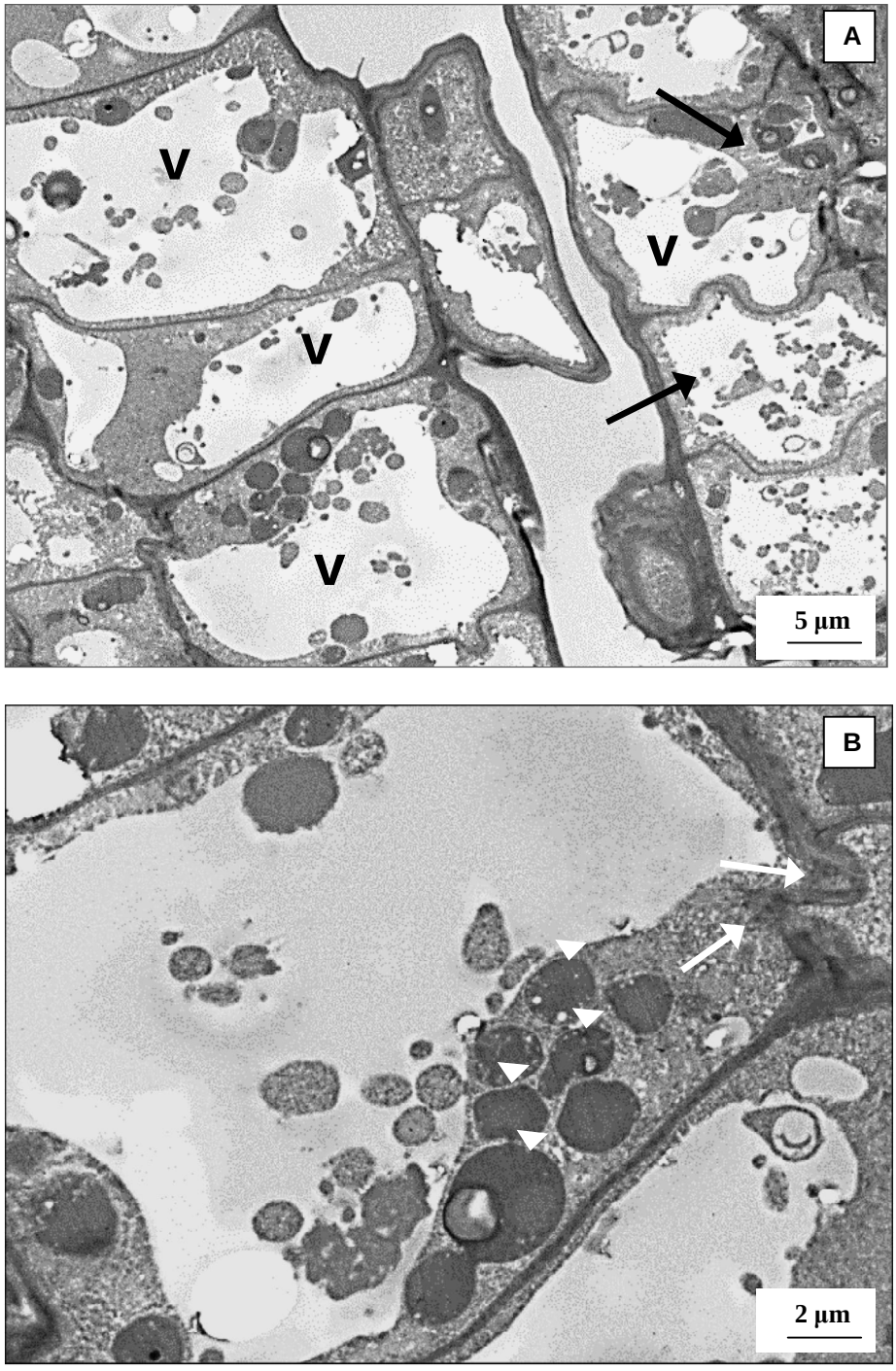

Fig. 4.26 Transmission electron micrographs of root tip cells of *A. marina* in the oiled treatment. (A) Cells of the cortex showing disorganization of cell contents (arrows) and large vacuoles (v) making up the bulk of the cell volume. (B) Cells of the cortex showing cell wall that appeared invaginated and perforated (arrows). Note oil deposits (arrowheads).

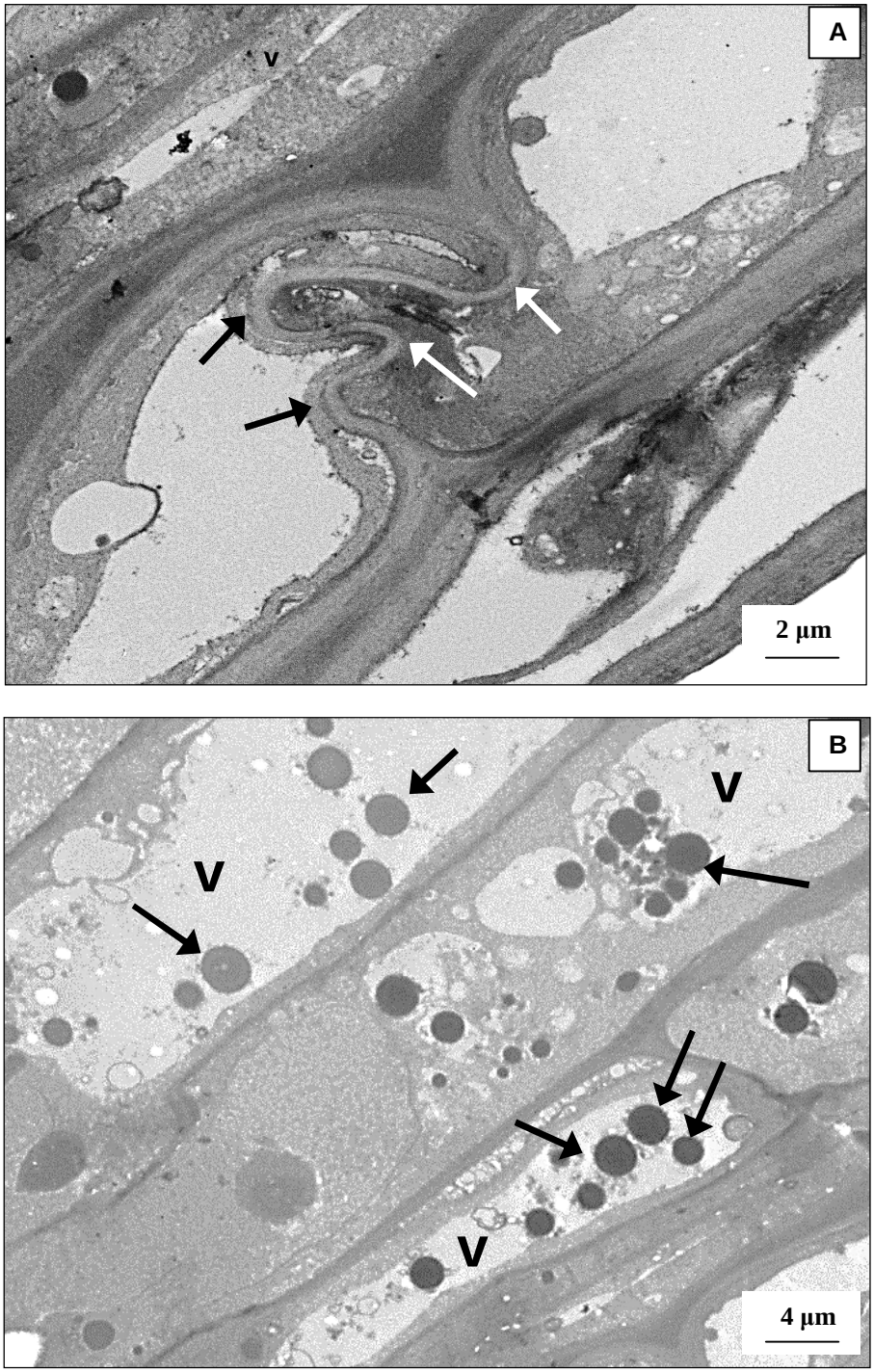

Fig. 4.27 Transmission electron micrographs of root tip cells of *A. marina* in the oiled treatment. (A) Parenchyma cell of phloem tissue showing invaginated cell wall (arrows). (B) Parenchyma cell of phloem tissue showing oil deposits (arrows) in large vacuoles (v).

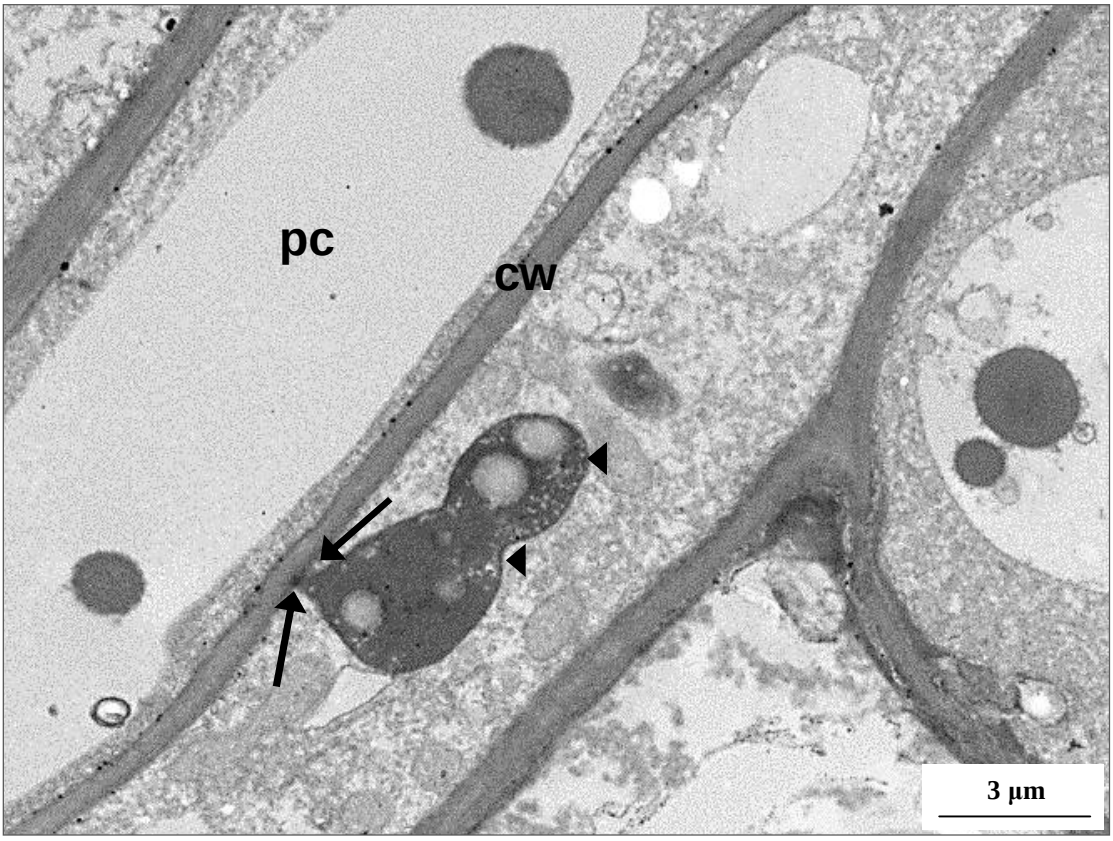

Fig. 4.28 Transmission electron micrograph of root tip cells of *A. marina* in the oiled treatment. Parenchyma cell (pc) of phloem tissue showing a large oil deposit (arrowheads) which appeared to leak out (arrows) of the cell wall (cw).

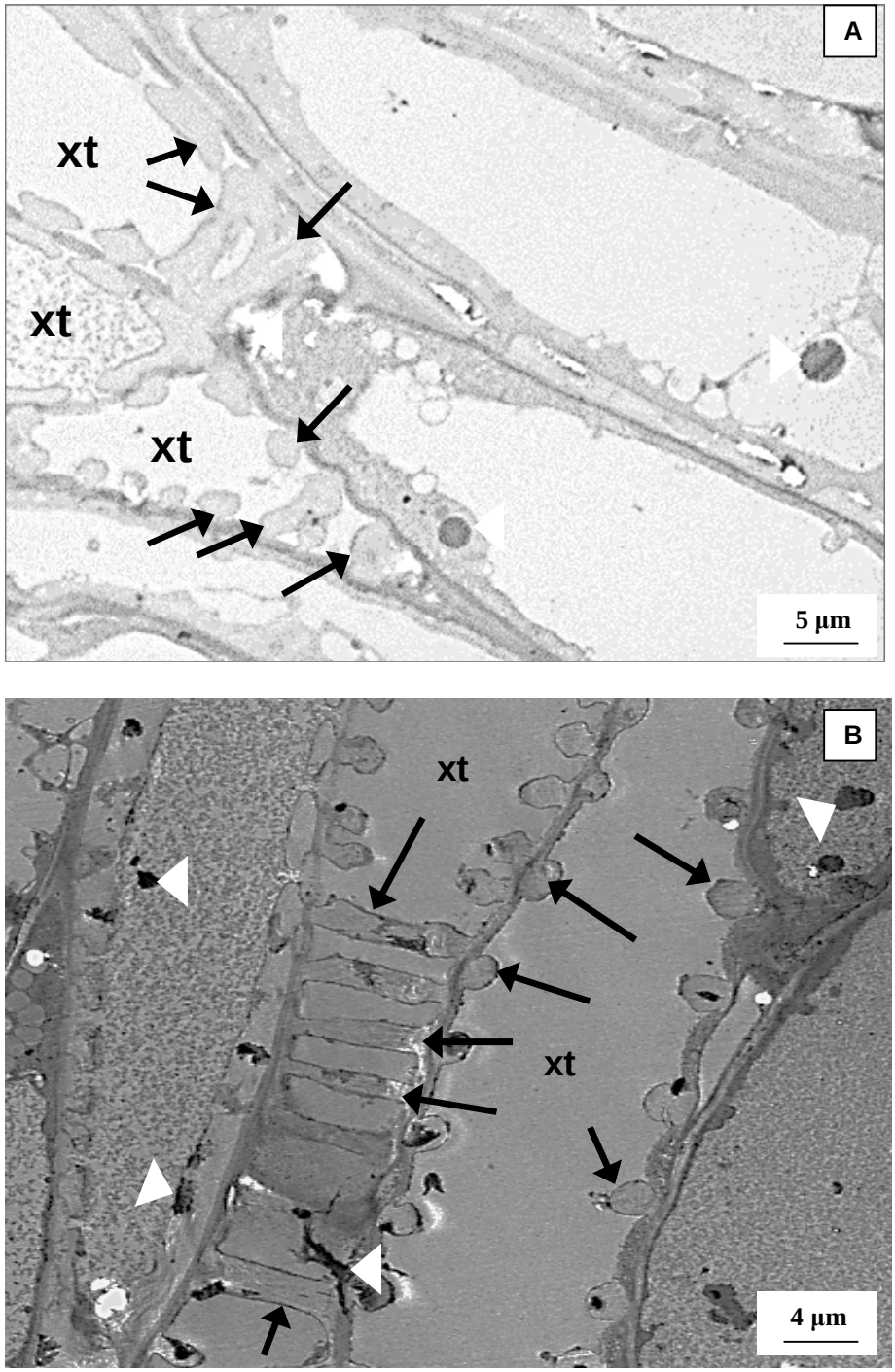

Fig. 4.29 Transmission electron micrographs of root tip cells of *A. marina* in the oiled treatment. (A and B) Xylem tracheids (xt) showing scalariform thickenings (arrows). Note oil deposits (arrowheads).

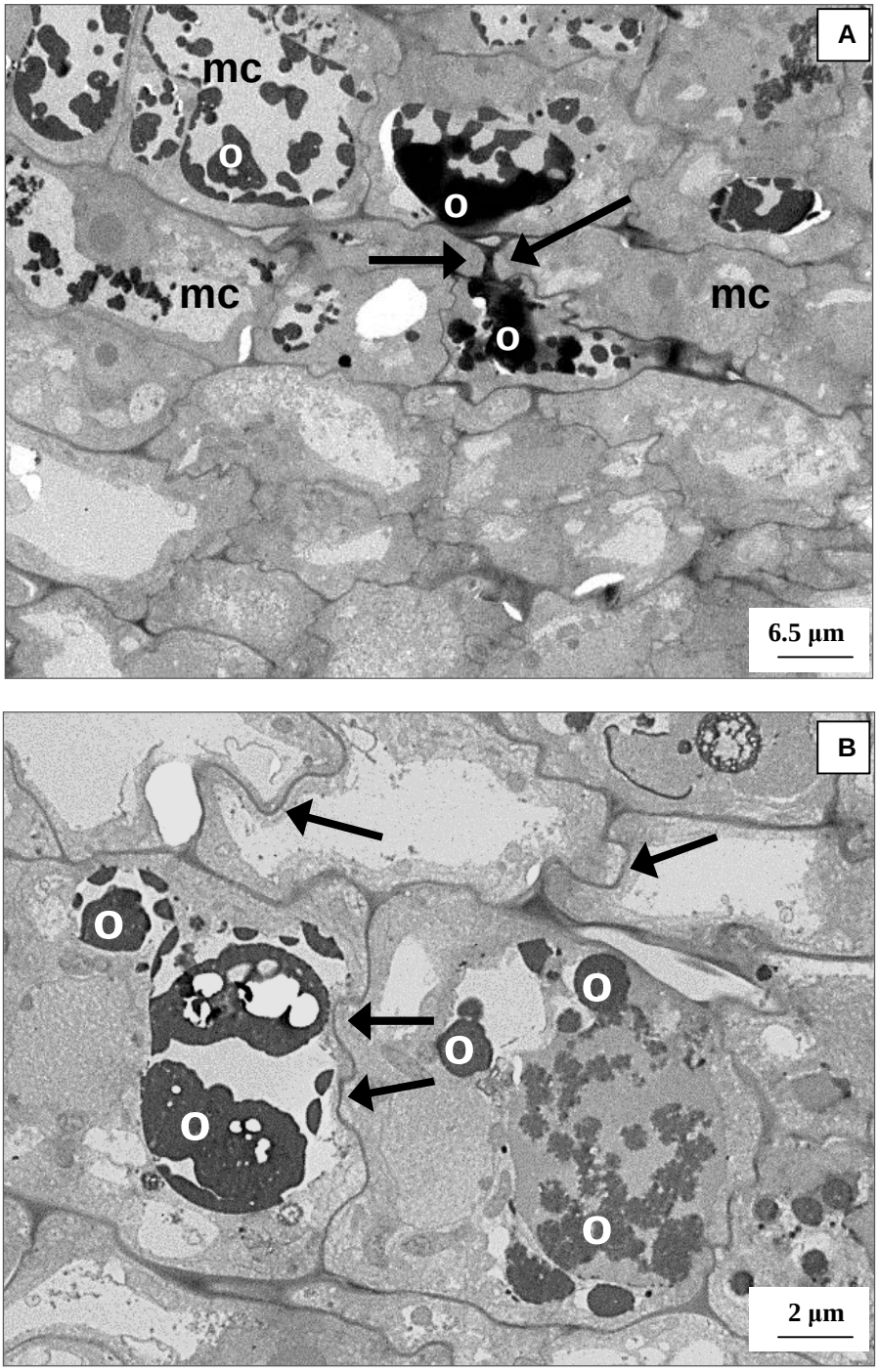

Fig. 4.30 Transmission electron micrographs of root tip cells of *B. gymnorrhiza* in the oiled treatment. (A) Cells of the meristematic tissue (mc) showing oil deposits (o). Note that cells appear to be deformed. Note also that oil appeared to infiltrate cells (arrows). (B) Meristematic cells showing oil deposits (o) and irregular invaginated cell walls (arrows).

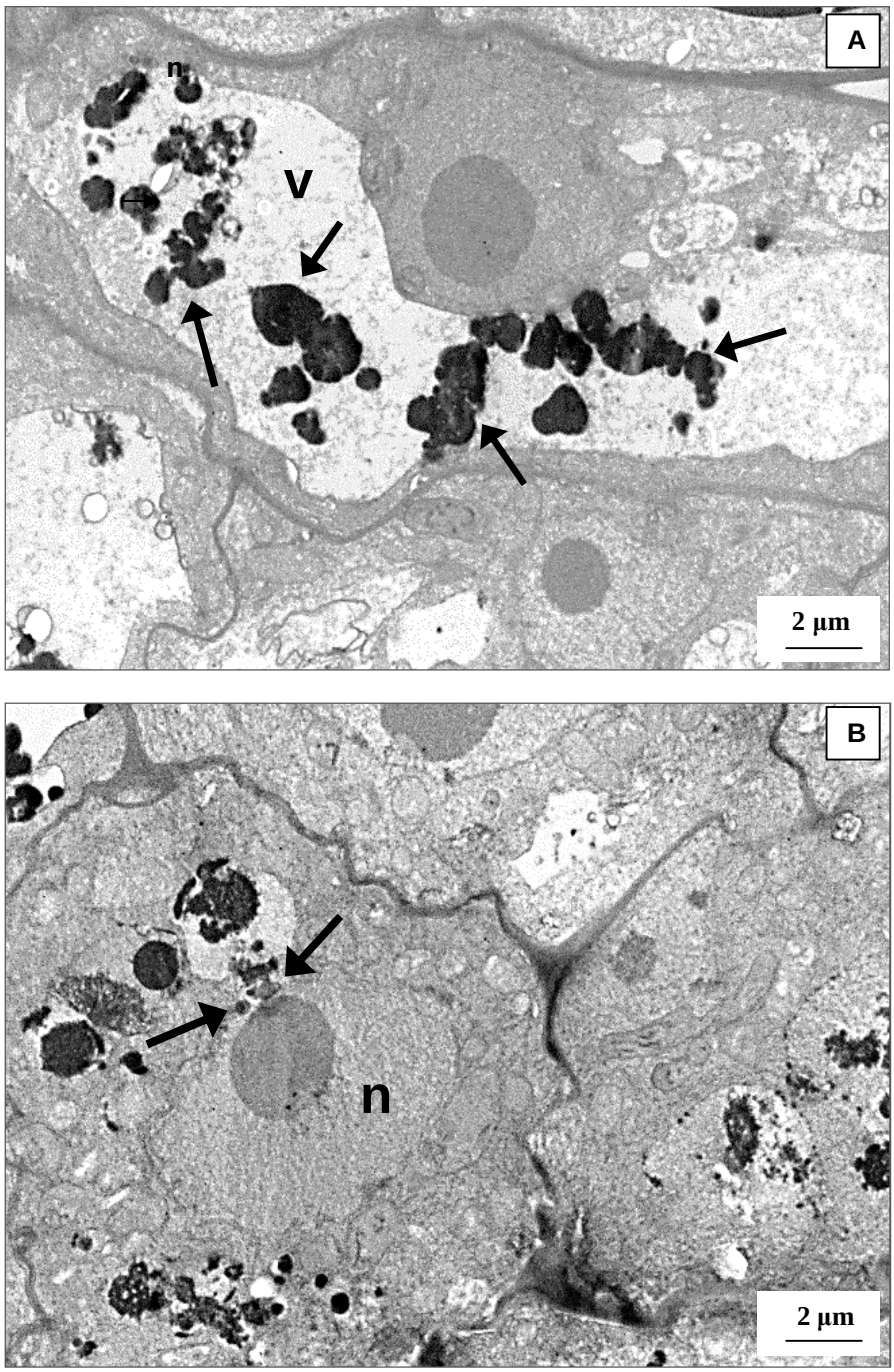

Fig. 4.31 Transmission electron micrographs of root tip cells of *B. gymnorrhiza* in the oiled treatment. (A) Cell of meristematic tissue showing oil deposits (arrows) inside large vacuole (v) occupying the bulk of the cell volume. (B) Cells of meristematic tissue showing nucleus (n) and nuclear membrane, which appeared to be perforated by oil (arrows).

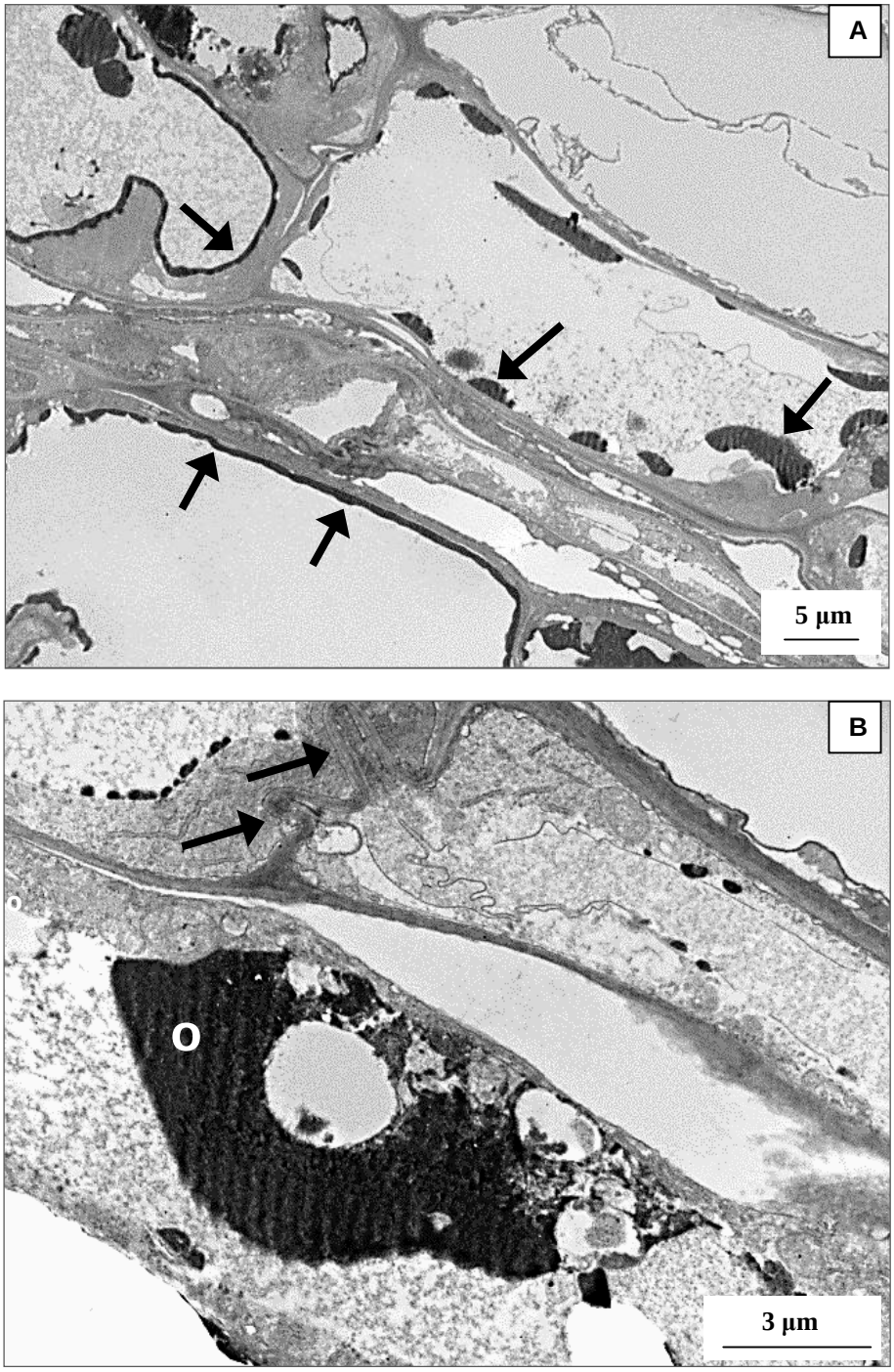

Fig. 4.32 Transmission electron micrographs of root tip cells of *B. gymnorrhiza* in the oiled treatment. (A) Phloem tissue showing parenchyma cells with oil deposits closely appressed to cell walls (arrows). (B) Phloem parenchyma cell showing oil deposit (o) and invaginated cell walls (arrows).

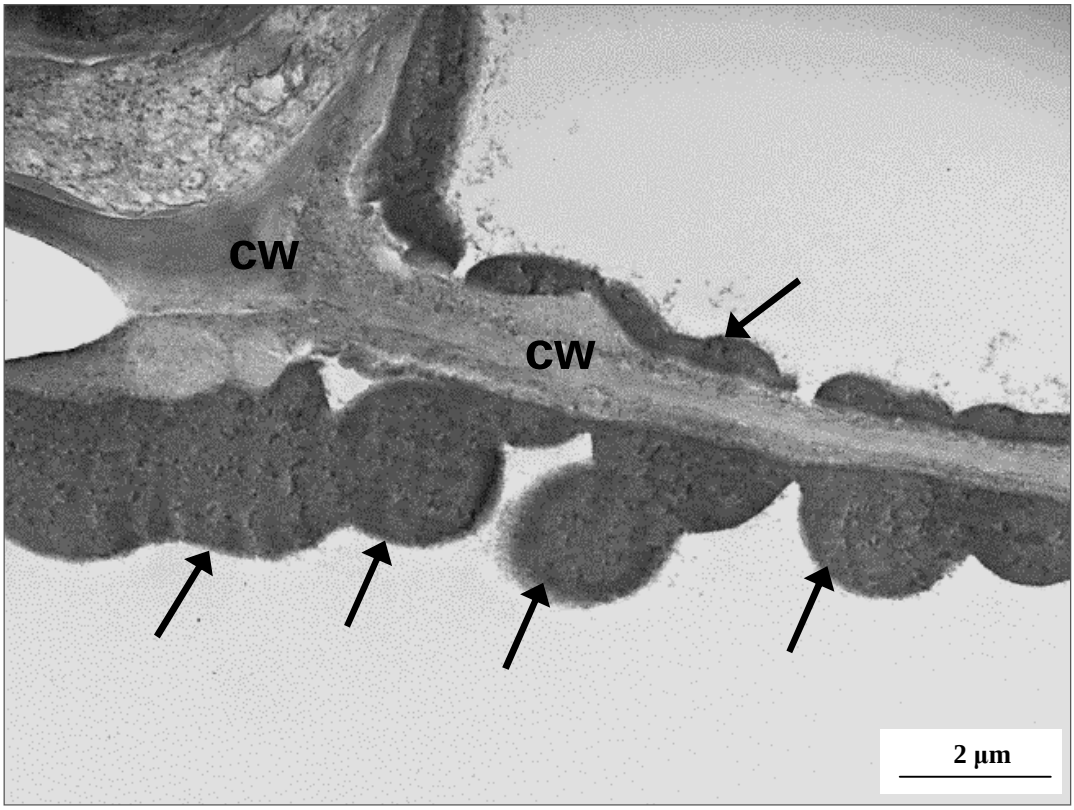

Fig. 4.33 Transmission electron micrograph of phloem parenchyma cell in *B. gymnorrhiza* in the oiled treatment showing oil deposits (arrows) closely appressed to the cell wall (cw).

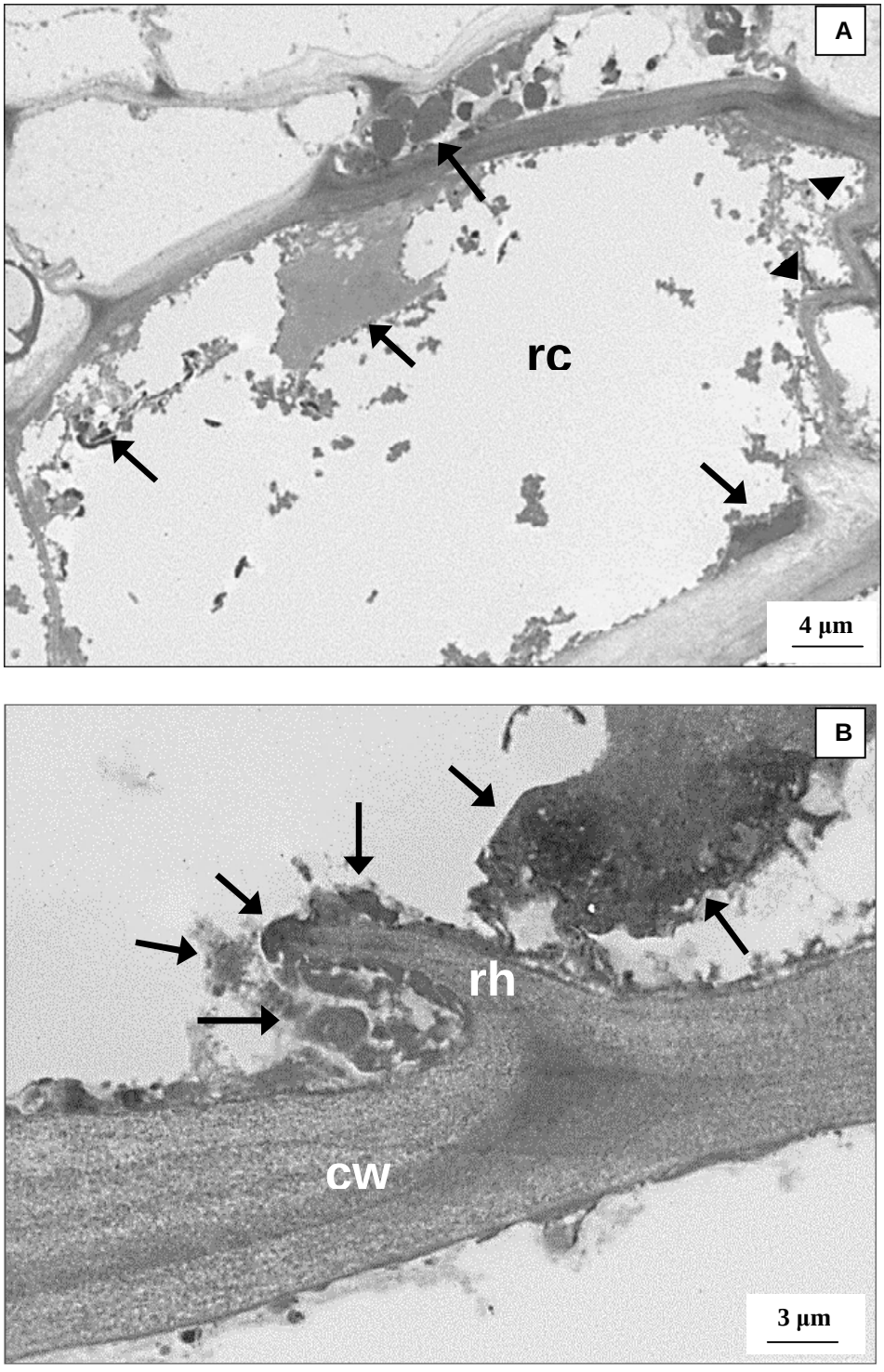

Fig. 4.34 Transmission electron micrographs of root tip cells of *R. mucronata* in the oiled treatment. (A) Root cap cell (rc) showing oil deposits (arrows). (B) Root cap cell showing root hair (rh) growing from cell wall (cw) with oil deposits around it (arrows).

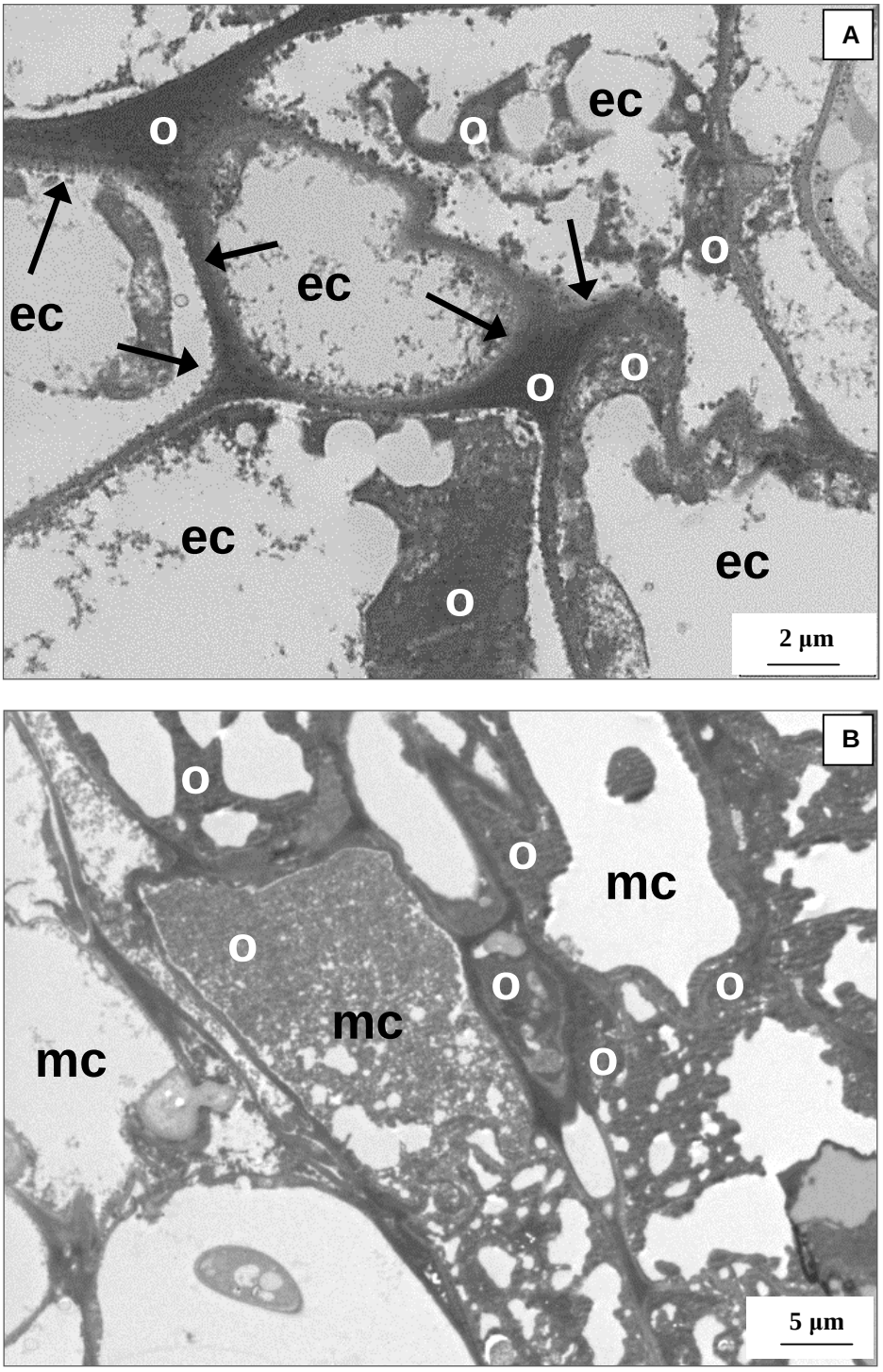

Fig. 4.35 Transmission electron micrographs of root tip cells of *R. mucronata* in the oiled treatment. (A) Epidermal cells (ec) with oil deposits (o). Note the cell walls appeared darkened with oil (arrows). (B) Cells of meristematic tissue showing oil deposits (o). Note that cells were distorted and appeared to be filled with oil.

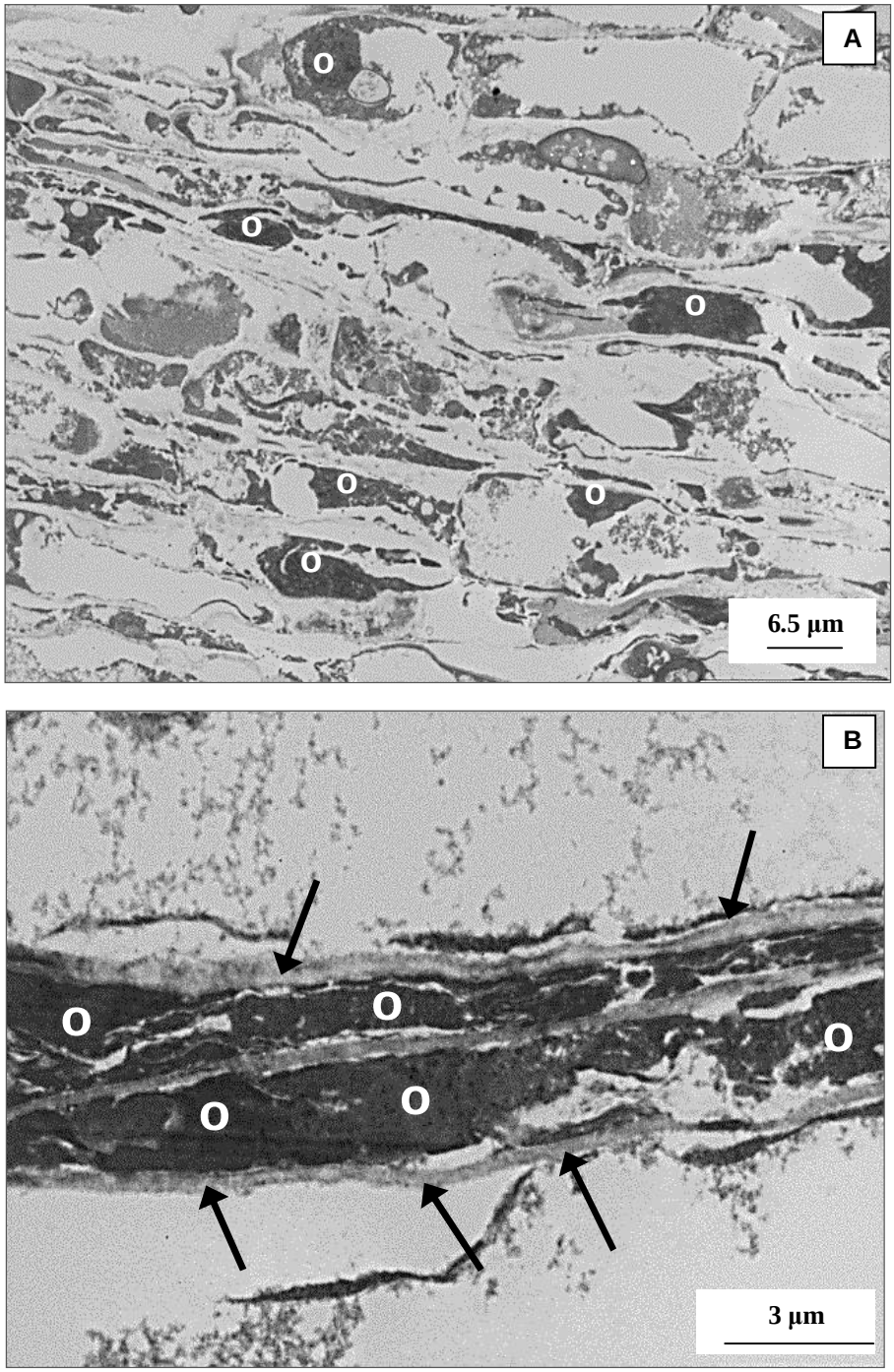

Fig. 4.36 Transmission electron micrographs of root tip cells of *R. mucronata* in the oiled treatment. (A) Phloem tissue showing deformed and distorted cells that are indistinguishable from each other. Note oil deposits (o). (B) Phloem tissue showing sieve tubes (arrows) filled with oil (o).

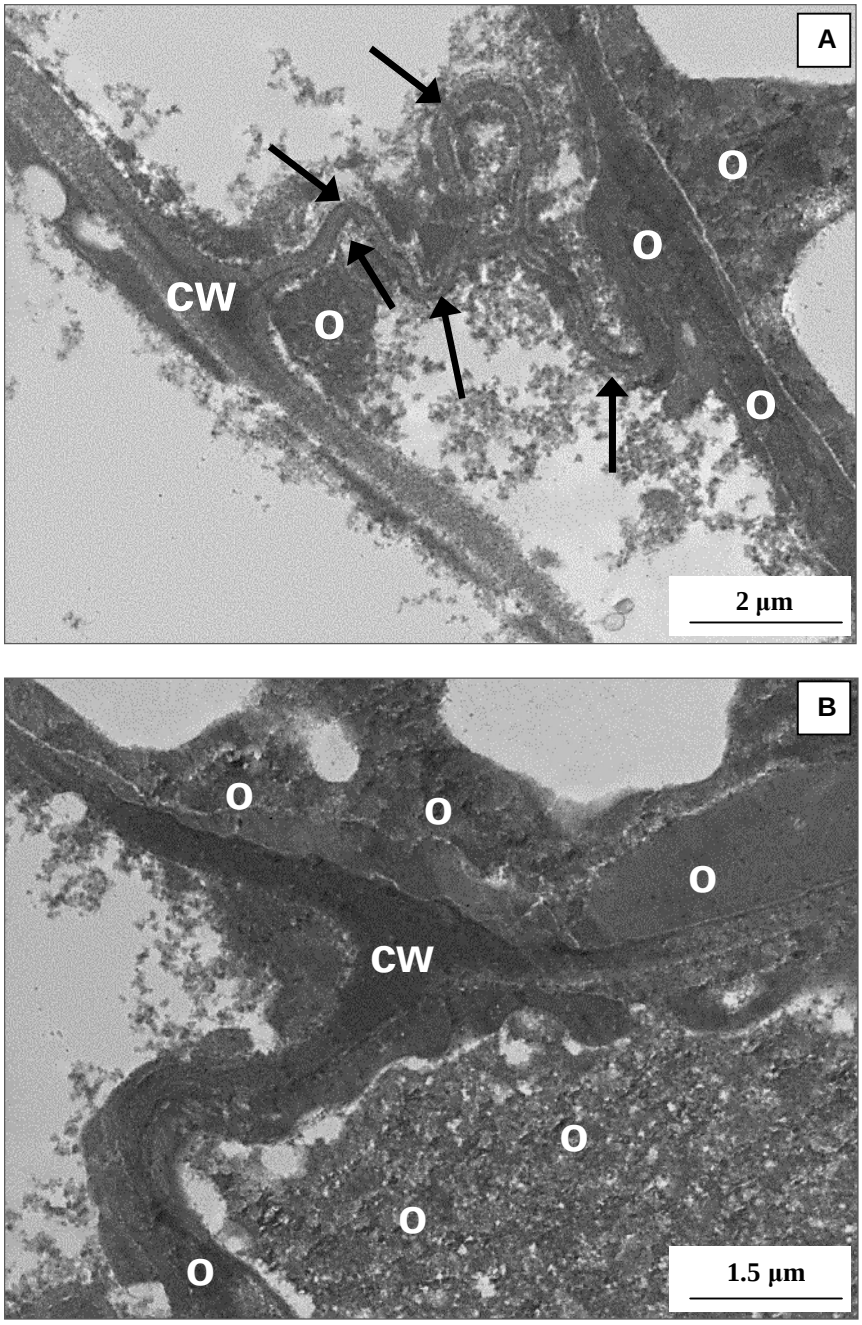

Fig. 4.37 Transmission electron micrographs of root tip cells of *R. mucronata* in the oiled treatment. (A) Phloem parenchyma cells showing oil deposits (o). Note the invaginated (arrows) cell wall (cw). (B) Phloem parenchyma cell showing close-up of oil deposits (o) within cells. Note that the cell wall appeared to be darkened by oil.

4.3.5 Transmission electron micrographs of leaf tissue

Ultrastructure of leaf cells in the control treatment

In the control, transmission electron micrographs of leaf cells in *A. marina* (Figs. 4.38 – 4.39), *B. gymnorrhiza* (Figs. 4.40 – 4.42) and *R. mucronata* (Figs. 4.43 – 4.45) showed typical healthy ultrastructure.

Palisade and spongy mesophyll cells were intact and had uniform cell walls in *A. marina* (Fig. 4.38A and B), *B. gymnorrhiza* (Figs. 4.40A and 4.42) and *R. mucronata* (Figs. 4.43A and B).

The cytoplasm formed a parietal layer against the cell wall in *A. marina* (Fig. 4.38A), *B. gymnorrhiza* (Figs. 4.40A) and *R. mucronata* (Figs. 4.43A).

Cells contained well-developed nuclei with nucleoli in *A. marina* (Fig. 4.38A), *B. gymnorrhiza* (Fig. 4.40B) and *R. mucronata* (Figs. 4.43B).

Elliptical chloroplasts populated the cytoplasm in *A. marina* (Fig. 4.39A), *B. gymnorrhiza* (Figs. 4.41B) and *R. mucronata* (Fig. 4.44A). Chloroplasts were abundant and well-developed with organized grana and integranal lamellae in *A. marina* (Fig. 4.39B), *B. gymnorrhiza* (Fig. 4.41B) and *R. mucronata* (Figs. 4.44A and 4.45).

Numerous small granules of starch were observed between lamellae in *A. marina* (Fig. 4.39B), *B. gymnorrhiza* (Fig. 4.41B) and *R. mucronata* (Figs. 4.44A and 4.45).

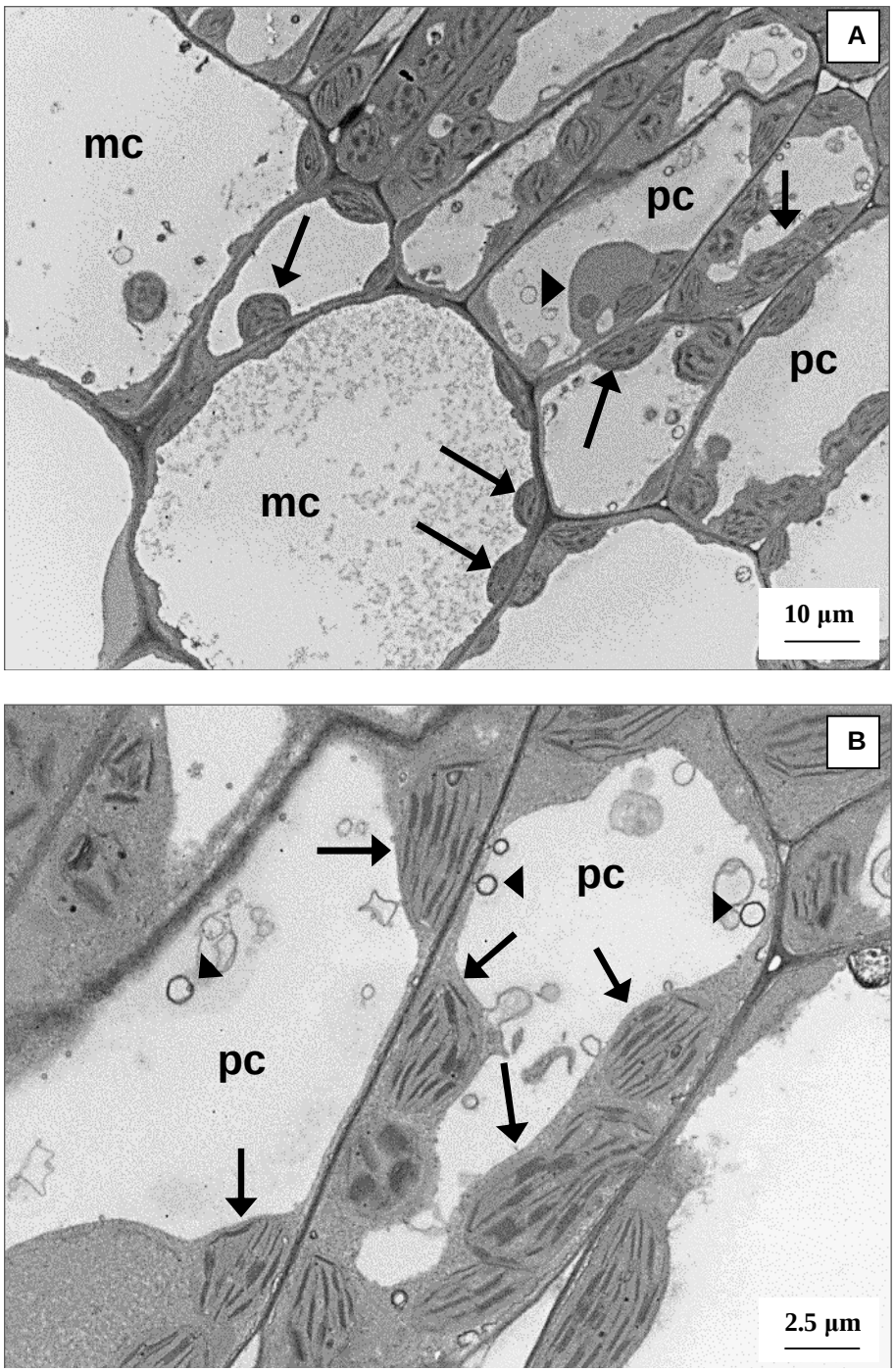

Fig. 4.38 Transmission electron micrographs of palisade and spongy mesophyll cells in leaf of *A. marina* from the control treatment. (A) Well defined palisade (pc) and spongy mesophyll (mc) cells. Note the nucleus (arrowhead) and abundance of chloroplasts (arrows). (B) Palisade cells (pc) showing several well-developed chloroplasts (arrows). Note few vesicles are present (arrowheads).

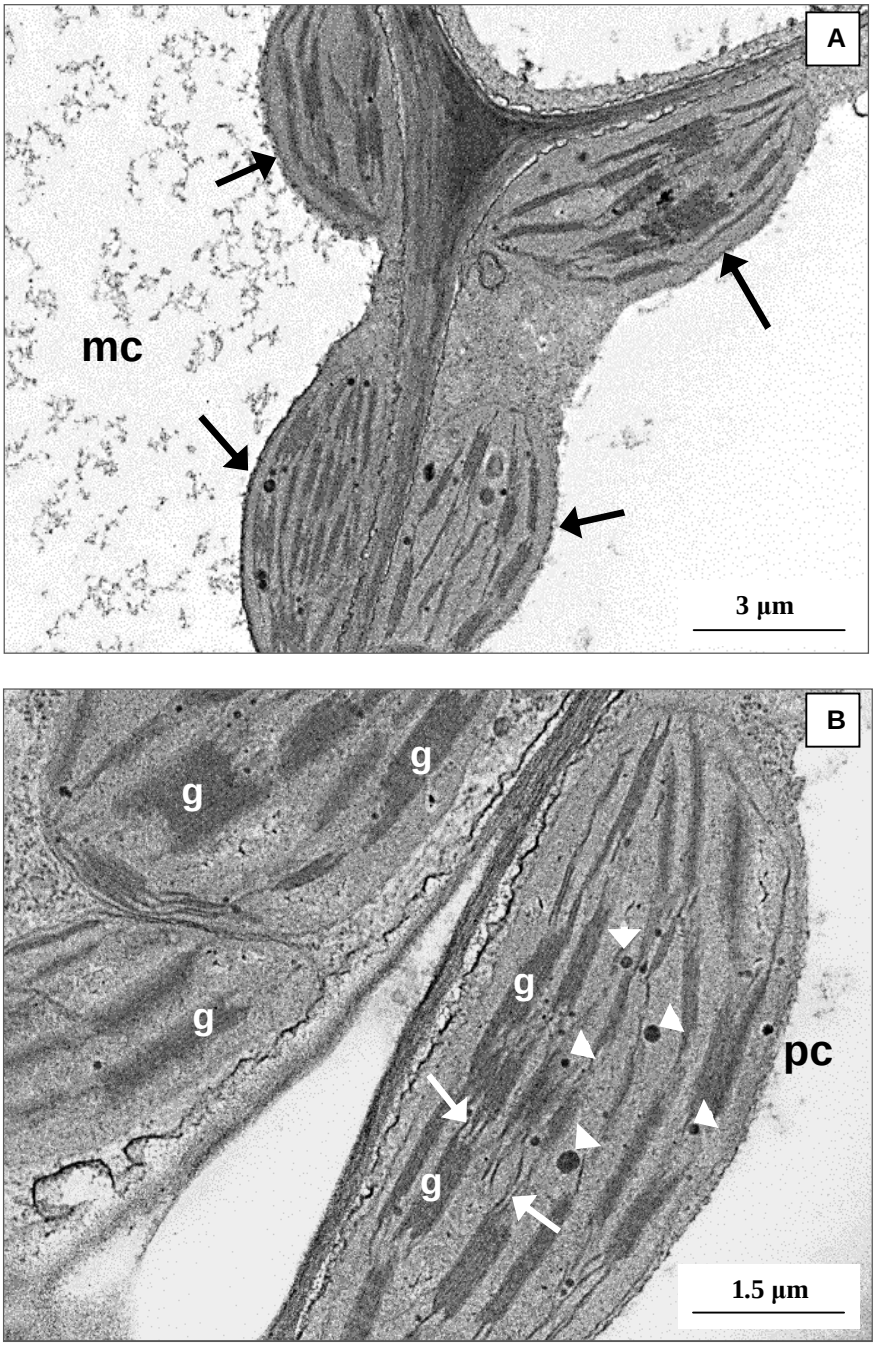

Fig. 4.39 Transmission electron micrographs of spongy mesophyll cell in leaf of *A. marina* from the control treatment. (A) Spongy mesophyll cell (mc) showing well-developed elliptical chloroplasts (arrows). (B) Chloroplast of palisade mesophyll cell (pc) showing well-defined granal stacks (g). Note several starch granules (arrowheads) are present. Note also closely appressed intergranal lamellae (arrows).

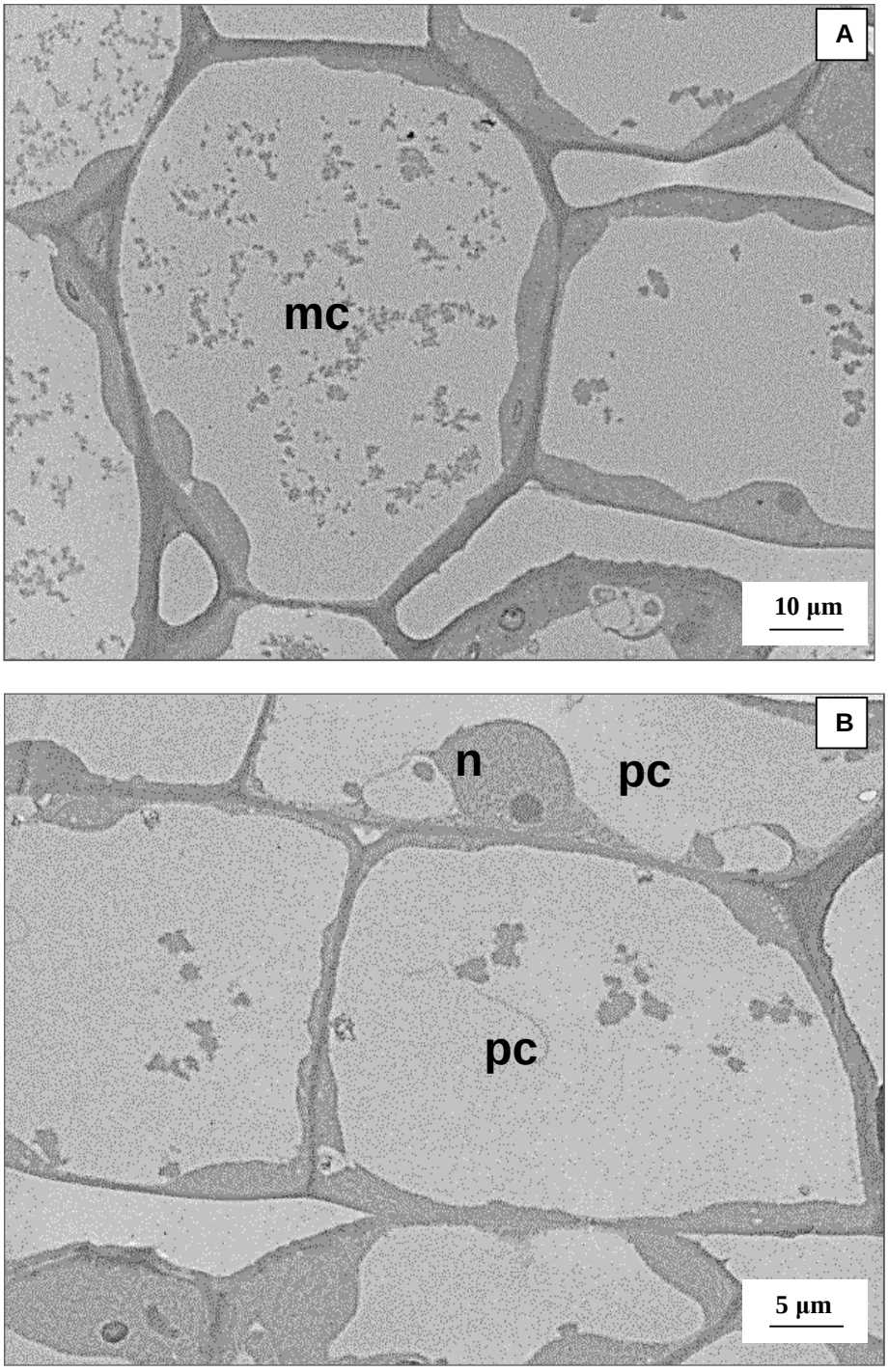

Fig. 4.40 Transmission electron micrographs of palisade and spongy mesophyll cells in leaf of *B. gymnorrhiza* from the control treatment. (A) Spongy mesophyll cell (mc). (B) Palisade mesophyll cell (pc). Note the nucleus (n).

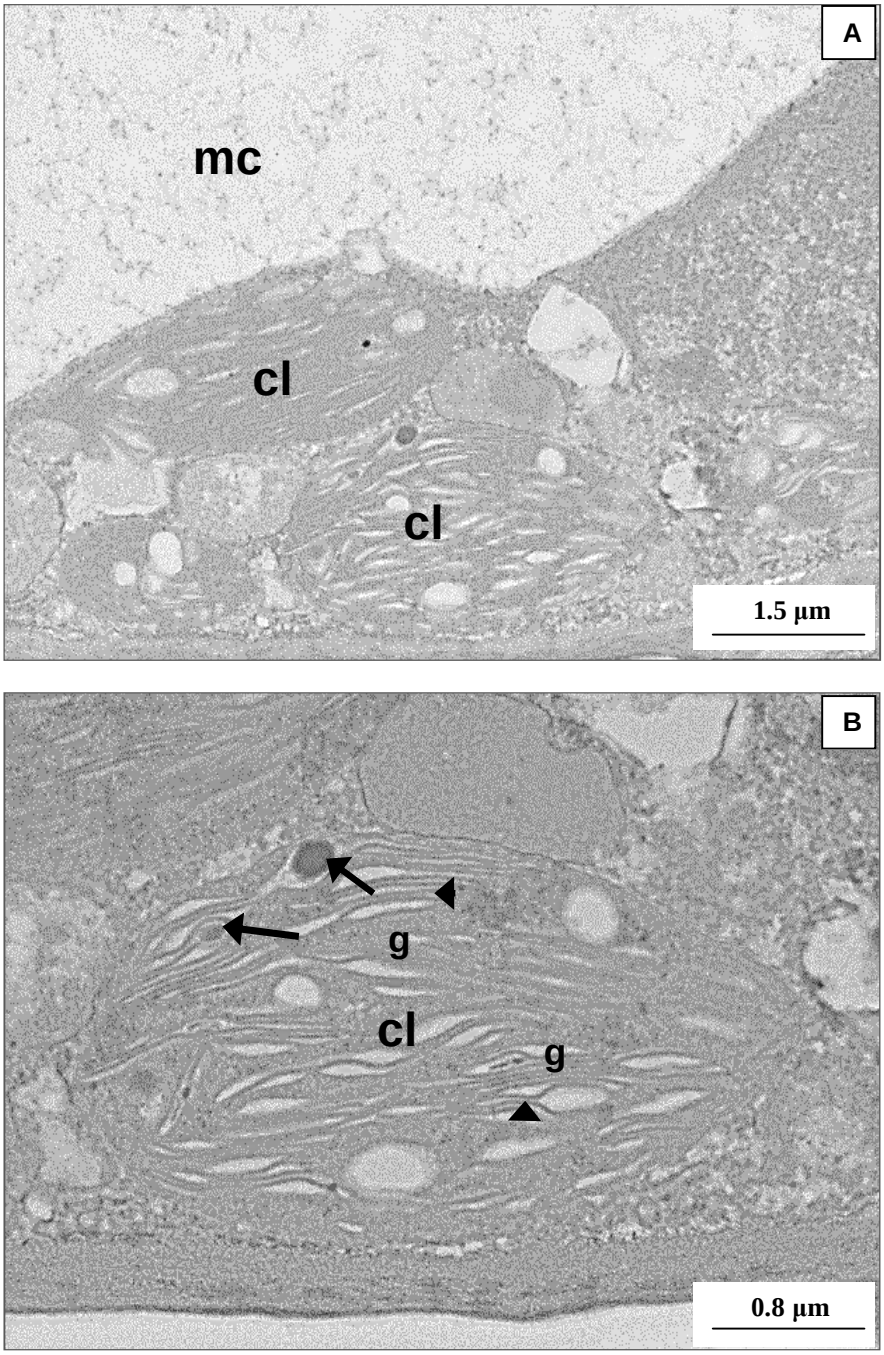

Fig. 4.41 Transmission electron micrographs of spongy mesophyll cell in leaf of *B. gymnorrhiza* from the control treatment. (A) Spongy mesophyll cell (mc) showing chloroplasts (cl). (B) Well-defined chloroplast (cl) of spongy mesophyll cell. Note starch granules (arrows), grana (g) and intergranal lamellae (arrowheads).

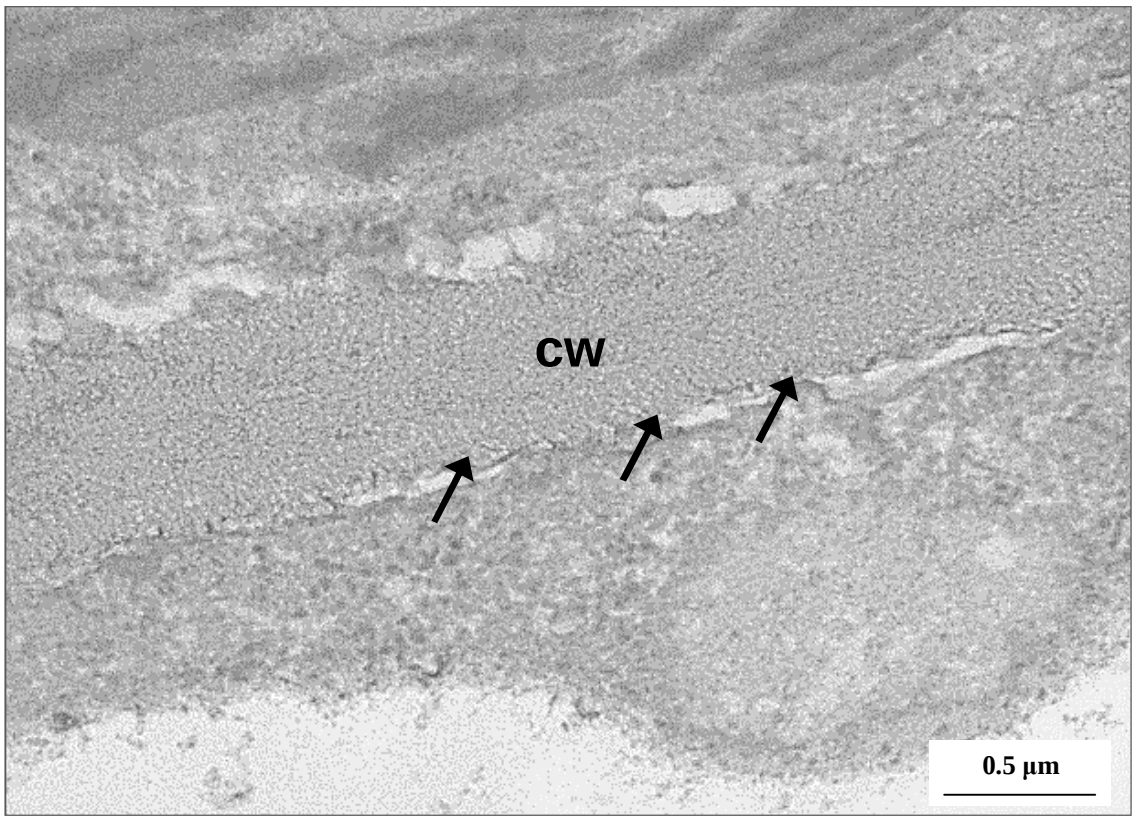

Fig. 4.42 Transmission electron micrograph of spongy mesophyll cell in leaf of *B. gymnorrhiza* from the control treatment showing well-defined cell wall (cw) and intact plasma membrane (arrows).

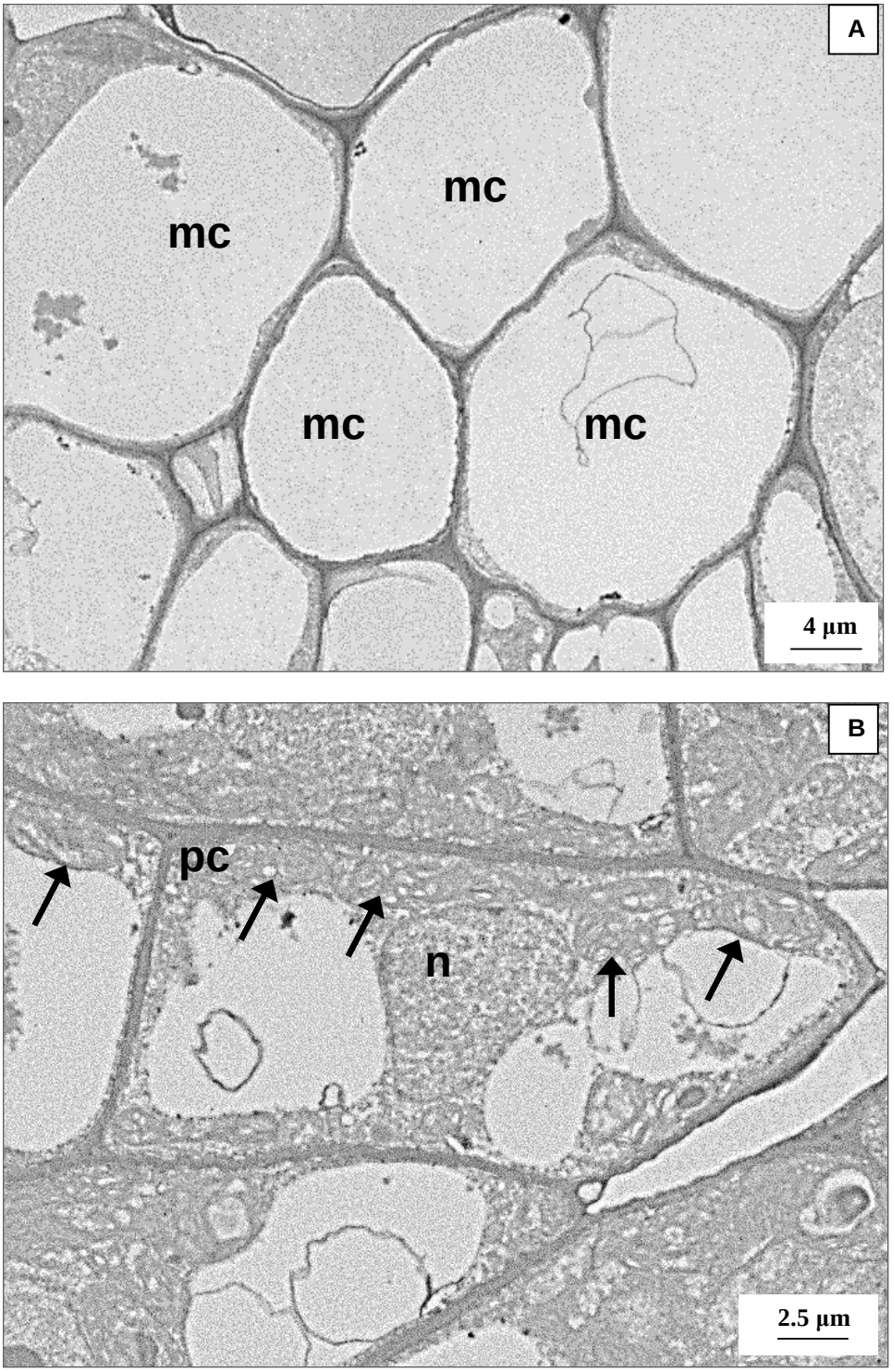

Fig. 4.43 Transmission electron micrographs of spongy mesophyll and palisade cells in leaf of *R. mucronata* from the control treatment. (A) Spongy mesophyll cells (mc). (B) Palisade mesophyll cells (pc) showing chloroplasts (arrows). Note the nucleus (n).

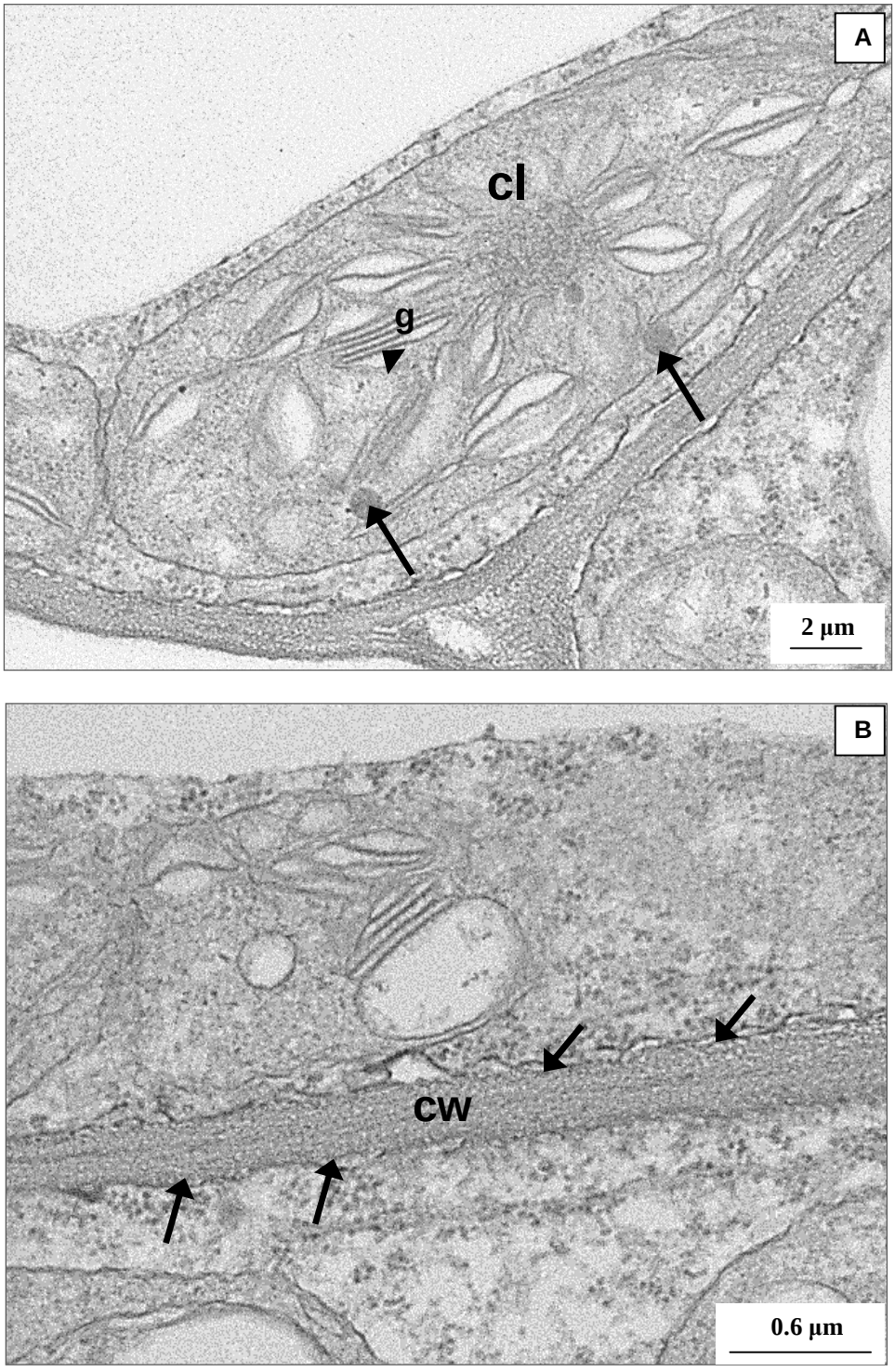

Fig. 4.44 Transmission electron micrographs of spongy mesophyll cell in leaf of *R. mucronata* from the control treatment. (A) Well-developed elliptical chloroplast (cl) showing thylakoids (arrowhead) of granum (g) and starch granules (arrows). (B) Spongy mesophyll cell showing well-defined cell wall (cw) and intact plasma membrane (arrows).

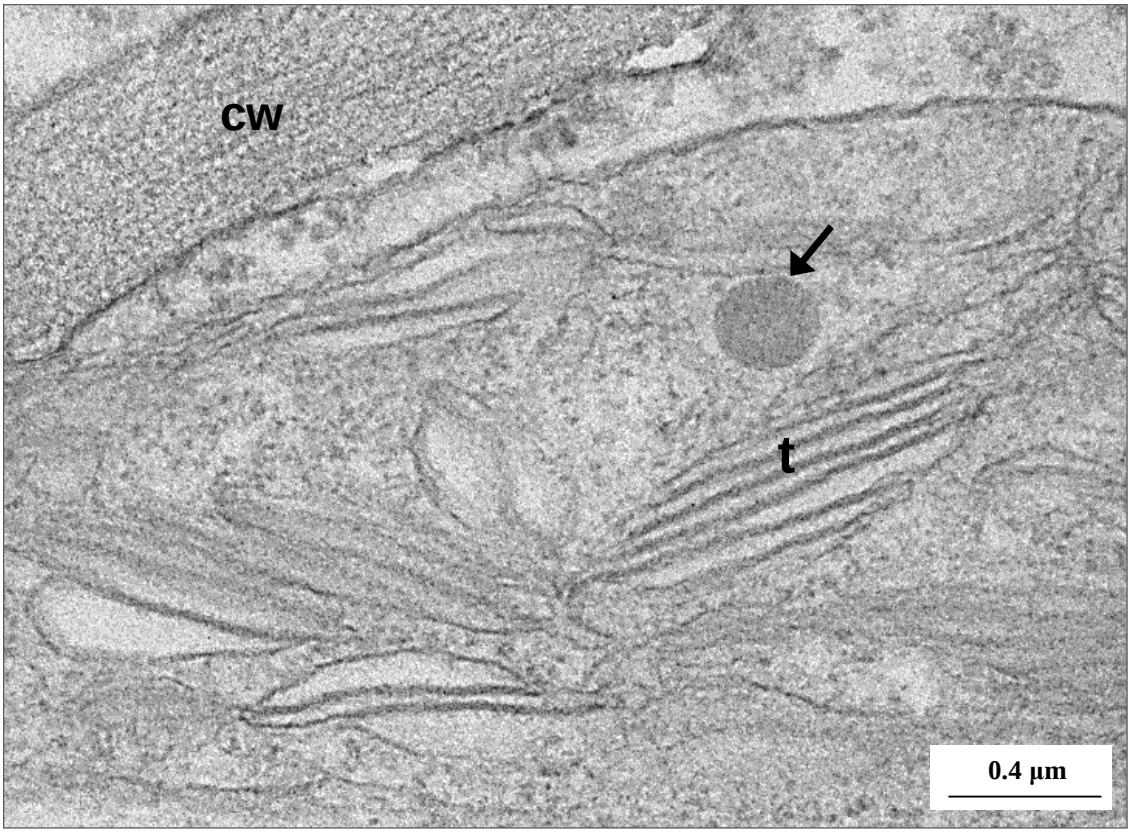

Fig. 4.45 Transmission electron micrograph of spongy mesophyll cell in leaf of *R. mucronata* from the control treatment. Note the prominent thylakoids (t) of granum and starch granule (arrow) of chloroplast adjacent to cell wall (cw).

Ultrastructure of leaf cells in the oiled treatment

In oiled treatments, transmission electron micrographs of palisade and spongy mesophyll cells from the leaves of *A. marina* (Figs. 4.46 – 4.47), *B. gymnorrhiza* (Figs. 4.48 – 4.49) and *R. mucronata* (Figs. 4.50 – 4.52) showed fine structural cell damage. Oil deposits were observed in palisade and spongy mesophyll cells in the leaves of *B. gymnorrhiza* (Fig. 4.48A) and *R. mucronata* (Fig. 4.50A). Oil deposits were observed in cell walls in *B. gymnorrhiza* (Fig. 4.49A) and *R. mucronata* (Fig. 4.52). Plasmolysis was observed in palisade cells of *B. gymnorrhiza* (Fig. 4.48B). In *R. mucronata,* multivesicular structures were visible in the cytoplasm and vacuoles of spongy mesophyll cells (Fig. 4.51A). In *A. marina,* vacuoles were large and irregular in shape in spongy mesophyll cells (Fig. 4.46).

Although no quantitative measurements were made, it was obvious that palisade and spongy mesophyll cells of leaves in the oiled treatments possessed fewer chloroplasts than those in the control. Chloroplasts were disorganized in the cytoplasm in *A. marina* (Fig. 4.46) and *R. mucronata* (Fig. 4.51B). Chloroplasts were displaced from the plasma membrane boundary in *A. marina* (Fig. 4.46) and *R. mucronata* (Fig. 4.52). The shapes of the chloroplasts were irregular in the oiled treatment in *R. mucronata* (Fig. 4.51B) compared to those in the control. Chloroplasts appeared to be dilated, distorted and degraded in *A. marina* (Fig. 4.47A and B), *B. gymnorrhiza* (Fig. 4.49B) and *R. mucronata* (Figs. 4.51B and 4.52).

Oil accumulated in chloroplasts in *R. mucronata* (Figs. 4.51 – 4.52). The ultrastructure of the photosynthetic apparatus, i.e. granal stacks and lamellae, appeared to be disorganized and disintegrated in *A. marina* (Fig. 4.47B), *B. gymnorrhiza* (Fig. 4.49B) and *R. mucronata* (Fig. 4.52). Thylakoids appeared to be dilated and lamellar separation was observed in *A. marina* (Fig. 4.47A and B), *B. gymnorrhiza* (Fig. 4.49B) and *R. mucronata* (Fig. 4.52). Granal stacks separated and thylakoids and intergranal lamellae appeared to be abnormally sinuous and not closely appressed to each other in *B. gymnorrhiza* (Fig. 4.49B) and *R. mucronata* (Fig. 4.52). In addition, large spaces were observed between grana and lamellae in chloroplasts in *A. marina* (Fig. 4.47A and B) and *B. gymnorrhiza* (Fig. 4.49B). The chloroplasts in *A. marina* (Fig. 4.47B), *B. gymnorrhiza* (Fig. 4.49B) and *R. mucronata* (Fig. 4.52) contained few or no starch granules.

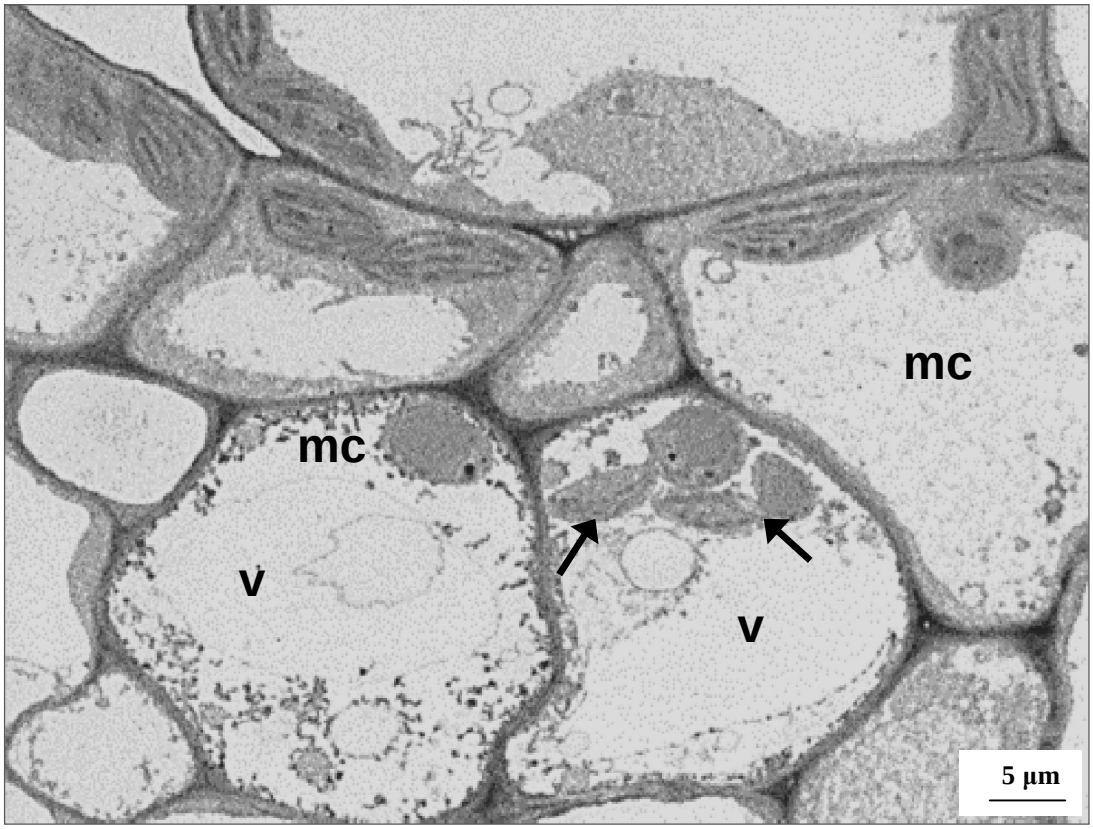

Fig. 4.46 Transmission electron micrograph of spongy mesophyll cells (mc) in leaf of *A. marina* from the oiled treatment. Note large vacuoles (v) and disorganization of chloroplasts (arrows).

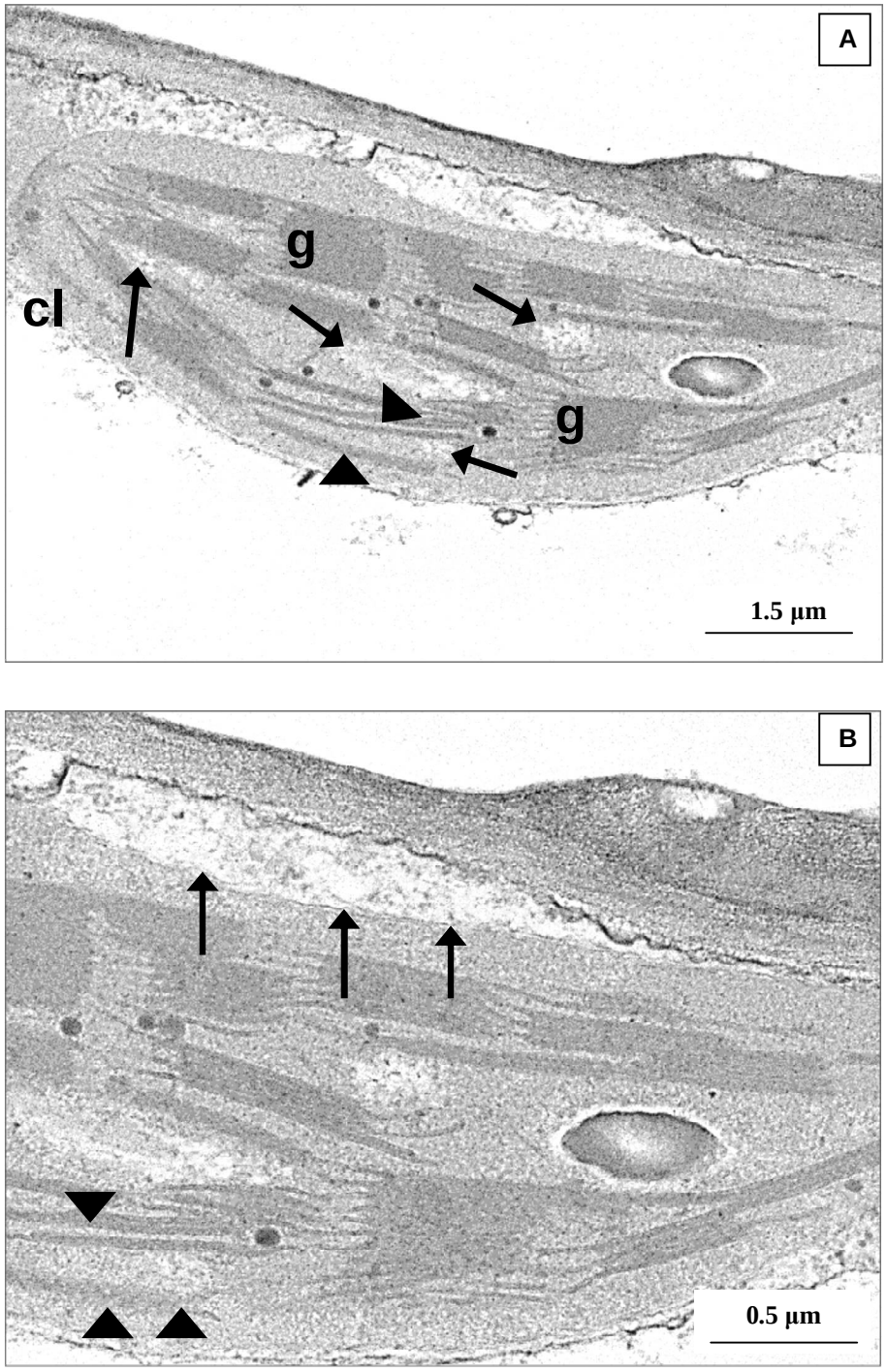

Fig. 4.47 Transmission electron micrographs of a chloroplast in spongy mesophyll cell in leaf of *A. marina* from the oiled treatment. (A) Chloroplast (cl) showing spaces (arrows) between grana and lamellae. Note lamellar separation (arrowheads). (B) Spongy mesophyll cell showing displacement of chloroplast from the plasma membrane (arrows). Note lamellar separation (arrowheads).

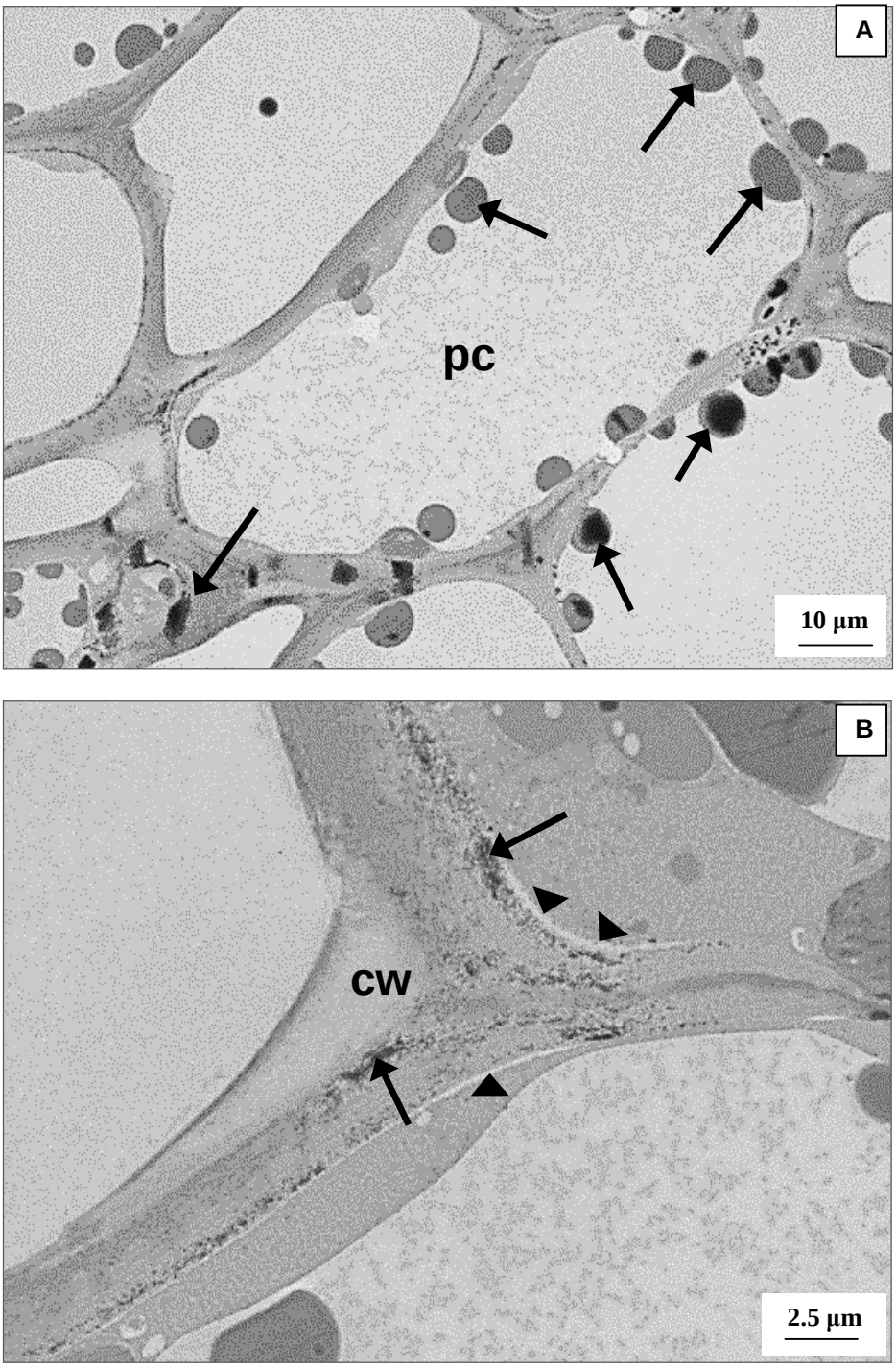

Fig. 4.48 Transmission electron micrographs of palisade mesophyll cells in leaf of *B. gymnorrhiza* from the oiled treatment. (A) Palisade mesophyll cell (pc) showing oil deposits (arrows). (B) Palisade mesophyll cell showing cell wall (cw) with oil deposits (arrows). Note plasmolysis (arrowheads).

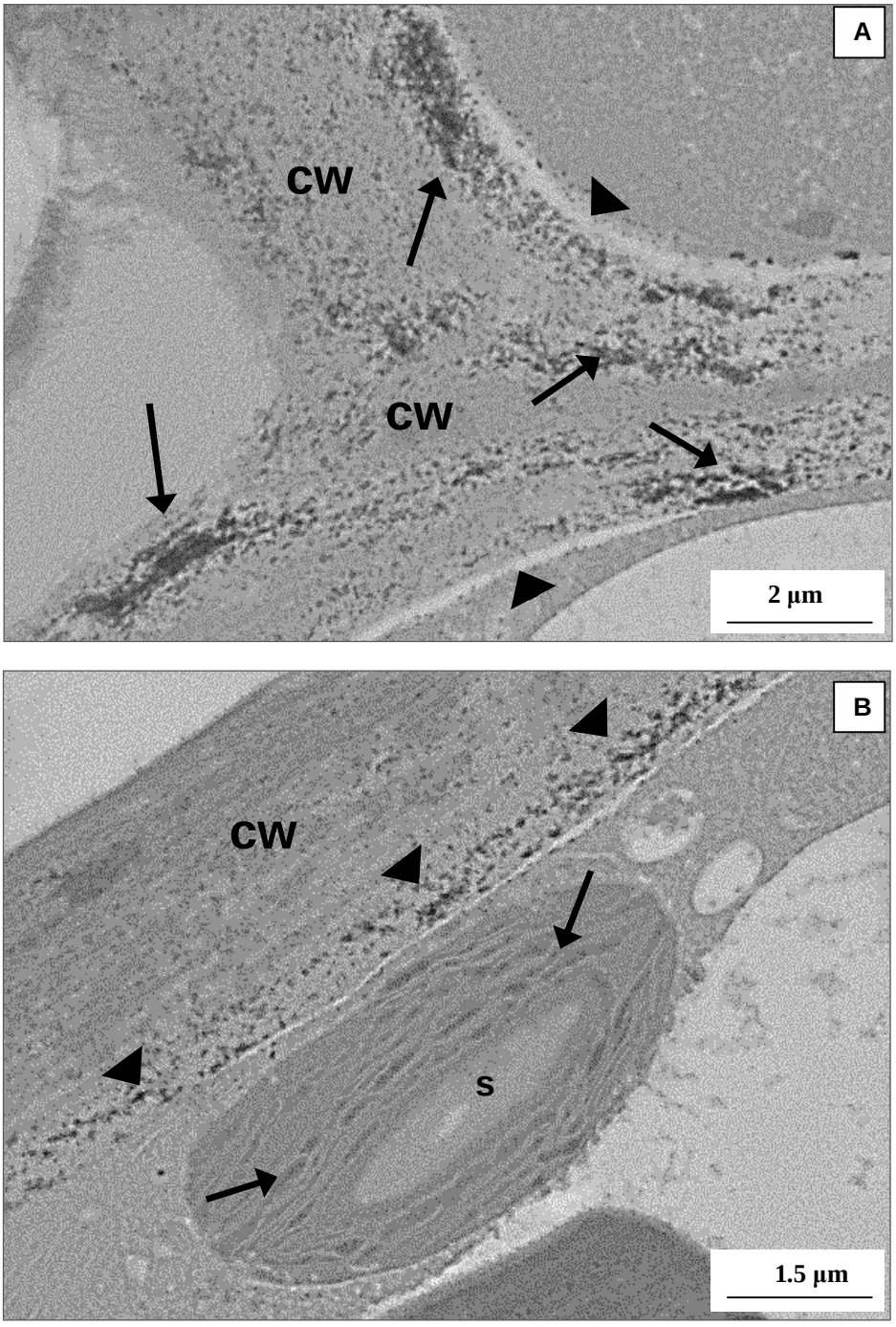

Fig. 4.49 Transmission electron micrographs of palisade mesophyll cells in leaf of *B. gymnorrhiza* from the oiled treatment. (A) Palisade mesophyll cell showing oil deposits (arrows) inside cell wall (cw). Note plasmolysis (arrowheads). (B) Chloroplast showing disintegrated grana (arrows) and large space (s) at the centre. Note oil deposits (arrowheads) in cell wall (cw). Note also the absence of starch granules.

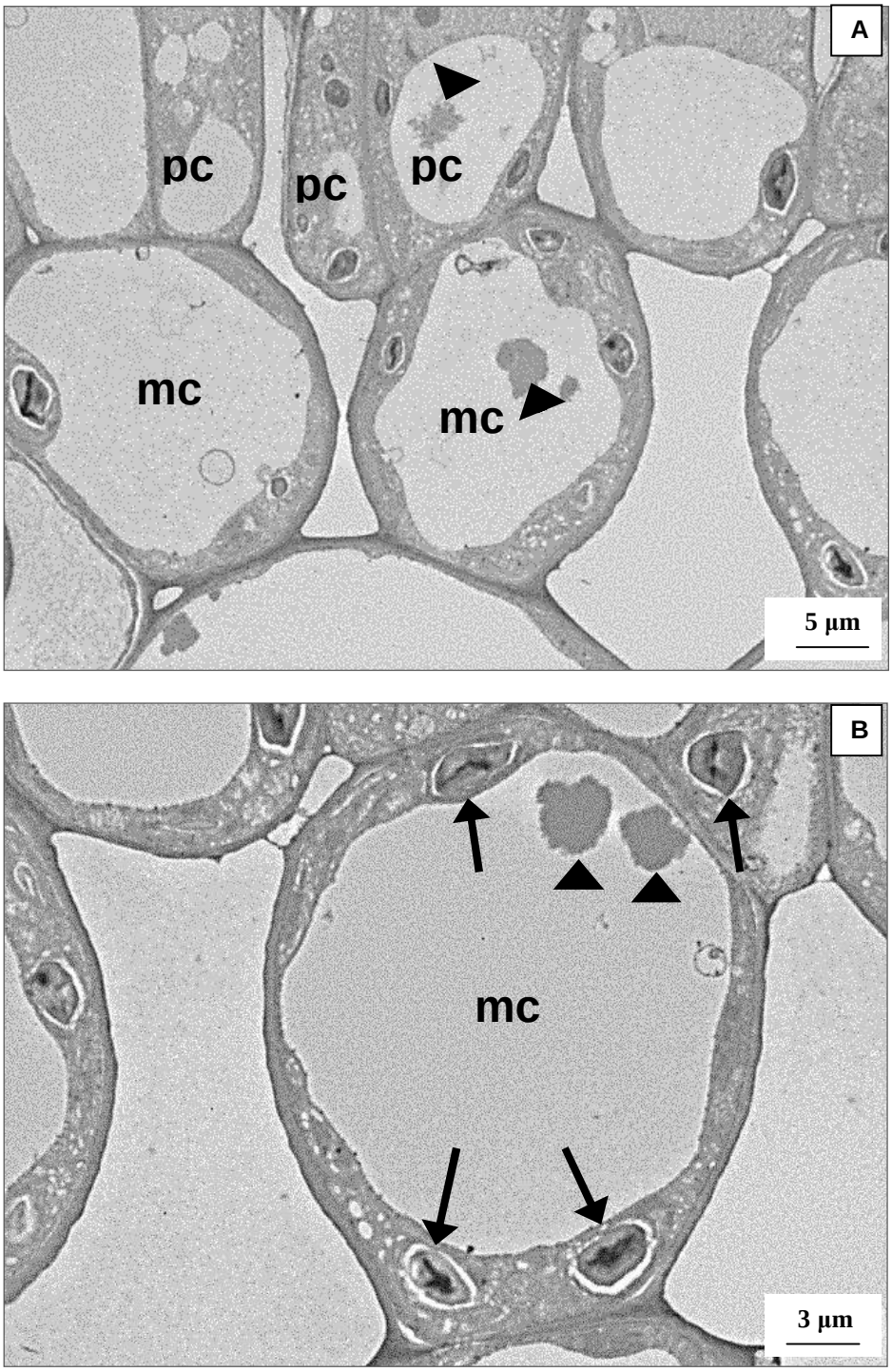

Fig. 4.50 Transmission electron micrographs of leaf cells of *R. mucronata* from the oiled treatment. (A) Spongy mesophyll (mc) and palisade cells (pc) showing oil deposits (arrowheads). (B) Spongy mesophyll cell showing oil deposits (arrowheads). Note oil deposits within chloroplasts (arrows).

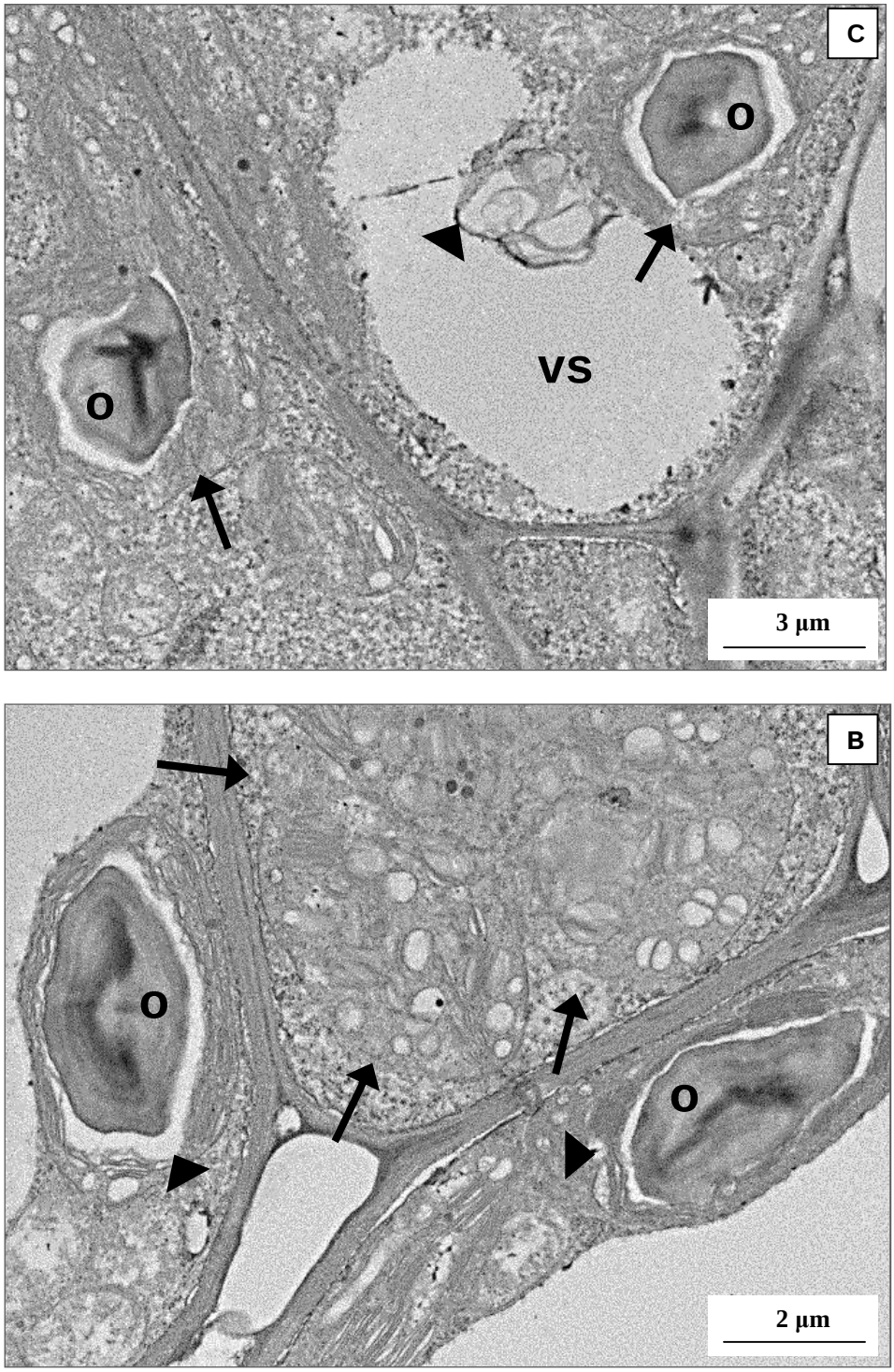

Fig. 4.51 Transmission electron micrographs of spongy mesophyll cell in leaf of *R. mucronata* from the oiled treatment. (A) Chloroplasts (arrows) showing oil deposits (o). Note multivesicular structure (arrowhead) occupied vacuolar space (vs). (B) Spongy mesophyll cell showing disorganized aggregated chloroplasts (arrows). Note the large oil deposits (o) within chloroplasts (arrowheads). Note the irregular shapes of chloroplasts (arrowheads).

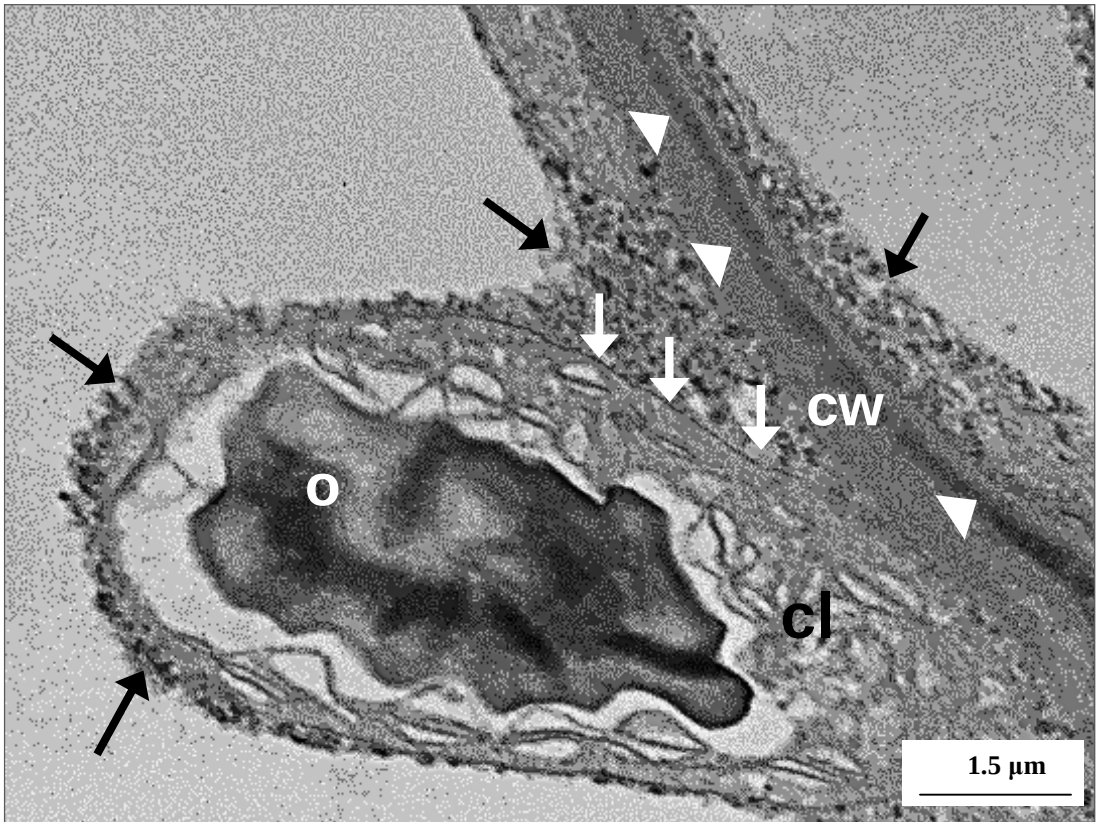

Fig. 4.52 Transmission electron micrograph of a chloroplast of spongy mesophyll cell in leaf of *R. mucronata* from the oiled treatment. Note the large oil deposit (o) making up the bulk of the volume of the chloroplast (cl). Note oil deposits (arrowheads) within cell wall (cw). Note also that oil deposits (black arrows) surrounded the chloroplast (cl). Note the displacement of the chloroplast (cl) from the plasma membrane (white arrows).

4.4 Discussion

This study demonstrated that PAHs entered mangrove roots and were translocated to shoots. Previous studies reported that oil was translocated from roots and accumulated in shoots of grasses (Parrish *et al*., 2006), vegetables (Jiao *et al*., 2007) and the mangrove *Kandelia candel* (L.) (Hong *et al*., 2009).

Concentrations of PAHs in roots and leaves

In oiled treatments, roots and leaves of all three species had high concentrations of low molecular weight PAHs (i.e. with two or three rings). Low molecular weight PAHs dissolve in water and enter plant roots (Wang *et al*., 2012). In oiled treatments, roots of all three species had a higher concentration of phenanthrene (three rings) than all other PAHs. Low molecular weight PAHs occur in higher concentrations in plant tissues because of their greater vapour pressure, water solubility and bioavailability (Jánská *et al*., 2006; Sojinu *et al*., 2010). Kang *et al*. (2010) demonstrated that phenanthrene had a high uptake and accumulation in ryegrass because it moved rapidly through the transpiration stream from roots to shoots.

In oiled treatments, PAHs with four and five rings were detected in roots of all three species. High molecular weight PAHs (i.e. four rings or more) are mostly associated with soil organic matter (Simonich and Hites, 1995; Sojinu *et al*., 2010). The strong adsorption of high molecular weight PAHs onto the root epidermis is suggested to prevent them from being drawn into the conducting tissue (Kipopoulou *et al*., 1999). However, benzo(a)pyrene (five rings), for example, which is listed as a persistent bioaccumulative and toxic chemical (Wilson and Jones, 1993; Binet *et al*., 2001), was found in oiled treatments within the roots of all species and also in the leaves of *A. marina*, showing that it is mobile and translocated. Previous studies reported high molecular weight PAHs in roots and shoots of saltmarsh plants (Meudec *et al*., 2006) and vegetables (Fismes *et al*., 2002).

In roots of oiled seedlings of *A. marina*, the total concentration of PAHs was about four times higher than those of *B. gymnorrhiza* and six times higher than *R. mucronata* (Table 4.4). This was probably due to oil exclusion in *B. gymnorrhiza* and *R. mucronata* (Suprayogi and Murray, 1999; Ke *et al*., 2011a). In oiled treatments, the roots of *B.*

gymnorrhiza had almost double the concentration of PAHs than those of *R. mucronata*. Additionally, oiled roots of *B. gymnorrhiza* had a higher concentration of pyrene (four rings) than *R. mucronata*. In a previous study, oiled *B. gymnorrhiza* accumulated more pyrene in its roots than *K. obovata* (Ke *et al*., 2003). Plants can exclude, metabolise or degrade PAHs (Wittig *et al*., 2003; Baek *et al*., 2004). The roots of *Rhizophora* sp. are known to exclude salt and organic compounds, including PAHs (Suprayogi and Murray, 1999; Ye and Tam, 2007). Lower bioaccumulation of PAHs in *R. mucronata* than the other two species could further be attributed to root exudation (Holmer *et al*., 1999). These exudates increase microbial activity around the roots which degrade PAHs in soil (Moreira *et al*., 2011).

The higher total concentration of PAHs in the roots of oiled *A. marina* compared to the other two species, was probably due to the ability of this species to synchronously take up salt and organic compounds (Suprayogi and Murray, 1999; Ye and Tam, 2007). The roots of *A. marina* probably have a greater ability to draw PAHs from the sediment through the transpiration stream compared to the two other species (Ke *et al*., 2011a). This characteristic probably enables greater accumulation of PAHs in tissues after root penetration (Ke *et al*., 2011a).

The roots of oiled *A. marina* also had a higher concentration of each of the 15 PAHs than the other two species. Pi *et al*. (2009) found that *A. marina* possessed the largest air spaces between aerenchyma cells in the root compared to seven other mangroves including *B. gymnorrhiza*. Their study also showed that *A. marina* possessed a thinner epidermis and hypodermis that provided a weak barrier against radial oxygen loss compared to other mangroves. In leaves of oiled *A. marina*, the total concentration of PAHs was 26 and 23 times higher respectively than those in *B. gymnorrhiza* and *R. mucronata* respectively (Table 4.4). In a previous study, *A. marina* was found to accumulate between two and six times more PAHs in the leaves than other mangroves including *R. mucronata* (Suprayogi and Murray, 1999). Furthermore, four and five ring PAHs were detected in leaves of oiled *A. marina* plants but not in the other two species. The larger air spaces, together with thinner epidermis and hypodermis, could account for the greater penetration and accumulation of PAHs in *A. marina* compared to the other two species.

In oiled treatments, leaves of *B. gymnorrhiza* only had four PAHs compared to 12 in *A. marina* and six in *R. mucronata*. The roots of *B. gymnorrhiza* plants contain closely packed root aerenchyma cells with smaller air spaces (Pi *et al.*, 2009). Furthermore, the root tips of *B. gymnorrhiza* have a thick epidermis that consists of greatly suberized cells that bind PAHs (Ke *et al*., 2003; Pi *et al.*, 2009). These characteristics restrict PAH entry into the roots of *B. gymnorrhiza* (Ke *et al*., 2011a) accounting for the lower accumulation.

Control treatments of all species had negligible concentrations of PAHs with two (naphthalene and acenaphthene) and three (phenanthrene) rings. The control and oil-treated plants in this experiment were grown in the glasshouse. As a precautionary measure, control and oil-treated plants were separated by about 3 m. Despite these efforts, low molecular weight PAHs could have entered control plants via the atmosphere, similar to previous studies (Watts *et al*., 2008; Desalme and Binet, 2011; Wang *et al*., 2012). Low molecular weight PAHs volatise and enrich the air even at a distance from oil-contaminated sediment (Wang *et al*., 2009; Yang *et al*., 2010) and enter foliage via stomata (Fismes *et al*., 2002; Yousseff and Ghanem, 2002) or diffusion through the epidermis (Wild *et al*., 2005).

In this study, the concentrations of PAHs in plant tissues varied amongst the three mangroves but identities of the compounds were identical. This suggests that the mechanisms controlling the distribution of PAHs in mangroves are similar. Similar findings were reported for the grasses, *Phragmites australis* and *Spartina alterniflora* and the sedge, *Scirpus mariqueter Tang. i*n an investigation of PAH accumulation in sediment oiled treatments (Wang *et al*., 2012).

Living and dead root tip cells

Fluorescein diacetate and propidium iodide were used to test for living and dead root tip cells. In control treatments, fluorescein diacetate exhibited bright green fluorescence in living root tip cells of all three species. Living cells have intact plasma membranes (Jones and Senft, 1985). Fluorescein diacetate is lipophilic (i.e. has an affinity for lipids) and membrane-permeable (Lassailly *et al*., 2010). Living cells are distinguished by the presence of intracellular esterases that hydrolyse fluorescein diacetate (Breeuwer *et al*., 1995; Battin, 1997). Fluorescein diacetate is hydrolyzed to highly polar fluorescent

compounds within living plant cells (Truernit and Haseloff, 2008). The polar nature of the hydrolyzed fluorescein diacetate makes it unable to diffuse across the plasma membrane and it is retained within living cells, thus exhibiting bright green fluorescence in the cytoplasm (Jones and Senft, 1985).

In oiled treatments, propidium iodide exhibited bright red fluorescence in dead root tip cells of all three species. Dead cells have non-intact or damaged plasma membranes (Jones and Senft, 1985; Steinkamp *et al*., 1999; Truernit and Haseloff, 2008). Propidium iodide can only enter cells with damaged membranes, whereupon it intercalates into double-stranded nucleic acids exhibiting bright red fluorescence (Steinkamp *et al*., 1999). In oiled treatments, the majority of root tip cells of all three species exhibited bright red fluorescence indicating that PAHs penetrated cell walls and membranes thereby killing cells.

The effects of oil on ultrastructure of root tip cells

Oiling of the sediment resulted in the disorganisation of root tip cells in all three species. The effects of oil on cell ultrastructure were similar in all three species. Various regions of the root including the root cap, meristem, cortex, and conducting tissue were adversely affected by oil.

Oil deposits accumulated in root tip cells. All PAHs adsorb onto roots first and then gradually diffuse into subcellular fractions of tissues (Alkio *et al*., 2005) before accumulating in cell walls, vacuoles and organelles (Gao and Zhu, 2004; Wild *et al*., 2005). Oil deposits filled the cytoplasm, vacuoles and cell walls. Anthracene was detected in epidermal cell walls and cytoplasm using TPEM (Two-Photons Excitation Microscopy) (Wild *et al*., 2005).

Electron micrographs of root tips in oiled treatments showed darkened cell walls and plasmodesmata were not visible as they were probably filled with oil deposits making them unrecognizable. PAHs are known to move through cells via plasmodesmata (Orcutt and Nielsen, 2000). Oil deposits infiltrated adjacent cells through deteriorated cell walls and membranes. Cell contents leaked out of cells with damaged cell walls and plasma membranes. The *lipophilic nature of PAHs* (Orcutt and Nielsen, 2000), *could have caused them to gravitate towards lipid components of the cell wall and*

membrane. PAHs accumulate in the plasma membrane and displace lipid molecules increasing membrane permeability (Gilfillan *et al*., 1989).

Furthermore, oil caused the fragmentation of organelles such as the nucleus, mitochondrion and endoplasmic reticulum. Similar effects were reported for maize root tip cells treated with nitrobenzene (Zaalishvili *et al*., 2002). The nucleus was penetrated by oil and nuclear membranes were invaginated and perforated. These nuclear abnormalities which are due to the toxic mutagenic effects of PAHs (Aina *et al*., 2006), leads to the death of cells (Fernandes *et al*., 2007). Oil caused nuclear abnormalities that led to the death of root cells of *Allium cepa* L. (Leme *et al*., 2008).

Phloem tissue was extensively damaged and cells were indistinguishable from each other. On the other hand, xylem tissue exhibited minimal damage from oiling. In a previous study on maize roots contaminated by lubricating oil, xylem tissue was found to be more intact than phloem (Grijalbo *et al*., 2013). In another study, bunker fuel oil applied to the base of debarked stems of *A. marina* resulted in the production of adventitious roots which was an adaptive response after damage to the phloem tissue (Naidoo *et al*., 2010). Scalariform thickenings of xylem vessel members probably reduced oil damage.

In all species, sieve tubes and parenchyma cells of phloem tissue accumulated large deposits of oil, due probably to the affinity of PAHs for lipids. Phloem exhibited greater adverse oil effects than the xylem. Phloem is a living tissue with cellular components and organelles that all contain lipid (Gao and Zhu, 2004; Kang *et al*., 2010). Xylem on the other hand is a dead tissue (Chen *et al*., 2013). As PAHs move through the conducting tissue apoplastically, they have an affinity for the lipid content of closely associated tissues (Meudec *et al*., 2006). Gao and Zhu (2004) found that the uptake of PAHs was dependent on the lipid content of plant roots. Previous studies demonstrated that PAHs accumulated in plant tissues containing high lipid content (Meudec *et al*., 2006; 2007). These studies showed that cellulose tissues, cell walls, membranes and all organelles containing lipid components, are primary domains for PAH accumulation after they enter root cells. Kang *et al*. (2010) found that the lipid content of cell organelles determined the degree of PAH accumulation.

The effects of oil on ultrastructure of leaf cells

Oil deposits were observed in leaf palisade and spongy mesophyll cell walls and cytoplasm in all three species. Aromatic hydrocarbons in vehicle emissions (e.g. benzene and xylene) damaged the mesophyll cells of spruce needles (Viskari *et al.*, 2000). Oil caused disorganization of chloroplasts in the cytoplasm. Chloroplasts are the first organelles to exhibit symptoms of physiological stress (Alscher *et al*., 1997). In this study, chloroplasts were degraded, distorted, dilated and irregularly-shaped. In previous studies, PAHs had adverse effects on the photosynthetic apparatus (Naidoo *et al*., 2010; Yin *et al*., 2011) and caused chlorophyll disintegration (Huang *et al*., 1996). Furthermore, chloroplasts were displaced from the plasma membrane boundary. In maize plants, lubricating oil caused degradation of the chloroplast structure and plasmolysis of the chloroplast membrane (Grijalbo *et al*., 2013). In this study, chloroplast lamellae were separated by oil and large spaces were observed between lamellae and grana. This indicated break down of the photosynthetic apparatus.

In *R. mucronata*, oil accumulated in chloroplasts and occupied the bulk of their volume resulting in irregular shapes. In this study, PAHs in leaf cell walls probably moved to and accumulated within chloroplasts. Kang *et al*. (2010) found that PAHs in the cell wall moved to and accumulate in organelles because of their high lipid content. The uptake and accumulation of PAHs by plants can also be related to the carbohydrate content of cells (Gao and Zhu, 2004; Zhang and Zhu, 2009). In chloroplasts, starch granules with high carbohydrate content (Kasperbauer and Hamilton, 1984) probably contributed to oil accumulation.

This study demonstrated that oil accumulated in root and leaf cells resulting in the disorganisation of organelles such as mitochondria, nuclei and chloroplasts. These organelles are responsible for cell vitality and are the sites of energy formation.

Chapter 5

Responses of *A. marina*, *B. gymnorrhiza* and *R. mucronata* to oil contamination at different salinities

Abstract

In this study, the responses of *A. marina*, *B. gymnorrhiza* and *R. mucronata* to oil at different salinities were investigated under glasshouse conditions. Healthy one year old seedlings were subjected to sediment oiling at 10% and 50% seawater for 12 months. In oiled treatments, 200 ml oil were added to the soil in each pot. Growth was better at 10% compared to 50% seawater in all species and significantly reduced in oiled treatments.

The effects of oil on salt secretion in *A. marina* were investigated by subjecting healthy one year old seedlings to sediment oiling at 0%, 10% and 50% seawater for three weeks. Sodium accumulated in the leaves in oiled treatments. Oiling increased the concentration of Na^+ in 50% seawater by 41% compared to the control while concentrations of K^+, Ca^{2+} and Mg^{2+} were significantly reduced by 72%, 100% and 100%, respectively. Oiling decreased the rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} in 50% seawater by 60%, 76%, 68% and 78%, respectively, compared to the control.

The effects of oil on salt secretion in *A. marina* during the day and night were compared in one year old seedlings subjected to control and oiled treatments for seven days. Sodium accumulated in the leaves of oiled seedlings during the day and night within 11 hours. Oiling increased Na^+ concentrations during the day and night by 42% and 73%, respectively, compared to the controls, while concentrations of K^+, Ca^{2+} and Mg^{2+} decreased by 48% - 78%. Oiling significantly reduced secretion rates of all ions compared to the controls during the day and night.

This study demonstrated that oiling reduced the growth of the three mangroves in 50% compared to 10% seawater. Oiling also reduced salt secretion in *A. marina* at both salinities.

Keywords:

Growth, mangroves, oil, salinity, salt secretion

5.1 Introduction

Mangroves inhabit intertidal zones with fluctuating salinities (Lovelock *et al*., 2004; Naidoo, 2006). High salinity in mangrove sediments is due to the presence of excessive amounts of salts (Parida and Das, 2005). An increase in salinity can lead to salt stress, even in halophytes, such as mangroves (Suarez *et al*., 1998).

Mangroves are morphologically and physiologically adapted to survive under saline conditions (Mizrachi *et al*., 1980; Naidoo and von Willert, 1995; Naidoo *et al.*, 2002). Species such as *K. obovata* and *R. mucronata* exclude salt by root ultrafiltration (Scholander, 1968; Naidoo, 1986). Some species such as *A. corniculatum* and *A. marina* secrete salt via salt glands present in their leaves (Naidoo, 1987; Ye *et al*., 2005; Naidoo *et al*., 2011). Others such as *B. gymnorrhiza* and *R. mucronata* accumulate salt in vacuoles and older leaves which are then shed (Steinke, 1999; Tam and Wong, 2000).

Mangroves also accumulate organic acids as osmotica to counter the toxic effects of salinity (Parida and Das, 2005). The amount of salt transported to the foliage is restricted by regulating water loss through stomatal control and avoiding salt accumulation in the plant (Naidoo and von Willert, 1995). In addition, mangroves decrease their water and osmotic potentials to maintain turgor at high salinity (Naidoo, 1987; Khan *et al*., 2000). Despite these adaptations, as salinity increases, carbon assimilation capacity, plant metabolism, growth and productivity are reduced (Allakhverdiev *et al*., 2000; Naidoo and Chirkoot, 2004).

The effects of salinity on mangroves have been studied in relation to leaf structure, stomatal conductance, rates of transpiration and photosynthesis (Naidoo *et al*., 2002; Patel *et al*., 2010; Hoppe-Speer *et al*., 2011). Salt stress decreases photosynthesis, protein synthesis and lipid metabolism (Hasegawa *et al*., 2000; Zhu, 2002). High salinity increases the activities of antioxidative enzymes (Takemura *et al*., 2000; Parida *et al*., 2004). Few studies have investigated the effects of salinity in response to oil contamination in mangroves (Page *et al*., 1985; Ke *et al*., 2011b).

Oil damages root membranes which adversely affect the ionic balance of plants and their ability to tolerate salinity (Gilfillan *et al*., 1989; Zhang *et al*., 2007a). Sediment

oiling at high salinity reduced germination in grasses (Youssef, 2002; Ibemesim, 2010) and mangroves (Ke *et al*., 2011b). Volatile fraction of Arabian crude oil impaired the salt secreting ability of *A. marina* (Youssef and Ghanem, 2002). This study aims to investigate the effects of salinity in combination with oil on the growth of *A. marina*, *B. gymnorrhiza* and *R. mucronata* seedlings and on salt secretion in *A. marina*.

5.2 Materials and methods

5.2.1 Plant material

Propagules of *A. marina*, *B. gymnorrhiza* and *R. mucronata* were collected and prepared for experiments as described in Chapters 2.2.1 and 4.2.1.

5.2.2 Growth experiment

5.2.2.1 Growth conditions

Sixteen one year old, healthy seedlings of *A. marina* (about 52 ± 10 cm in height), *B. gymnorrhiza* (about 61 ± 6 cm in height) and *R. mucronata* (about 62 ± 7 cm in height) were selected for the growth experiment. Pots containing uniform, actively growing plants were placed in large circular troughs (70 cm diameter x 18 cm height) and subjected to oiling treatments at 10% and 50% seawater (or 3.8 and 19 practical salinity units) for 12 months. Oil (200 ml) was carefully poured onto the soil surface of each pot. Four replicate pots were allocated to each treatment in a completely randomised design. Salinity treatments were introduced gradually at increments of 10% seawater every two days. Pots were irrigated from below and seawater renewed every five days.

5.2.2.2 Plant growth measurements

Measurements of plant height and number of leaves were determined monthly.

5.2.2.3 Chlorophyll content

Measurements of chlorophyll content were determined monthly as described in Chapter 2.2.3. There were six measurements per replicate.

5.2.3 Salt secretion experiments

5.2.3.1 Growth conditions

Effects of oil at different seawater salinities

Twenty four one year old, healthy seedlings of *A. marina* of uniform height were selected to determine the effects of oil on salt secretion at different salinities. Pots were placed in troughs and maintained identically to the growth experiment. Pots were subjected to control and oiling treatments at 0%, 10% and 50% seawater for three weeks. The experimental design was identical to the growth experiment (5.2.2.1). Pots were irrigated from below and seawater renewed every two days.

Effects of oil in the light and dark

Eight one year old, healthy seedlings of *A. marina* of uniform height were selected to determine the effects of light and darkness on salt secretion of oiled seedlings. Pots were placed in troughs and maintained identically to the growth experiment. Pots were subjected to oiling treatments at 10% seawater for seven days. Oil (200 ml) was carefully poured onto the soil surface of each pot. Four replicate pots were allocated to each treatment identical to the growth experiment. Pots were irrigated from below and seawater renewed daily.

5.2.3.2 Salt secretion and ion analysis

Effects of oil at different seawater salinities

After three weeks of treatment, six mature leaves from each replicate were washed with double distilled water to remove previously secreted salt from the leaf surface,

labelled and blotted dry. After five days, secreted salts from each leaf were rinsed for 20 seconds with 20 ml double distilled water and the wash water sealed in air-tight vials. The rinsed leaves were excised from the plant and leaf areas determined by photocopying fresh leaves and scanning the images into a computer using image analysis software, SIS Pro Softward, version 3:1. The fresh mass of leaves was determined on a Toledo scale, dried to constant mass at 70°C and weighed again. The dried leaves were milled before ion analysis. Concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} were determined by atomic absorption using mid-infrared spectroscopy (Bruker Tensor 27, FTIR Spectrometer with HTS/XT).

Effects of oil in the light and dark

In the light and dark experiments, six leaves from each replicate were washed to remove previously secreted salts as described before. In the light experiment, leaves were washed at 06h00, labelled and after 11 hours (at 17h00) rinsed to collect secreted salts. In the dark experiment, leaves were washed at 17h00 to remove previously secreted salts and labelled. Seedlings were then covered partially with black plastic bags which allowed for air movement but excluded light. Seedlings were then placed in a dark room. After 11 hours (06h00 the following morning), seedlings were removed from darkness and the leaves rinsed to collect secreted salts. The rinsed leaves from both light and dark were excised and leaf area, mass and ion concentrations determined as before.

Salt secretion was calculated using the formula:

For Na^+ and K^+: $\{[meq\ L^{-1} / LA] \times 1000\} / t$

For Ca^{2+} and Mg^{2+}: $\{[\frac{meq\ L^{-1}}{2} / LA] \times 1000\} / t$

where meq L^{-1} = the concentration of the ion in meq L^{-1}, LA = leaf area in m^2, t = duration of the experiment in seconds.

5.2.4 Data analyses

Means and standard errors were calculated for all measurements. Resulting data were tested for normality using the Kolmogorov-Smirnov test and subjected to two-way ANOVA and Tukey's multiple comparisons test ($P \leq 0.05$) using GraphPad Prism Version 6.05 (GraphPad Software, Inc., USA). Other data were subjected to unpaired *t*-tests using MINITAB version 16 (Minitab Statistical Software, MINITAB Inc., USA).

5.3 Results

5.3.1 The effects of salinity on oil contamination

A. marina

The effects of seawater salinity in the absence of oil

In the controls, plant height increment (final plant height – initial) was highest in 10% seawater and 25% lower in 50% seawater (Fig. 5.1A). There were no significant differences in change in number of leaves (final number of leaves – initial) and chlorophyll content between 10% and 50% seawater treatments (Fig. 5.1B and C).

The effects of oiling at different seawater salinities

Plant height increment (Fig. 5.1A), change in number of leaves (Fig. 5.1B) and chlorophyll content (Fig. 5.1C) were highest in the control and significantly lower in the oiled treatments at 10% and 50% seawater. Decreases in these parameters with oiling were higher at 50% compared to 10% seawater.

B. gymnorrhiza

The effects of seawater salinity in the absence of oil

In the controls, plant height increment (Fig. 5.2A) and change in number of leaves (Fig. 5.2B) were highest in 10% seawater and significantly lower in 50% seawater, by 42%

and 31%, respectively. There were no significant differences in chlorophyll content between the 10% and 50% seawater treatments (Fig. 5.2C).

The effects of oiling at different seawater salinities

Plant height increment (Fig. 5.2A) and change in number of leaves (Fig. 5.2B) were highest in the control and significantly lower in the oiled treatments at 10% and 50% seawater. Decreases in these parameters with oiling were higher at 50% compared to 10% seawater.

There were no differences in chlorophyll content (Fig. 5.2C) between the oiled treatment and control at 10% seawater. In the oiled treatments, decreases in chlorophyll content were higher at 50% compared to 10% seawater.

R. mucronata

The effects of seawater salinity in the absence of oil

In the controls, plant height increment (Fig. 5.3A), change in number of leaves (Fig. 5.3B) and chlorophyll content (Fig. 5.3C) were highest in 10% seawater and significantly lower in 50% seawater by 52%, 40% and 24%, respectively.

The effects of oiling at different seawater salinities

Plant height increment (Fig. 5.3A), change in number of leaves (Fig. 5.3B) and chlorophyll content (Fig. 5.3C) were highest in the control and significantly lower in the oiled treatments at 10% and 50% seawater. Decreases in number of leaves and chlorophyll content with oiling were higher at 50% compared to 10% seawater.

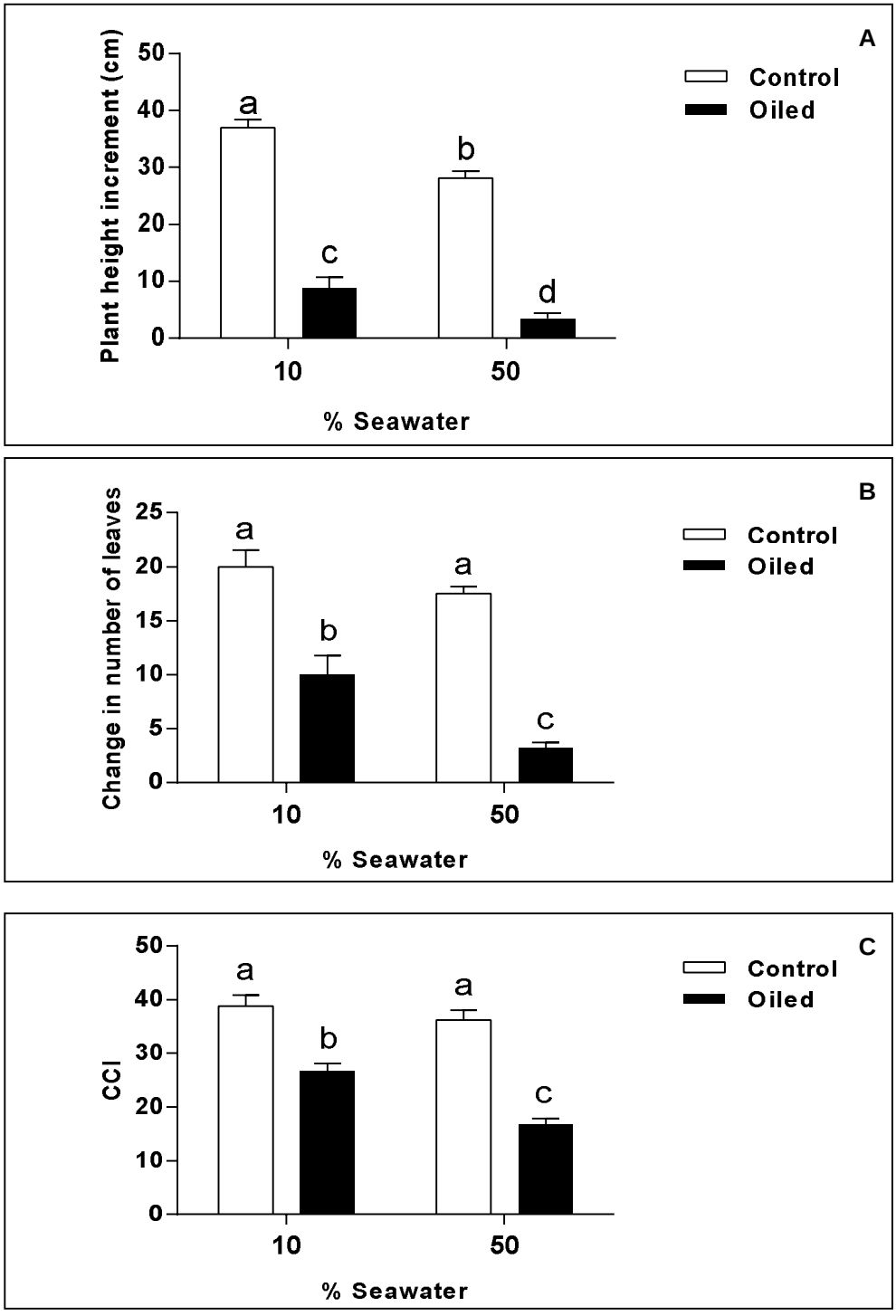

Fig. 5.1 Effects of oil at different salinities on plant height (A), number of leaves (B) and chlorophyll content index (CCI) (C) in *A. marina*. Measurements were taken monthly for 12 months in control and oiled treatments. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

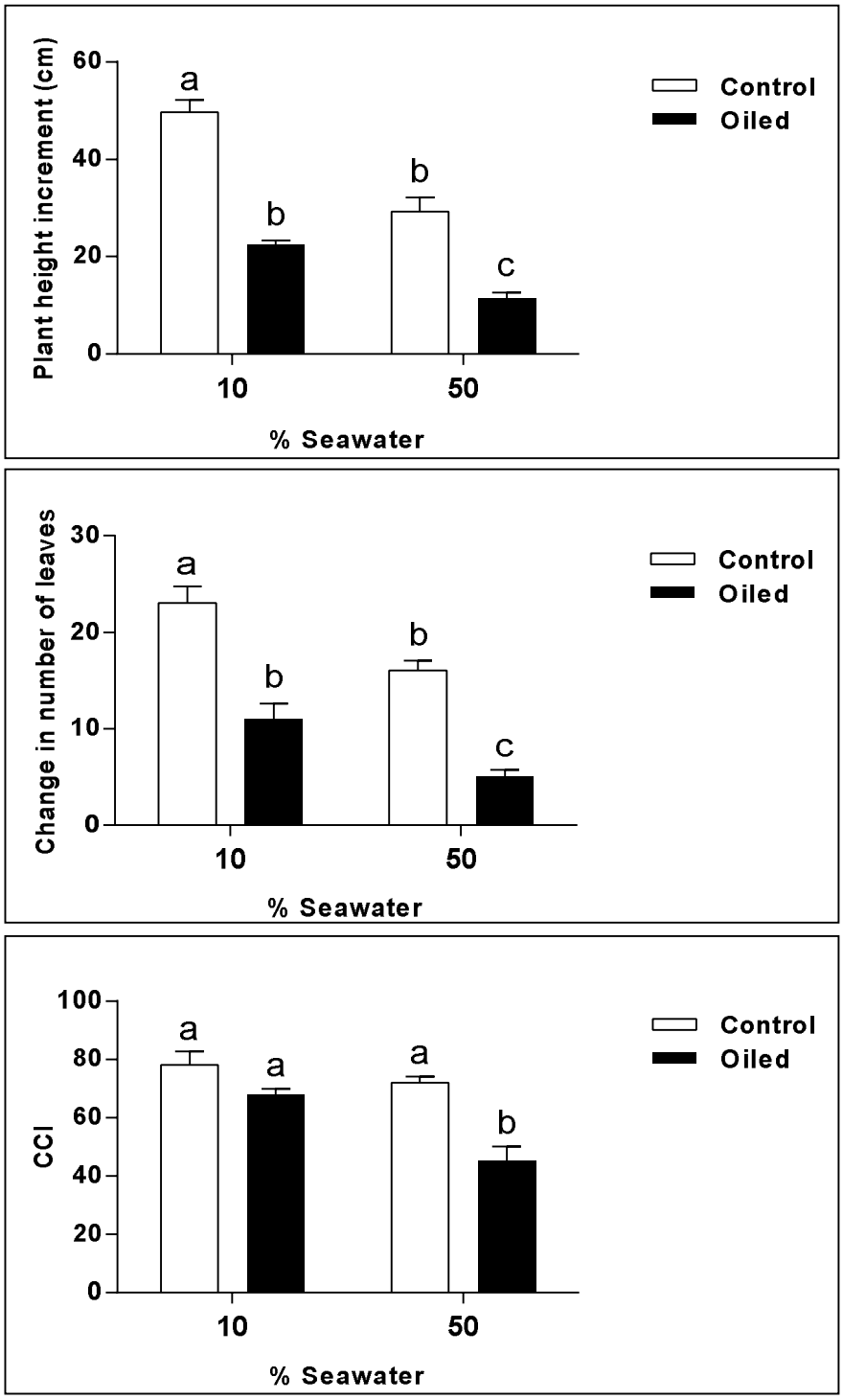

Fig. 5.2 Effects of oil at different salinities on plant height (A), number of leaves (B) and chlorophyll content index (CCI) (C) in *B. gymnorrhiza*. Measurements were taken monthly for 12 months in control and oiled treatments. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

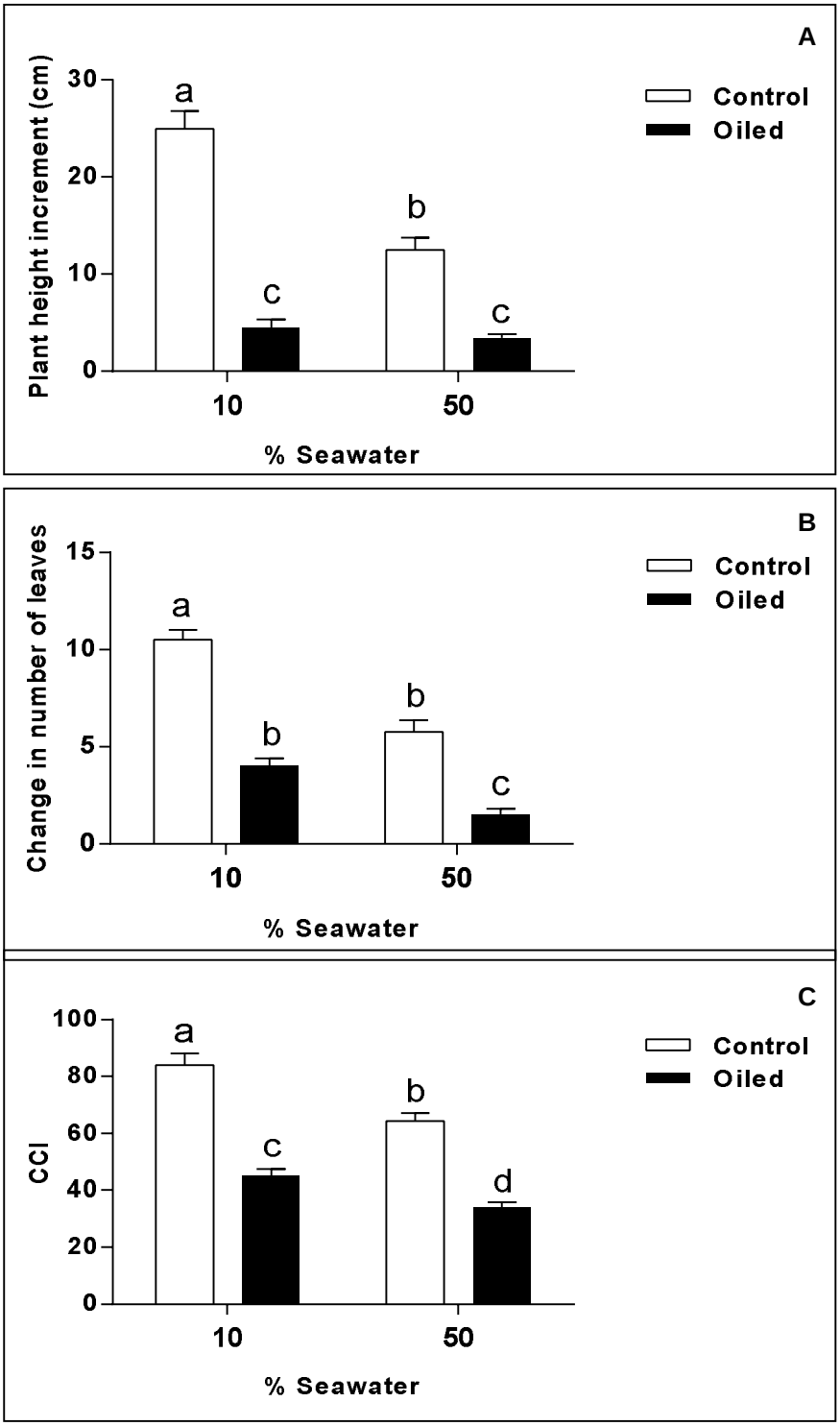

Fig. 5.3 Effects of oil at different salinities on plant height (A), number of leaves (B) and chlorophyll content index (CCI) (C) in *R. mucronata*. Measurements were taken monthly for 12 months in control and oiled treatments. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

5.3.2 The effects of salinity and oil on concentration of ions in *A. marina*

In all treatments, the dominant cation in the leaves was Na^+.

The effects of different seawater salinities in the absence of oil

In the absence of oil, concentration of Na^+ (Fig. 5.4A) was least in 0% seawater and higher in 10% seawater by 58%, while there were no significant differences in concentrations of K^+ (Fig. 5.4B), Ca^{2+} (Fig. 5.5A) and Mg^{2+} (Fig. 5.5B). In the absence of oil, concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} increased significantly with salinity increase from 0% to 50% seawater by 80%, 55%, 67% and 56%, respectively.
In the absence of oil, concentrations of Na^+, Ca^{2+} and Mg^{2+} increased significantly with salinity increase from 10% to 50% seawater by 53%, 51% and 46%, respectively, while there was no significant difference in the concentration of K^+. In the absence of oil, Na^+/K^+ ratios were least in 0% seawater (0.83), 1.36 in 10% and 1.91 in 50% seawater (Fig. 5.6).

The effects of oiling at different seawater salinities

Oiling decreased the concentration of K^+ (Fig. 5.4B) by 75% in 10% seawater compared to the control while there were no significant differences in the concentrations of Na^+ (Fig. 5.4A), Ca^{2+} (Fig. 5.5A) and Mg^{2+} (Fig. 5.5B). Oiling increased the concentration of Na^+ in 50% seawater by 41%, compared to the control while concentrations of K^+, Ca^{2+} and Mg^{2+} decreased by 72%, 100% and 100%, respectively.

In the oiled treatments, the concentration of Na^+ increased by 65% with increase in salinity from 0% to 10% seawater while there were no significant differences in concentrations of K^+, Ca^{2+} and Mg^{2+}. In the oiled treatments, the concentration of Na^+ was least in 0% seawater and higher in 50% seawater by 81%, while there were no significant differences in the concentrations of K^+, Ca^{2+} and Mg^{2+}. In oiled treatments, the concentration of Na^+ increased by 45% with increase in salinity from 10% to 50% seawater while there were no significant differences in the concentrations of K^+, Ca^{2+} and Mg^{2+}. Na^+/K^+ ratios in oiled treatments in 0%, 10% and 50% seawater were 2.33, 9.78 and 9.09, respectively (Fig. 5.6).

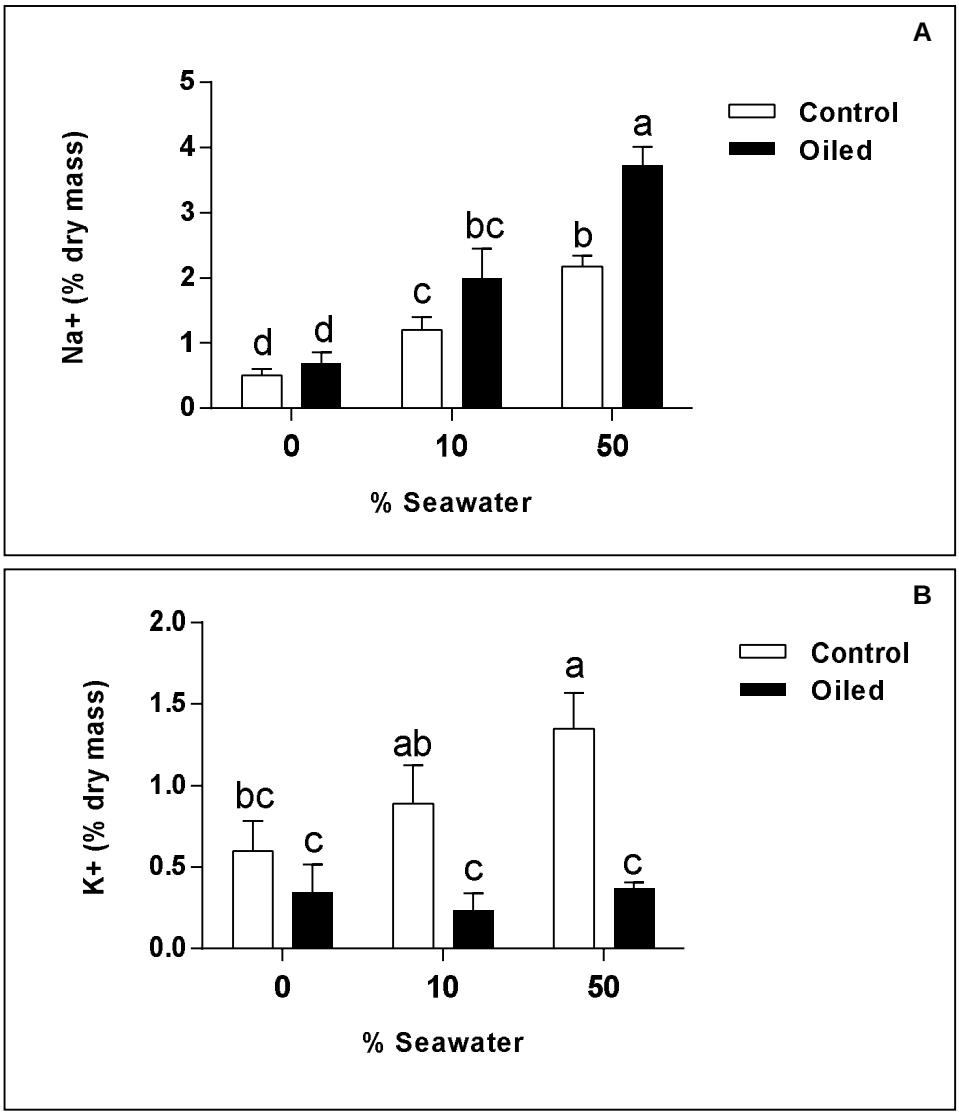

Fig. 5.4 Effects of oil at different salinities on the concentration of Na^+ (A) and K^+ (B) in *A. marina* after three weeks of treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

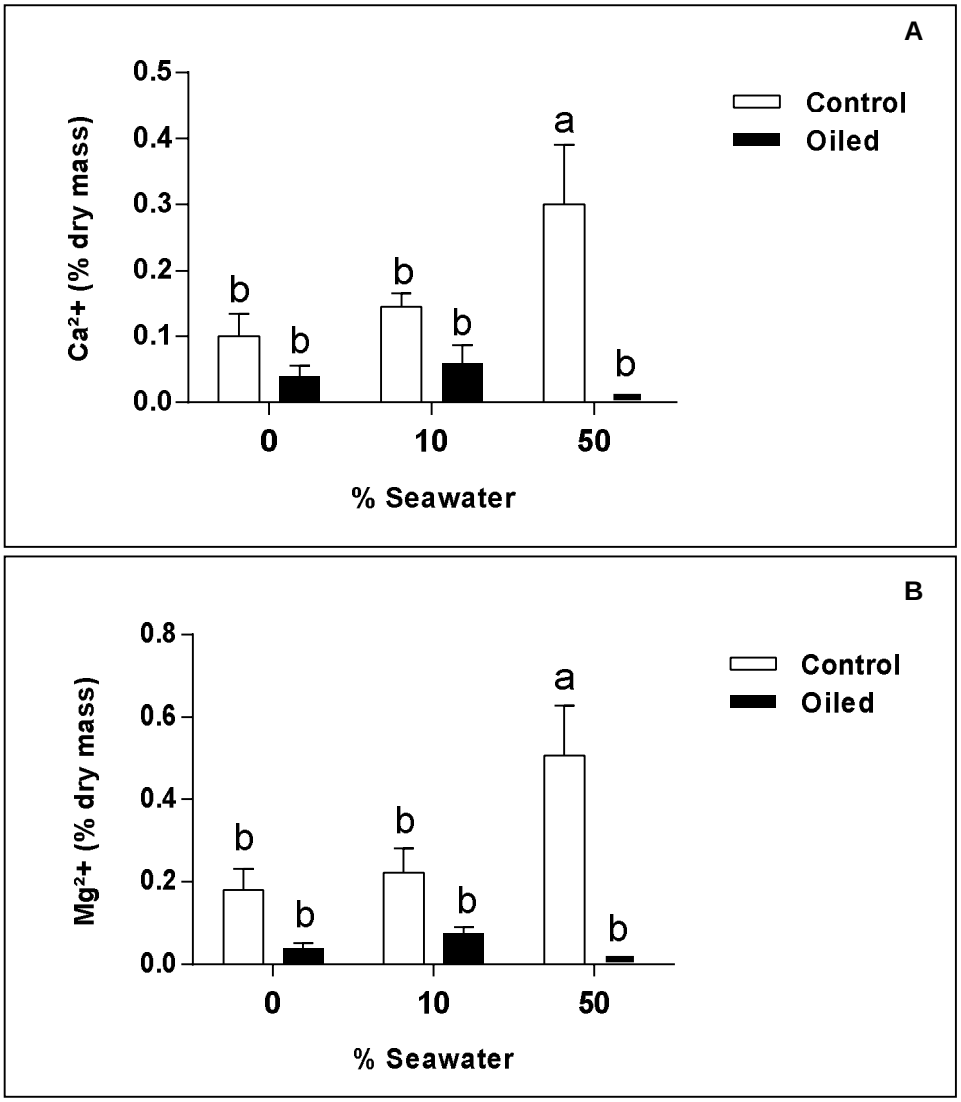

Fig. 5.5 Effects of oil at different salinities on the concentration of Ca^{2+} (A) and Mg^{2+} (B) in *A. marina* after three weeks of treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

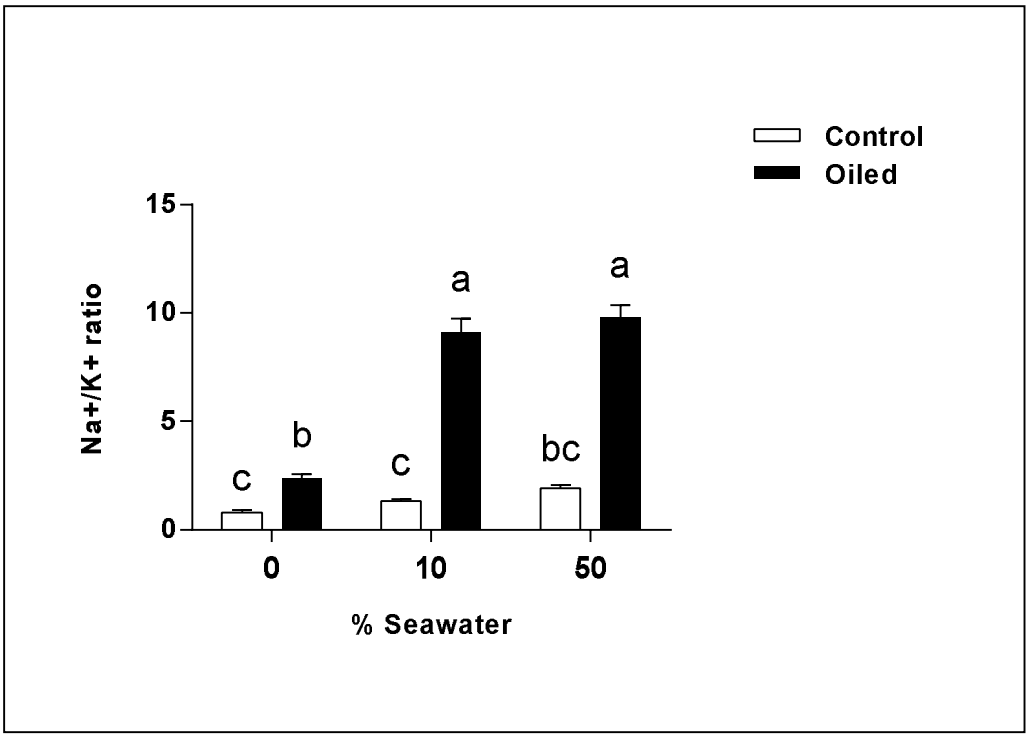

Fig. 5.6 Effects of oil at different salinities on the Na^+/K^+ ratio in *A. marina* after three weeks of treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

5.3.3 The effects of salinity and oil on salt secretion in *A. marina*

The effects of different seawater salinities in the absence of oil

There were no significant differences in rates of secretion of Na^+, K^+ (Figs. 5.7A and B), Ca^{2+} and Mg^{2+} (Figs. 5.8A and B) between 0% and 10% seawater.

In the 50% seawater treatment, rates of secretion of Na^+ and Mg^{2+} increased by 67%, and 63%, respectively, compared to 10% seawater. There were no significant differences in rates of secretion of K^+ and Ca^{2+} between the 10% and 50% seawater treatments.

In the 50% seawater treatment, rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} increased significantly by 78%, 43%, 52% and 75%, respectively, compared to 0% seawater.

The effects of oiling at different seawater salinities

There were no significant differences in rates of secretion of Na^+, K^+ (Figs. 5.7A and B), Ca^{2+} and Mg^{2+} (Figs. 5.8A and B) between the oiled and control treatments at 0% or 10% seawater.

Oiling decreased rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} in 50% seawater by 60%, 76%, 68% and 78%, respectively, compared to the control.

There were no significant differences in rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} in the oiled treatments at 0%, 10% or 50%.

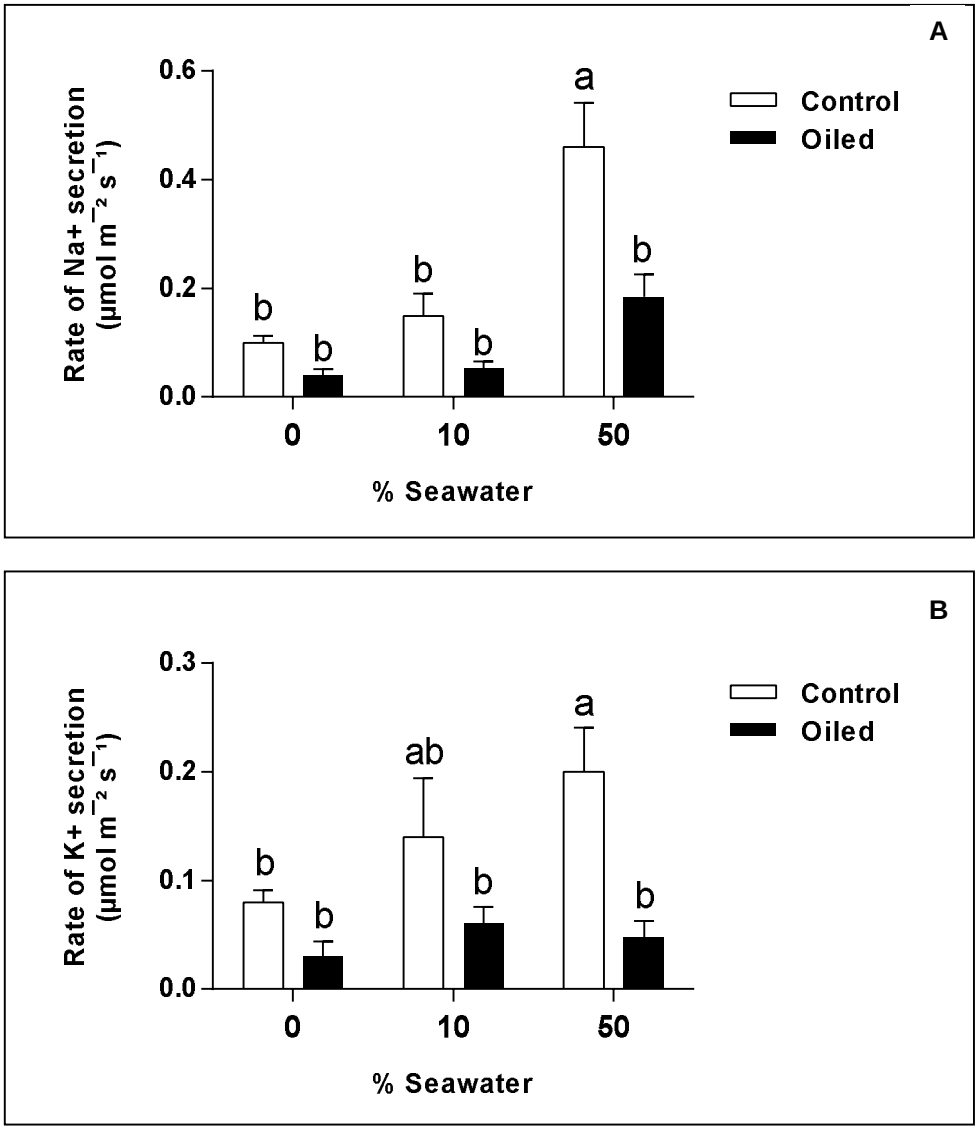

Fig. 5.7 Effects of oil at different salinities on secretion of Na^+ (A) and K^+ (B) in *A. marina* after three weeks of treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

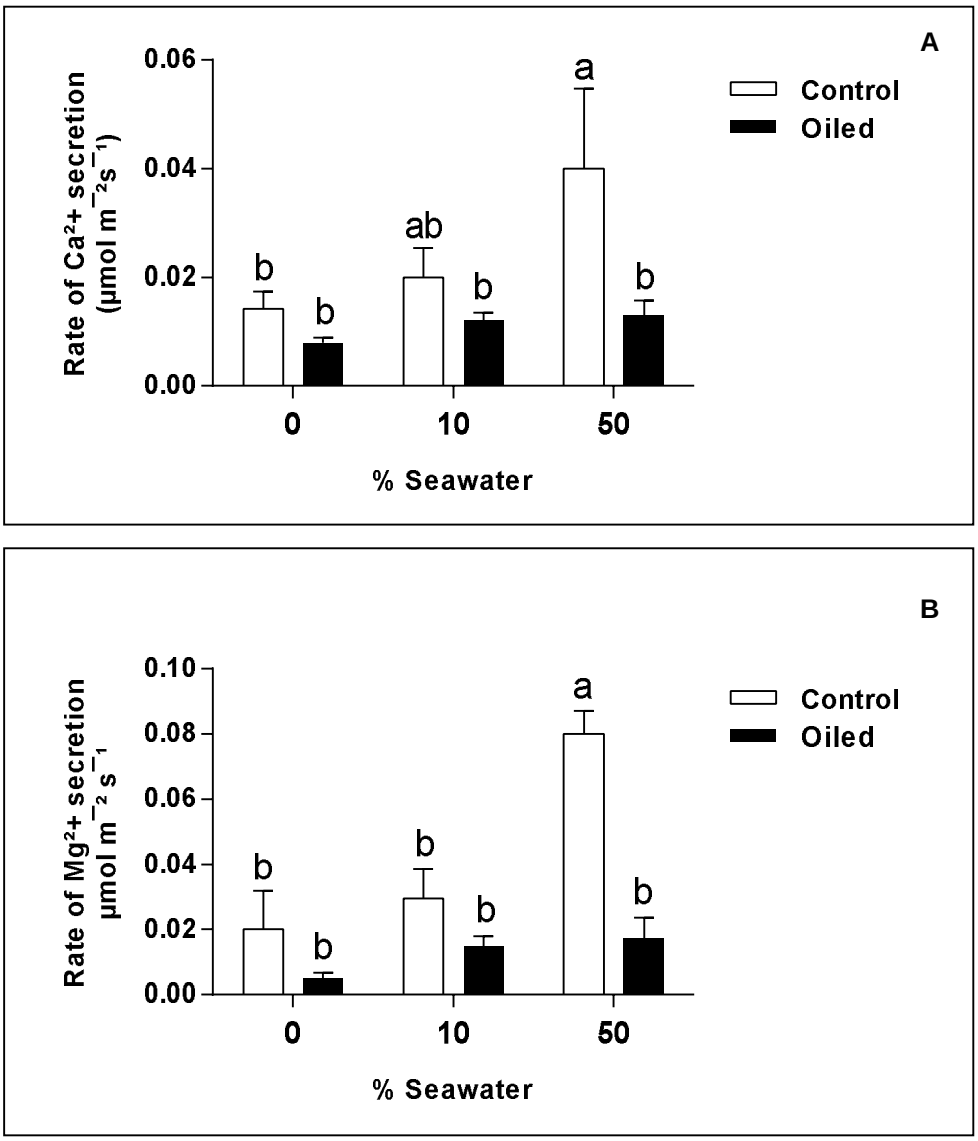

Fig. 5.8 Effects of oil at different salinities on secretion of Ca^{2+} (A) and Mg^{2+} (B) in *A. marina* after three weeks of treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

5.3.4 The effect of oil on concentrations of ions in *A. marina* during the day and night

Sodium accumulated in the leaves in oiled treatments during the day and night.

Light experiment

In the oiled treatment, the concentration of Na^+ was 42% higher compared to the control while concentrations of K^+, Ca^{2+} and Mg^{2+} were significantly lower by 74%, 48% and 78%, respectively (Fig. 5.9).

Na^+/K^+ ratios were 1,21 in the control and 8,17 in the oiled treatment (Fig. 5.11).

Dark experiment

In the oiled dark treatment, the concentration of Na^+ was 73% higher compared to the control while concentrations of K^+, Ca^{2+} and Mg^{2+} were significantly lower by 68%, 60% and 71%, respectively (Fig. 5.10).

Na^+/K^+ ratios were 1,33 in the control and 7,63 in the oiled treatment (Fig. 5.12).

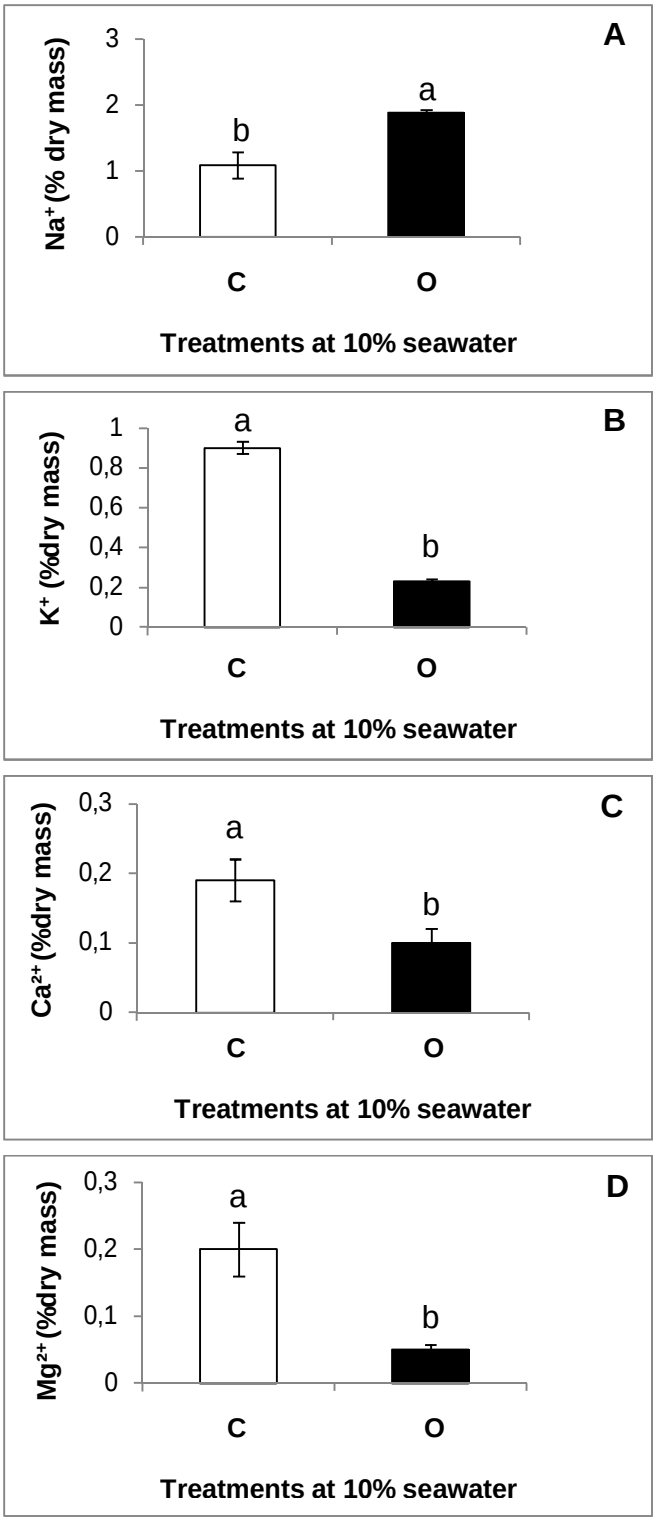

Fig. 5.9 Effects of oil on the concentration of Na^+ (A), K^+ (B), Ca^{2+} (C) and Mg^{2+} (D) in *A. marina* in the light experiment after seven days of treatment, C = control and O = oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using unpaired *t*-tests.

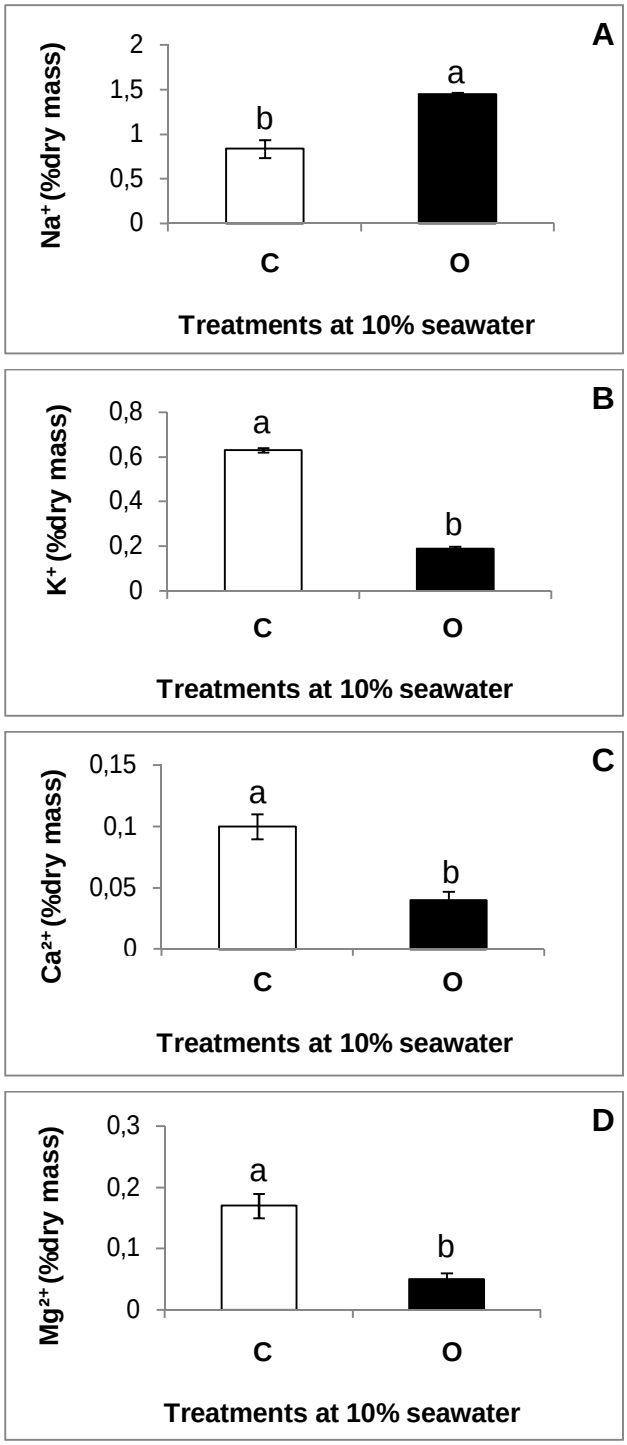

Fig. 5.10 Effects of oil on the concentration of Na^+ (A), K^+ (B), Ca^{2+} (C) and Mg^{2+} (D) in *A. marina* in the dark experiment after seven days of treatment, C = control and O = oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using unpaired *t*-tests.

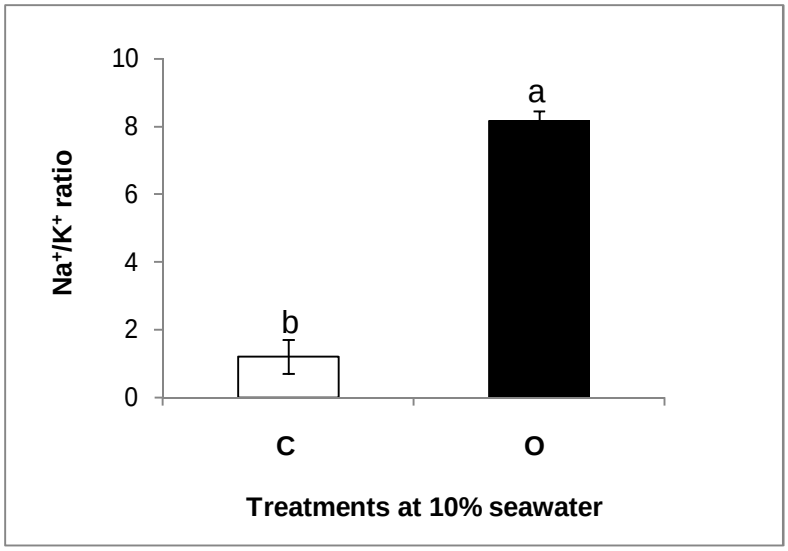

Fig. 5.11 Effects of oil on the Na^+/K^+ ratio in *A. marina* in the light experiment after seven days of treatment, C = control and O = oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at P ≤ 0.05 using unpaired *t*-tests.

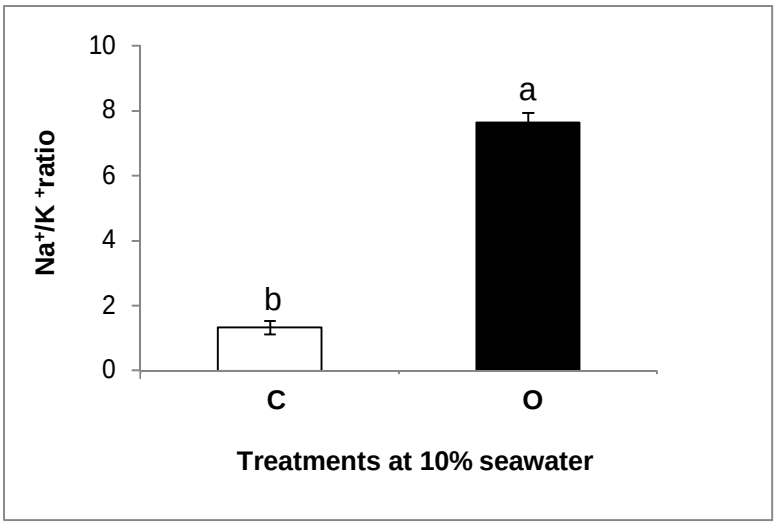

Fig. 5.12 Effects of oil on the Na^+/K^+ ratio in *A. marina* in the dark experiment after seven days of treatment, C = control and O = oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at P ≤ 0.05 using unpaired *t*-tests.

5.3.5 The effect of oil on salt secretion in *A. marina* during the day and night

Salt secretion was highest in the control and significantly lower in the oiled treatments in both light and dark experiments.

Light experiment

In the light experiment, rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} were highest in the control and significantly lower in the oiled treatment by 54%, 56%, 67% and 49%, respectively (Fig. 5.13).

Dark experiment

In the dark experiment, rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} were highest in the control and significantly lower in the oiled treatment by 56%, 69%, 52% and 66%, respectively (Fig. 5.14).

In the absence of oil, rate of secretion of Na^+ was 31% higher in the dark compared to the light while there were no significant differences in rates of secretion of K^+, Ca^{2+} and Mg^{2+}. There were no significant differences in the rates of secretion of Na^+, K^+, Ca^{2+} and Mg^{2+} in the oiled treatment between light and darkness.

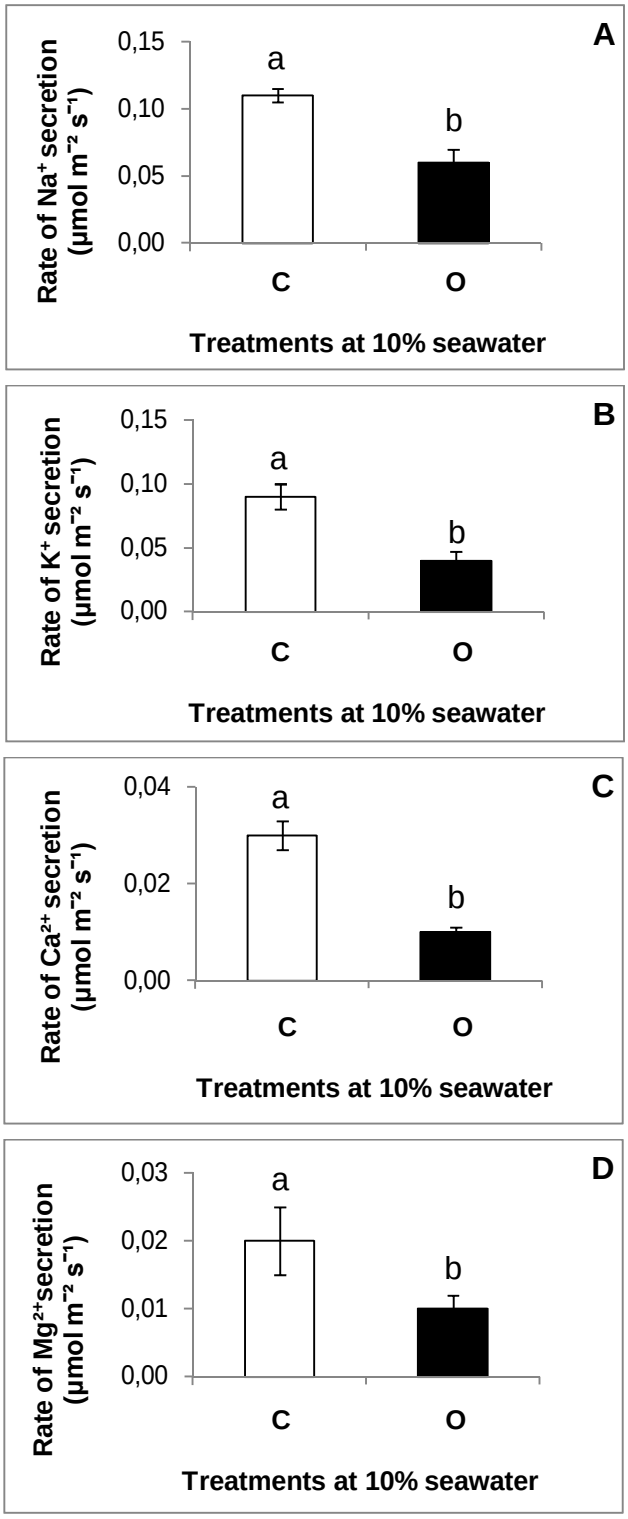

Fig. 5.13 Effects of oil on secretion of Na^+(A), K^+ (B), Ca^{2+} (C) and Mg^{2+} (D) in *A. marina* in the light experiment after seven days of treatment, C = control and O = oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using unpaired *t*-tests.

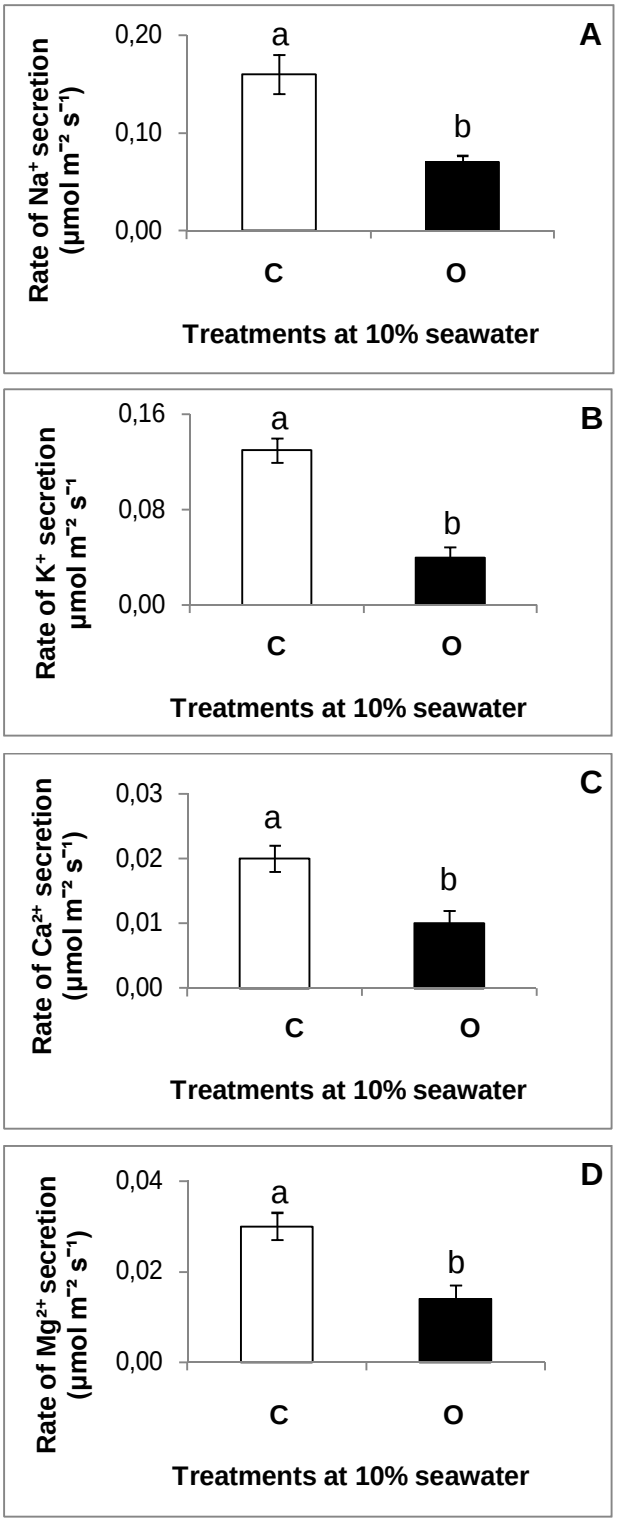

Fig. 5.14 Effects of oil on secretion of Na^+(A), K^+ (B), Ca^{2+} (C) and Mg^{2+} (D) in *A. marina* in the dark experiment after seven days of treatment, C = control and O = oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using unpaired *t*-tests.

5.4 Discussion

5.4.1 The effects of salinity and oil on growth

Optimal growth of mangroves is in the range of 10% to 45% seawater (Aziz and Khan, 2001; Ye *et al*., 2005). Seawater salinity greater than 45% leads to reduced growth (Naidoo, 2006; Patel *et al*., 2010; Hoppe-Speer *et al*., 2011).

Growth in all species was higher at 10% seawater and decreased at 50%. In this study, salt stress at 50% seawater probably decreased metabolism and cell division leading to reduced growth as reported previously (Hayashi and Murata, 1998; Hasegawa *et al*., 2000; Parida and Das, 2005). Seawater salinity greater than 40% reduced growth in *A. marina* (Patel *et al*., 2010), *A. corniculatum* and *A. ilicifolius* (Ye *et al*., 2005). Optimal growth for *Rhizophora apiculata* Bl., *Rhizophora stylosa* Griff., *R. mangle* (Aziz and Khan, 2001; Biber, 2006) and *R. mucronata* (Hoppe-Speer *et al*., 2011) was reported to be between 23% and 40% seawater and reduced at higher salinities. Optimal growth for *B. gymnorrhiza* was reported to be between 30% and 40% seawater and reduced at higher salinities (Basak *et al*., 2004).

The accumulation of inorganic ions such as Na^+ and Cl^- in cell walls reduces cell turgor, expansion and growth (Parida and Das, 2005). Although the regulation of water loss through stomatal control in mangroves reduces the amount of salt transported to the foliage (Naidoo and von Willert, 1995), salinity greater than 40% seawater decreases stomatal conductance, thereby decreasing transpiration and growth (Youssef and Ghanem, 2002; Parida and Das, 2005). The results of this study support previous reports that 50% seawater reduces growth in the three species investigated.

The number of leaves in *B. gymnorrhiza* and *R. mucronata* were higher at 10% seawater and decreased at 50%. This was probably due to leaf-shedding, a coping mechanism during salt stress in halophytes (Krauss *et al*., 2008). Salt excluders like *B. gymnorrhiza* and *R. mucronata* accumulate salt in older leaves which are then shed (Steinke, 1999). Leaf production in *R. mucronata* was previously reported to decrease at a salinity of 47% seawater (Hoppe-Speer *et al*., 2011).

There were no significant differences in number of leaves and chlorophyll content in *A. marina* between 10% and 50% seawater. *Avicennia marina* is a highly salt tolerant pioneer species (Naidoo, 2006). In this study, *A. marina* was more salt tolerant than *B. gymnorrhiza* and *R. mucronata* probably because of the ability to secrete salts via glands in the leaves (Ye *et al*., 2005; Jayatissa *et al*., 2006; Patel *et al*., 2010). Additionally, ion exclusion at the root level in *A. marina* was reported to be an effective barrier for restricting solute transport to the leaves (Youssef and Ghanem, 2002; Naidoo *et al*., 2014).

Increase in salinity from 10% to 50% seawater reduced chlorophyll content in *R. mucronata*. In mangroves under salt stress, chlorophyll content decreases as a result of chlorophyll degradation (Steinke *et al*., 1993; Flores-Santiago *et al*., 2013). At 50% seawater, decreases in osmotic potential probably caused Na^+ ions to leak into the cytosol (Papageorgiou *et al*., 1998) disrupting photosynthesis (Allakhverdiev *et al*., 2000) by decreasing chlorophyll content (Parida *et al*., 2003). Reduced chlorophyll content reduces energy trapping efficiency, carbon fixation and ultimately growth (Parida and Das, 2005; Naidoo *et al*., 2011; Khaleghi *et al*., 2012).

Greatest reduction in growth occurred when plants were exposed to two stressors in combination, (salinity of 50% seawater and oil). Oil decreases sediment permeability and dissolved oxygen concentrations (Suprayogi and Murray, 1999; Pereira *et al*., 2002) which exacerbates salt stress. Oil probably disrupted ion and water relations that further reduced plant growth as reported previously (Gilfillan *et al*., 1989; Pezeshki *et al*., 2000). High Na^+ concentrations in the soil prolong the residence time of oil by inhibiting the metabolic activity of microorganisms that degrade PAHs (Walker and Colwell, 1975) thereby increasing toxicity (Rhykerd *et al*., 1995).

Salinity of 50% seawater, in combination with oil, reduced plant height, number of leaves and chlorophyll content in all species. Oil probably damaged root cell walls and membranes that led to excessive salt accumulation in plant tissues resulting in reduced growth (Page *et al*., 1985). Salinity of 40% seawater in combination with oil caused reduced growth and biomass in the grass, *Paspalum conjugatum* Bergius (Ibemesim, 2010). In previous studies, *B. gymnorrhiza* and *A. corniculatum* exhibited reduced growth and biomass and increased SOD activity in sediment oiled treatments at 35% compared to 15% seawater (Ke *et al*., 2011b). In the oiled treatments, *R. mucronata*

exhibited necrosis of leaves and propagules, in addition to leaf shedding. Oil probably caused sub-lethal biochemical and molecular damage and mortality of cells (Duke and Watkinson, 2002; Zhang *et al*., 2007a).

Decreased chlorophyll content was probably a result of PAHs negatively affecting the photosynthetic apparatus of chloroplasts (Naidoo *et al*., 2010; Yin *et al*., 2011) and degrading chlorophyll (Huang *et al*., 1996). In maize plants, lubricating oil degraded the chloroplast structure (Grijalbo *et al*., 2013). Furthermore, oil causes Na^+ to accumulate in leaves of mangroves (Page *et al*., 1985; Gilfillan *et al*., 1989). High concentrations of Na^+ in leaf cells induce swelling of thylakoids, disintegration of the lamellae and chloroplast envelope, thereby reducing photosynthetic capacity (Naidoo *et al*., 2011).

5.4.2 The effects of salinity and oil on concentrations of ions in *A. marina*

Concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} in leaves increased with increase in salinity from 0% to 50% seawater. The accumulation of inorganic ions is commonly observed in mangroves at high salinity (Naidoo, 1985; Gilfillan *et al*., 1989; Parida and Das, 2005). Salinity of 50% seawater probably caused roots to absorb greater amounts of salt resulting in an increase in transportation of ions from root to shoot via the transpiration stream as reported previously (Tattini *et al*., 1995). Many studies have shown that *A. marina* accumulates more Na^+ ions than other mangroves such as *R. stylosa, R. mucronata* and *B. gymnorrhiza* growing in nutrient solutions with exactly the same salt composition (Clough, 1984; Naidoo, 1985; Medina, 1999).

Salt secretors like *A. marina* absorb salt from the soil to maintain a water potential that is more negative than the soil, thereby increasing ion concentrations in root and leaf cells (Papageorgiou *et al*., 1998). This creates a higher osmotic potential difference between plant cells and soil water enabling water to enter cells through the plasma membrane via osmosis (Parida *et al*., 2004). Furthermore, *A. marina* possesses a thin endodermis that allows a greater amount of salt to pass into root cells than other mangroves (Pi *et al*., 2009).

Concentrations of Na^+ in leaves were significantly higher in oiled treatments compared to the controls. This probably occurred because oil causes excessive Na^+ accumulation in plant tissues due to damaged root cell walls and membranes, as reported previously

(Page *et al*., 1985; Gilfillan *et al*., 1987; 1989; Ke *et al*., 2011b). Mangroves can control the amount of salt entering the plant by ultrafiltration that occurs at the interface of the root cell surface (Youssef and Ghanem, 2002; Basyuni *et al*., 2012). Root tissue therefore, plays a pivotal role in controlling the content of ions such as Na^+ in the whole plant (Munns, 2005). Ultrafiltration in *A. marina* is the most important process for reduction of salt uptake in which 80% of salt absorbed by the root through the transpiration stream is excluded (Medina, 1999; Youssef and Ghanem, 2002). Salt glands remove 40% of the remaining quantities of salt that enter the shoot, which is 8% of that eliminated at the root level (Waisel *et al*., 1986; Ball, 1988).

Oil disrupts the cellular lipid membranes in root tissues (Ye and Tam, 2007; Zhang *et al*., 2007a) allowing excess salt to enter the foliage. Medina (1999) found that *A. marina* root cells may have more permeable plasma membranes (based on greater salt concentrations in the xylem sap) than other mangroves including *Rhizophora stylosa*. PAHs are lipophilic and accumulate in root cell plasma membranes (Kang *et al*., 2010) disrupting the lipid components (phytosterols) that regulate ion permeability (Hartmann, 1998). Oil-induced permeability of the plasma membrane probably reduced the tolerance of *A. marina* to 50% seawater.

In oiled treatments, the Na^+/K^+ ratios were significantly higher (> 7) than the controls (< 2), due to increased Na^+ and decreased K^+ concentrations. The concentrations of Na^+ and K^+ ions are important in determining the level of oil-induced stress in mangroves (Gilfillan *et al*., 1987). High Na^+ uptake interferes with the absorption of other nutrient ions, especially K^+, leading to K^+ deficiency (Ball *et al*., 1987). High Na^+/K^+ ratios (4 - 7) have been reported previously for mangroves under salt stress (Waisel *et al*., 1986; Parida and Das, 2005; Naidoo, 2010). In oil-damaged roots, the concentration of Na^+ was elevated in *A. marina* leading to deleterious effects as described previously for other salt-secretors (Gilfillan *et al*., 1987; 1989; Parida and Das, 2005).

Oil has been reported to disrupt Na^+/K^+ ratios. Page *et al*. (1985) reported a Na^+/K^+ ratio of 8.5 in the leaves of sediment oiled mangroves. High Na^+/K^+ ratios were found in the leaves of sediment oiled *Spartina alterniflora* (a salt-secretor) (Gilfillan *et al*., 1989) and other grasses (Ibemesim, 2010) under salt stress. K^+ is a major cation that serves as a reference for Na^+ accumulation in plant tissues (Page *et al*., 1985). High Na^+/K^+ ratios in mangroves result in Na^+ toxicity (Sümer *et al*., 2004; Wakeel *et al*., 2011) and

impaired cell metabolism (Parida and Das, 2005; Patel *et al*., 2010; Naidoo, 2010). The high Na^+/K^+ ratios in this study are probably a result of oil-induced increases in permeability of the root plasma membranes (Page *et al*., 1985; Gilfillan *et al*., 1989).

Oiling significantly reduced the concentrations of K^+, Ca^{2+} and Mg^{2+} in the leaves of *A. marina*. High Na^+ absorption disrupts the absorption of other nutrient ions, such as K^+, Ca^{2+} and Mg^{2+} resulting in deficiency of these ions, as reported previously (Parida and Jha, 2010). Similar results were obtained by Suprayogi and Murray (1999) in which Kuwait crude oil added to the sediment significantly reduced the concentrations of Mg^{2+} and K^+ in *A. marina*. Magnesium plays an important role in chlorophyll structure and in the export of photosynthates (Marschner and Cakmak, 1989). The accumulation of Na^+ and the subsequent decrease in K^+, Ca^{2+} and Mg^{2+} leads to a reduction in quantum yield of PSII thereby decreasing photosynthesis (Ball *et al*., 1987; Parida *et al*., 2003).

5.4.3 The effects of oil on salt secretion in *A. marina*

Salt secretion was higher in 50% seawater compared to 10%. The rate of secretion increases as the salt content in the foliage increases (Waisel *et al*., 1986; Naidoo and von Willert, 1995). Seawater salinity greater than 40% increased the amount of salt entering the roots and therefore increased salt secretion, as reported previously (Jayatissa *et al*., 2006; Patel *et al*., 2010). Salt glands are important in controlling the ionic composition of photosynthesizing leaves (Waisel *et al*., 1986). Some reports suggest that the salt glands of *A. marina* are more efficient at secreting salts than those of other salt secreting mangroves such as *Aegiceras corniculatum* (Gordon, 1993; Ye *et al*., 2005; Jayatissa *et al*., 2006).

In this study, oiling reduced leaf salt secretion. Oil probably damaged salt glands resulting in accumulation of salt in tissues as suggested previously (Youssef and Ghanem 2002; Ke *et al*., 2011b). Youssef and Ghanem (2002) found that volatile fractions of Arabian crude oil reduced salt secretion in *A. marina*. Their study suggested that oil damaged the plasma membranes of the salt glands decreasing secretion. Oil could have displaced phytosterols in the plasma membranes of salt glands reducing their capacity to secrete salt, as described previously for root cells during salt exclusion (Gilfillan *et al*., 1989; Zhang *et al*., 2007a).

Oil taken up by roots accumulates in leaf tissue via the transpiration stream (Meudec *et al*., 2006; 2007). PAHs detected in mesophyll cells of spruce needles disrupted cell metabolism (Viskari *et al*., 2000). PAHs probably entered salt gland membranes from surrounding mesophyll cells, by diffusion across cellular membranes and through plasmodesmata (Orcutt and Nielsen, 2000). Furthermore, oil passed through intercellular spaces and aerenchyma channels in leaves of *Phragmites australis,* whereas water normally does not (Armstrong *et al*., 2009). The l*ipophilic nature of PAHs could have caused them to gravitate towards lipid components of the cell wall and membrane of salt glands as described previously for root cells* (Gao and Zhu, 2004; Meudec *et al*., 2006; 2007).

PAHs could have decreased plasma membrane integrity of salt glands. PAHs change membrane structure in microorganisms by causing expansion and increased fluidity, compromising the capacity of the membrane to regulate ions (Sikkema *et al*., 1994). This adversely affects the functioning of membranes altering homeostasis (e.g. pH balance, ion permeability, and respiration) (Gauthier *et al*., 2014). PAHs such as fluoranthene and pyrene were reported to decrease membrane integrity in trout cells (Schirmer *et al*.,1998). PAHs could have adversely affected the function of the plasma membrane in salt glands as an ion-selective barrier and matrix for important enzymes, as reported previously for the mussel, *Mytilus edulis* (Antoun, 2011).

PAHs are known to be cytotoxic (Kang *et al*., 2010). Certain salt gland characteristics could make them sites for PAH accumulation and thus toxicity. The salt glands of *A. marina* comprise of collecting, stalk and secretory cells (Salama *et al.*, 1999). The stalk and secretory cells contain numerous minute vacuoles (Drennan *et al*., 1987) while the collecting cells have large central vacuoles. As the leaf matures, the glands of the adaxial and abaxial surfaces become highly vacuolated (Drennan and Berjak, 1982). Previous chemical studies demonstrated that PAHs accumulate in vacuoles of ryegrass root and leaf cells disrupting cell metabolism (Gao and Zhu, 2004; Wild *et al*., 2005). Accumulation of PAHs in salt gland vacuoles could have had a similar effect.

Furthermore, salt reaching the leaves is accumulated in vacuoles in mangroves as well as other halophytes (Harvey *et al*., 1981). This ensures that salt sensitive enzymes in the dense cytoplasm are isolated from high salt concentrations (Medina, 1999). Organic osmolytes, such as cyclitols and amino acids are synthesized at high Na^+

concentrations to ensure cytoplasm hydration for enzyme function (Medina, 1999) as described previously in mangroves (Popp *et al*., 1984). Oil probably accumulated in the vacuoles of salt glands resulting in Na^+ accumulation in the cytoplasm, leading to dehydration and destruction of enzymes and decreased salt secretion.

The stalk and secretory cells of salt glands contain numerous endoplasmic reticulum and mitochondria (Drennan *et al*., 1987). Kang *et al*. (2010) found that phenanthrene and pyrene primarily accumulated in organelles of ryegrass roots. PAHs could have accumulated in the organelles of stalk and secretory cells. Drennan *et al*. (1987) suggested that salt could move from the stalk cell to the secretory cells via the plasmodesmata. The plasmodesmata could provide a pathway (Orcutt and Nielsen, 2000) for PAHs to the stalk and secretory cells of the salt glands. PAHs could have accumulated in organelles such as mitochondria disrupting sites of energy formation, thereby decreasing salt secretion. The large number of mitochondria and endoplasmic reticulum in the gland cells suggest that salt secretion is an energy requiring process (Drennan *et al*., 1987). In order to maintain ion selectivity and membrane stability, large amounts of photosynthetic energy are required for salt secretion (Medina, 1999). Reduced chlorophyll content and reduced energy trapping efficiency by oil could have contributed to decreased salt secretion.

The outer walls of stalk and secretory cells of salt glands are surrounded by a cutinized layer that creates a large collecting compartment (Salama *et al*., 1999). PAHs could have degraded this layer (entering the collecting compartment), as reported previously for the cuticle of spruce needles (Sauter and Pambor, 1989). Furthermore, this collecting compartment is a cavity (Salama *et al*., 1999; Drennan *et al*., 1987) which could also serve as sites of oil accumulation and toxicity within the salt gland.

In the absence of oil, Na^+ secretion was higher during the night compared to the day as reported previously (Drennan and Pammenter, 1982; Waisel *et al*., 1986; Naidoo, 2010). The salt that is accumulated during the day is secreted at night (Parida and Das, 2005). In oiled treatments, there were no differences in salt secretion during the day and night, probably due to oil-damage to the salt glands.

This study demonstrated that the adverse effects of 50% seawater were exacerbated by the presence of oil, resulting in reduced growth as described previously (Youssef,

2002; Ke *et al*., 2011b). This study also showed that Na^+ accumulated in leaf cells of *A. marina* due to oiling. The salt glands of *A. marina* are highly selective and eliminate large quantities of salt (Jayatissa *et al*., 2006; Patel *et al*., 2010; Naidoo, 2010). However, oil probably damaged the plasma membranes and organelles of collecting, stalk and secretory cells of salt glands. Oil probably impaired the functioning of the salt glands at 50% seawater leading to accumulation of Na^+ and disruption of the uptake of essential elements such as K^+, Ca^{2+} and Mg^{2+}.

Chapter 6

The effects of oil on soil meiofauna

Abstract

In this study, the effects of oil on species abundance, richness and community structure of free living nematodes were investigated in microcosms. Mangrove sediment containing natural nematode assemblages were collected from the Isipingo estuary and transferred in buckets to an airconditioned glasshouse. Microcosms (350 ml plastic jars) were filled with mangrove sediment and subjected to oiling (with or without fertiliser) for four weeks. In the oiled treatments 15 ml oil and 5 ml/L fertiliser were added to 200 g soil. Fertiliser consisted of N, P and K (3:2:5). Nematodes were extracted after the experimental period and identified to genus or species level. In the unfertilised oiled treatment, nematode abundance and species richness were significantly reduced by 87% and 53%, respectively, compared to the control. In the fertilised oiled treatment, nematode abundance and species richness increased by 56% and 30% respectively. Five species, *Monhystrella* sp., *Prodesmodora* sp., *Plectus* sp., *Tobrilus* sp. and *Fictor* sp. were present in the control but absent in oiled treatments and characterised as oil-intolerant. The species from the family Leptolaimidae increased in the unfertilised oiled treatment by 78% compared to the control and was characterised as opportunistic. In all treatments, the dominant species was *Ethmolaimus* sp. The seven species present in the oiled treatments were characterised as oil-resistant (i.e. tolerant to oiling) and resilient. Species such as *Rhabditis* sp., *Koerneria* sp. and *Rotylenchus* sp. survived oiling due to the addition of fertiliser. Furthermore, the addition of fertiliser increased reproduction in species from the family Leptolaimidae. Out of the 16 nematode taxa identified in this study; seven were oil-resistant and nine oil-intolerant. This study demonstrated that oiling eliminated species that were oil-intolerant and favoured those that were resistant and resilient, thereby altering the free living nematode community structure.

Keywords:

Community structure, fertiliser, nematode abundance, oil, species richness

6.1 Introduction

Coastal marine ecosystems around the world are contaminated by PAHs (Louati *et al*., 2001). The major sources of PAH contamination in the oceans are spills of crude oil, discharges of refined fuels and offshore production (Kennish, 1992). Changes in the community structure of macro- and meiofauna have been widely used to determine the effects of anthropogenic contamination in aquatic environments (Munawar *et al*., 2003; Rubal *et al*., 2009). Many studies have investigated the impacts of PAHs on benthic communities around the world (Gesteira and Dauvin, 2000; Suderman and Thistle, 2003; Mazzella *et al*., 2007).

Oil contamination in macrofauna has been more commonly studied because larger organisms are easier to sample and identify (Netto and Gallucci, 2003). However, it is important to investigate soil meiofauna and their responses to oil contamination because these organisms are closely associated with the sediment (Kennedy and Jacoby, 1997). Soil meiofauna therefore constitute an important link in the benthic food web by providing ecosystem services such as sediment bio-turbation, recycling of organic matter and consumption of bacteria and microalgae (Christie and Berge, 1995; Coull *et al*., 1995). Additionally, meiofauna are an important food source for juvenile fish that use intertidal habitats as nurseries (Nozais *et al*., 2005; Cibic *et al*., 2009).

Soil meiofauna such as nematodes are a widespread, taxonomically and functionally diverse group (Albuquerque *et al.*, 2007; Wei *et al*., 2012). Nematode communities are common biological indicators of soil health because of their role in essential ecological processes including nutrient cycling and decomposition (Ingham *et al*., 1985; Beare *et al*., 1992). Nematodes are an ideal group for microcosm experiments because they are small and abundant in soil (Suderman and Thistle, 2003). Furthermore, nematodes reproduce continuously and are sediment bound throughout their life history which makes them easy to maintain under experimental conditions (Neher, 2001; Blakely *et al*., 2002; Gyedu-Arabio and Baird, 2006).

Nematodes exhibit rapid responses to many toxicants because of their short life cycles (Guo *et al*., 2001; Santos *et al*., 2010). Many studies have investigated the effects of PAHs on natural free-living nematodes in field (Feder and Blanchard, 1998; Thompson *et al*., 2007) and laboratory experiments (Beyrem *et al*., 2007; Lindgren *et al*., 2012).

The intensity of oil pollution effects are dependent on the type and quantity of the oil (Mahmoudi *et al*., 2005), the bioavailability of PAHs in the soil (Langston and Spence, 1994), the type of sediment (Di Toro *et al*., 1991) and on the nematode species (Powell *et al*., 2005).

The majority of spilled oil that is not cleaned by human intervention undergoes degradation by soil microorganisms (Leahy and Colwell, 1990). The rate of oil degradation is limited by low levels of biologically available N and P (Hozumi *et al*., 2000). Bioremediation of oil spills has involved the use of fertilisers (Head and Swannell, 1999; Nikolopoulou and Kalogerakis, 2013) which are effective in biodegradation of PAHs because they maintain nutrient concentrations at high levels in contaminated sediments (Xu *et al*., 2005). The addition of fertilisers can increase the rate of oil degradation in sediments by three to five times (Atlas, 1995; Swannell *et al*., 1996). Studies on the responses of meiofauna to the addition of fertilisers in oil contaminated sediments are limited (Schratzberger *et al*., 2003; Sundbäck *et al*., 2010).

Studies on oil pollution in South Africa have focussed on the impact of spills on beach meiofauna (Fricke *et al*., 1981), shellfish (O'Donoghue and Marshall, 2003; Degger *et al*., 2011) and penguins (Wolfaardt *et al*., 2009). Most studies have concentrated on the effects of heavy metals on meiofauna (Watling and Watling, 1988; Gyedu-Ababio *et al*., 1999; Gyedu-Ababio, 2011). Studies on the effects of PAHs on nematodes in South African mangrove sediments are non-existent. This study aims to use experimental microcosms to investigate the effects of oil, with or without fertiliser, on natural nematode abundance, species richness and community structure.

6.2 Materials and methods

6.2.1 Collection of nematodes

Mangrove sediment containing natural nematode assemblages were collected from the Isipingo estuary. Sediment was removed from a depth of 15 cm, transferred to a bucket and transported to an airconditioned glasshouse. Sediments were homogenised by

gentle stirring with a spatula before they were used for oil contamination experiments. Soil characteristics for the Isipingo estuary are indicated in Table 2.2, Chapter 2.

6.2.2 Experimental microcosms

Microcosms consisted of 350 ml plastic jars, sterilized with ethanol, washed with double distilled water and filled with 250 g of sediment. Microcosms were subjected to control and oiled treatments (with or without fertiliser). There were four replicates in each treatment.

Mass of wet sediments was determined on a Toledo scale. Oiled treatments consisted of 200 g untreated natural mangrove sediment and 50 g oil-treated sediment. Sediment used for oil contamination was frozen overnight in a chest freezer at -20 °C and thawed the next day. This process was repeated over three days in order to kill any existing microorganisms (Austen *et al*., 1994). Fifty grams of the frozen and thawed sediment were contaminated by 15 ml of oil. The oil was mixed into the sediment with a spatula. In the control, 200 g untreated natural mangrove sediment and 50 g frozen and thawed sediment were combined.

In the fertilised oiled treatment, 200 g untreated natural mangrove sediment and 50 g oil-treated sediment were combined with an organic liquid fertiliser (Biotrissol, Germany), the specifications of which are indicated in Table 6.1. Fertiliser (5 ml) was diluted with double distilled water (1 L) and the mixture sprayed onto the sediment surface weekly. All treatments were kept moist by spraying seawater at 10 psu daily onto the soil surface. Jars were unsealed throughout the duration of the experiment to allow for ventilation. Microcosms were maintained in a glasshouse at 25 °C (day) and 18 °C (night) for four weeks. The properties of the bunker fuel oil used in this study are indicated in Table 2.1, Chapter 2.

6.2.3 Nematode extraction

Nematode extraction was undertaken with the centrifugal flotation method in which sucrose solution was the separating agent (Lackey and May, 1971). Live nematodes that passed through a 1 mm mesh sieve and retained on a 40 µm sieve were collected (Vitiello and Dinet, 1979). Nematodes were placed in 5% formalin and counted under a

LEICA MZ16 stereo microscope (Leica Microsystems, USA). Genus and species identification were undertaken with a compound oil immersion microscope (Olympus BH2, Japan) using the pictorial keys of Platt and Warwick (1983, 1988), and Warwick *et al*. (1998).

6.3 Data analyses

Means and standard errors were calculated for all measurements. Resulting data were tested for normality using the Kolmogorov-Smirnov test and subjected to two-way ANOVA and Tukey's multiple comparisons test ($P \leq 0.05$) using GraphPad Prism Version 6.05 (GraphPad Software, Inc., USA). Other data were subjected to one-way ANOVA and Tukey-Kramer multiple comparisons test ($P \leq 0.05$) using MINITAB version 16 (Minitab Statistical Software, MINITAB Inc., USA).

Table 6.1
Specifications of fertiliser used in the study (Biogrow Chemicals, 2007).

		Value
pH		6.4
N	%	3
P	%	2
K	%	5
Cu	mgL^{-1}	10
Fe	mgL^{-1}	3.2
Mn	mgL^{-1}	0.55
Zn	mgL^{-1}	0.2
B	mgL^{-1}	0.18
Ca	mgL^{-1}	50
Mg	mgL^{-1}	105
S	mgL^{-1}	85
Na	mgL^{-1}	100

6.4 Results

As nematodes in mangroves in South Africa have not been investigated previously, most species were assigned to genus and some to family (Table 6.1).

Species were ranked from highest dominance [1] (based on their abundance in each treatment), to lowest [5] (Table 6.1). Of the 15 species present in the control, *Ethmolaimus* sp. exhibited highest dominance (Fig. 6.2).

6.4.1 Effects of oil on nematodes

Nematode abundance (Fig. 6.1) and species richness (Fig. 6.2) were highest in the control and significantly lower in the unfertilised oiled treatment by 87% and 53%, respectively.

In the unfertilised oiled treatment, *Monhystera* sp. (13%), *Ethmolaimus* sp. (40%) and species from the family Leptolaimidae (40%) exhibited the highest abundance. In the unfertilised oiled treatment, *Hemicycliophora typical, Hemicycliophora ripa*, *Panagrolaimus* sp., and species from the family Areaolaimida had similar abundance (2 - 5%) (Fig. 6.2). The seven species present in the unfertilised oiled treatment were characterised as oil-resistant and resilient (Millward *et al*., 2004; Mahmoudi *et al*., 2005).

In the unfertilised oiled treatment, *Ethmolaimus* sp. and *Monhystera* sp. decreased in abundance by 33%, 58%, respectively, compared to the control (Fig. 6.2).

In the unfertilised oiled treatment, abundance of species from the family Leptolaimidae increased by 79%, compared to the control and was characterised as opportunistic (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010) (Fig. 6.2).

The nine species present in the control but absent in the unfertilised oiled treatment, namely, *Rhabditis* sp., *Monhystrella* sp., *Prodesmodora* sp., *Plectus* sp., *Desmodora* sp., *Tobrilus* sp., *Koerneria* sp., *Fictor* sp., and *Rotylenchus* sp. (Fig. 6.2) were characterised as oil-intolerant (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010).

Effects of oil on adults and juveniles

In the unfertilised oiled treatment, adults of five species were present, namely, *Ethmolaimus* sp. (50%), *Monhystera* sp. (7%), *Hemicycliophora ripa* (4%) and species from the families Leptolaimidae (36%) and Areaolaimida (4%) (Fig. 6.3).

Juvenile abundance of *Monhystera* sp. was highest in the control and significantly lower in the unfertilised oiled treatment by 50% (Fig. 6.4). In the unfertilised oiled treatment the species from the family Leptolaimidae exhibited the highest juvenile abundance (46%), while juveniles were absent in the control (Fig. 6.4). In the unfertilised oiled treatment, the juvenile abundance of *Ethmolaimus* sp. was 63% lower than the control (Fig. 6.4).

6.4.2 Effects of oil with added fertiliser on nematodes

Nematode abundance (Fig. 6.1) and species richness (Fig. 6.2) were highest in the control and significantly lower in the fertilised oiled treatment by 69% and 33% respectively.

The addition of fertiliser to oiled treatments increased nematode abundance and species richness by 56% and 30%, respectively, compared to the unfertilised oiled treatment.

In the fertilised oiled treatment, *Ethmolaimus* sp. (51%) and species from the family Leptolaimidae (27%), exhibited the highest abundance. In the fertilised oiled treatment, *Monhystera* sp., *Hemicycliophora typica*, *Hemicycliophora ripa*, *Rhabditis* sp., *Panagrolaimus* sp., *Desmodora* sp, and *Rotylenchus* sp. and *Koerneria* sp. had similar abundance (1 - 6%) (Fig. 6.2).

Abundance of *Ethmolaimus* sp. and species from the family Leptolaimidae increased in the fertilised oiled treatment by 37% and 83% respectively, compared to the control (Fig. 6.2).

Three species, namely, *Rhabditis* sp., *Koerneria* sp. and *Rotylenchus* sp. were absent in the unfertilised oiled treatment but present in the fertilised oiled treatment and in the

control and characterised as oil-sensitive (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010) (Fig. 6.2). One species, *Desmodora* sp. was absent in the unfertilised oiled treatment and control but present in the fertilised oiled treatment.

Five species, *Monhystrella* sp., *Prodesmodora* sp., *Plectus* sp., *Tobrilus* sp. and *Fictor* sp (Fig. 6.2) were absent in both oiled treatments but present in the control and characterised as oil-intolerant (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010).

Effects of oil with added fertiliser on adults and juveniles

In the fertilised oiled treatment, adults of six species were present, namely, *Ethmolaimus* sp. (57%), the species from the family Leptolaimidae (32%), *Monhystera* sp. (4%), *Desmodora* sp. (2%), *Koerneria* sp. (4%) and *Hemicycliophora ripa* (2%) (Fig. 6.3).

In the fertilised oiled treatment, adult abundance of *Ethmolaimus* sp. and species from the family Leptolaimidae increased significantly by 44% and 75% compared to the control (Fig. 6.3).

In the control, *Monhystera* sp. exhibited the highest juvenile abundance, which was 60% lower in the fertilised oiled treatment (Fig. 6.4). In the fertilised oiled treatment, species from the family Leptolaimidae exhibited the highest juvenile abundance (25%), while juveniles were absent in the control (Fig. 6.4).

In the fertilised oiled treatment, juveniles of *Rhabditis* sp. (8%) and *Rotylenchus* sp. (8%) were present, but absent in the unfertilised oiled treatment (Fig. 6.4). There were no significant differences in juvenile abundance of *Ethmolaimus* sp. between the treatments. Juveniles of species from the family Areaolaimida, *Desmodora* sp., and *Koerneria* sp. were absent in the control and both oiled treatments (Fig. 6.4).

Table 6.1 Species richness of nematodes from the Isipingo estuary after four weeks of treatment, ranked (1 - 5) according to abundance, C = control, O = unfertilised oiled, O + F = fertilised oiled.

Taxon	Abbreviation	Treatment		
		C	O + F	O
Ethmolaimus sp.	E	1	1	1
Monhystera sp.	M1	2	3	2
Leptolaimidae	L	3	2	1
Hemicycliophora typica	H1	3	4	3
Hemicycliophora ripa	H2	5	4	3
Panagrolaimus sp.	P1	5	4	3
Prodesmodora sp.	P2	5	0	0
Plectus sp.	P3	5	0	0
Areaolaimida	A	5	0	3
Desmodora sp.	D	0	4	0
Tobrilus sp.	T	5	0	0
Koerneria sp.	K	5	4	0
Fictor sp.	F	5	0	0
Monhystrella sp.	M2	4	0	0
Rhabditis sp.	R1	5	4	0
Rotylenchus sp.	R2	5	4	0

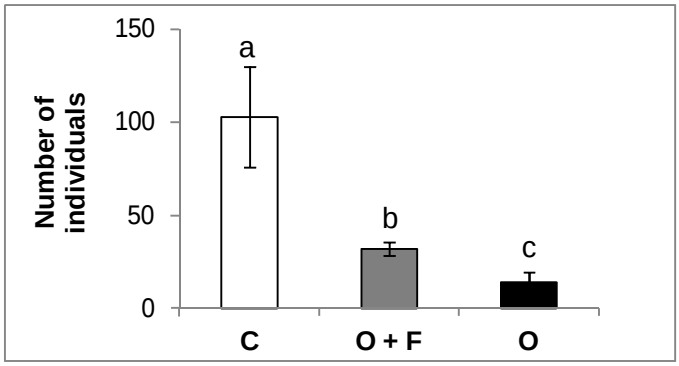

Fig. 6.1 Effects of oil and added fertiliser on nematode abundance in mangrove sediment. Measurements were taken after four weeks of treatment, C = control, O + F = fertilised oiled, O = unfertilised oiled. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using Tukey-Kramer multiple comparisons test.

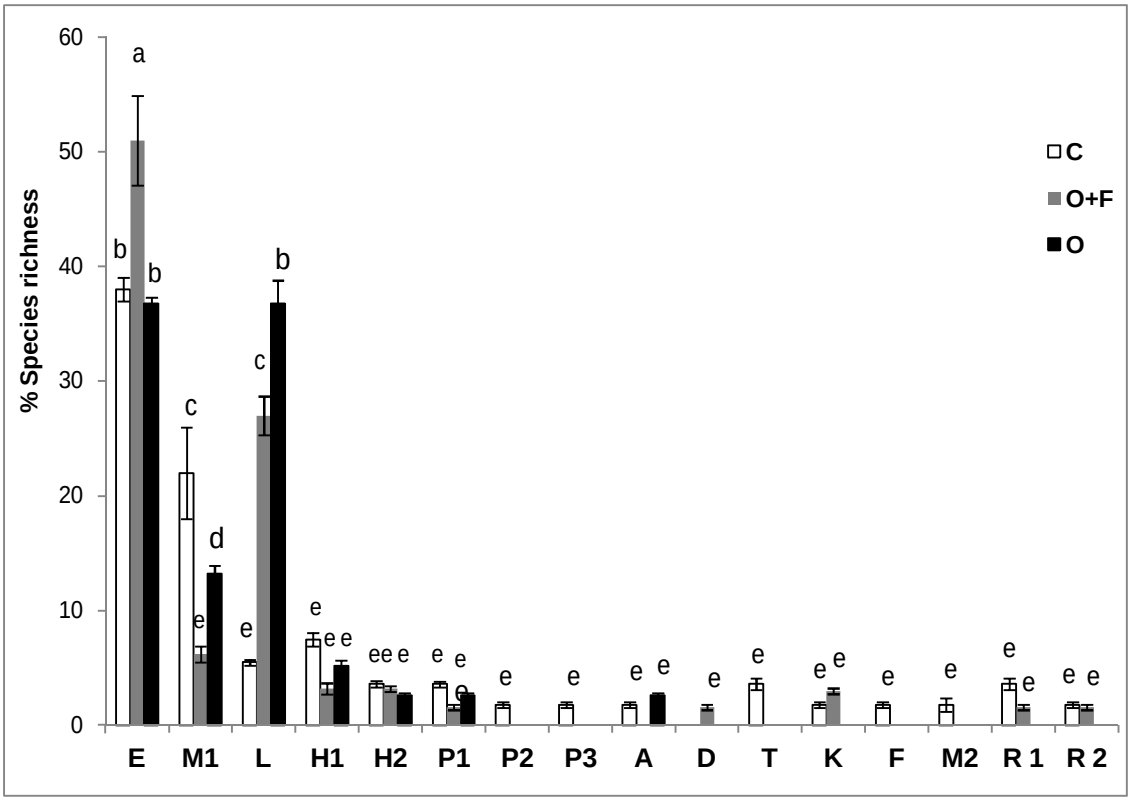

Fig. 6.2 Effects of oil and added fertiliser on species richness in mangrove sediment. Measurements were taken after four weeks of treatment, C = control, O + F = fertilised oiled, O = unfertilised oiled. Species names on the X-axis are listed in Table 6.1 (E1 to R2). No bars indicate absence of species in treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

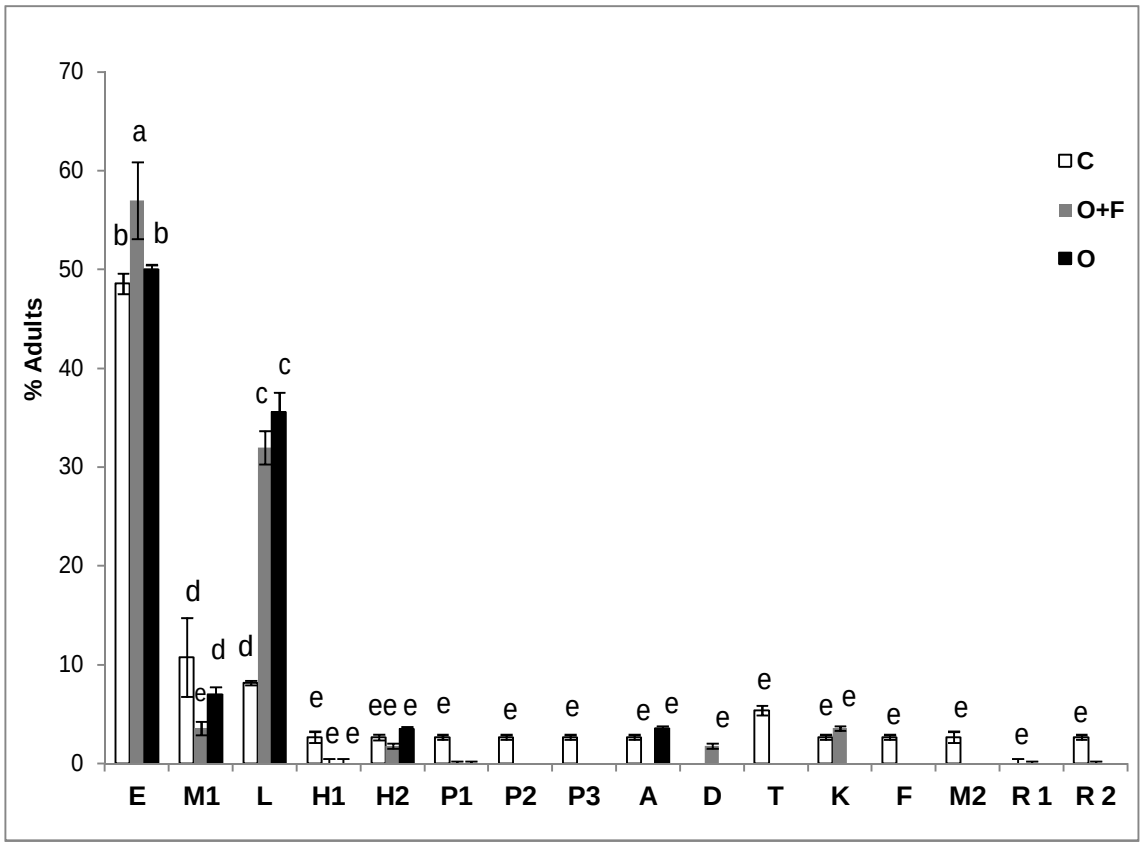

Fig. 6.3 Effects of oil and added fertiliser on adult nematode abundance in mangrove sediment. Measurements were taken after four weeks of treatments, C = control, O + F = fertilised oiled, O = unfertilised oiled. Species names on the X-axis are listed in Table 1 (M1 to R2). No bars indicate absence of species in treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at $P \leq 0.05$ using two-way ANOVA and Tukey's multiple comparisons test.

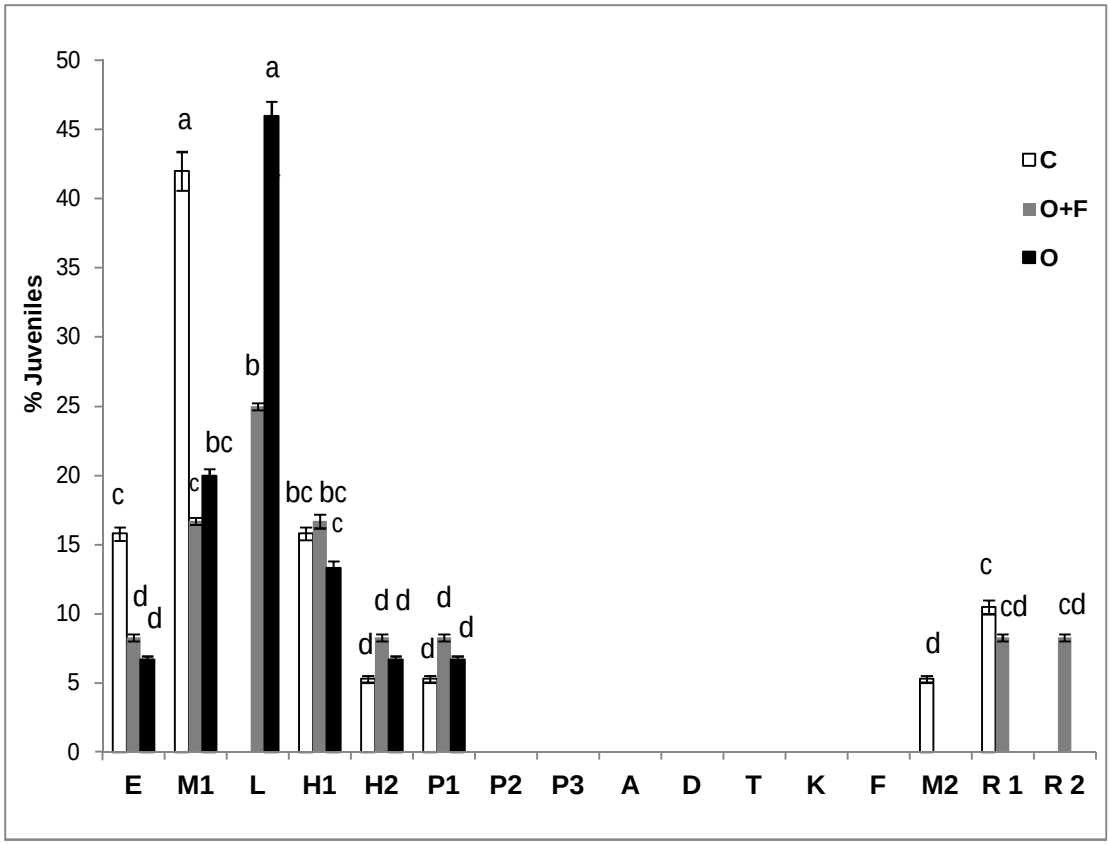

Fig. 6.4 Effects of oil and added fertiliser on juvenile nematode abundance in mangrove sediment. Measurements were taken after four weeks of treatment, C = control, O + F = fertilised oiled, O = unfertilised oiled. Species names on the X-axis are listed in Table 1 (M1 to R2). No bars indicate absence of species in treatment. Means ± standard error are given, n = 4. Bars with different letters are significantly different at P ≤ 0.05 using two-way ANOVA and Tukey's multiple comparisons test.

6.5 Discussion

The nematode assemblage-level approach to determine, monitor and assess the negative effects of PAHs has been validated in many studies (Moreno *et al*., 2009; Beyrem *et al*., 2010; Lv *et al*., 2011). In this study, natural populations of nematodes from the Isipingo estuary responded negatively to oil contamination in experimental microcosms.

6.5.1 The effects of oiling on nematode community structure

Oiling significantly decreased nematode abundance and species richness. Although nematodes are generally resistant to disturbance, many studies have found that they are particularly sensitive to PAHs (Moreno *et al*., 2009; Lv *et al*., 2011; Lindgren *et al*., 2012). Nematodes, because of their close association with the sediment, are directly in contact with oil contaminated soil particles (Blakely *et al*., 2002). Their permeable cuticles, and lack of a protective casing (calcium or silicate) that other meiofauna, such as copepods possess, makes them vulnerable to PAH penetration and toxicity, resulting in decreased abundance (Stringer *et al*., 2012). PAHs diffuse passively across membranes as a result of their non-polar and lipophilic nature (Gauthier *et al*., 2014). PAHs adversely affect membrane structure, causing expansion and increased fluidity that impairs cellular function and results in cell death (Sikkema *et al*., 1994). Benzene was reported to increase membrane fluidity in the bacterium, *Rhodococcus sp.* (Gutierrez *et al*., 1999) and disrupt ion transport and decrease Ca^{2+} in the mussel, *Mytilus edulis* (Borseth *et al*., 1995). Furthermore, PAHs are cytotoxic, causing intracellular damage in nematodes by preventing the synthesis of proteins that are important for both damage repair and survival (Liuzzi *et al*., 2012).

Previous studies showed that nematode abundance and species richness decreased with diesel (Lindgren *et al*., 2012), crude (Lv *et al*., 2011) and lubricating oil (Beyrem *et al*., 2010) added to the sediment. In this study, oil probably depleted dissolved oxygen in the soil decreasing nematode activities such as sediment bio-turbation and recycling of organic matter, as reported previously (Aller and Aller, 1992; Neira *et al*., 2001; Sundbäck *et al*., 2010). The addition of pyrene to sediment, was shown to decrease grazing activity of nematodes (Petersen *et al*., 2009; Sunbäck *et al*., 2010). Reduced grazing activity increases the anoxic zone in the sediment, as the nematodes no longer

oxygenate the sediment through their bio-turbation (Sochová *et al*., 2006; Lindgren *et al*., 2012). Anoxia of the sediment, as a result of oiling, probably contributed to the decrease in abundance and species richness in this study. Anoxia in soil alters nematode community structure by reducing competition and favouring resilient species (Elmgren *et al*., 1983).

Four species decreased with oiling. Previous studies found that sensitive species decrease with oiling (Morena *et al*., 2009; Sundbäck *et al*., 2010; Lindgren *et al*., 2012). Individual species vary in their sensitivity to PAHs at sub-lethal concentrations (Austen and McEvoy, 1997; Carman *et al*., 2000; Millward *et al*., 2004). In marine nematodes, regulation of water and salt content of the body is achieved by renette glands, present under the pharynx (White *et al*., 1986). PAHs disrupt the functioning of cell membranes of microorganisms as ion-selective barriers and matrix for enzymes (Sikkema *et al*., 1995; Wilke, 1997). PAH-induced damage to nematode membranes could have contributed to sensitivity in saline mangrove soil. Furthermore, specialized excretory systems are not well developed in nematodes and nitrogenous wastes are lost by diffusion through the outer membrane of the body (White *et al*., 1986). PAHs could have contributed to waste build up in nematodes thereby increasing sensitivity.

Nine species present in the control were absent in the oiled treatment. Five oil-intolerant species were absent in both oiled treatments. The elimination of species by oil was reported in several studies (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010). In a previous study, crude oil eliminated 15 genera that were oil-intolerant (Lv *et al*., 2011). PAHs are neurotoxic (Tang *et al*., 2003). Nematodes have nervous systems (ganglia from which nerves run throughout the body), that are connected to the gut, reproductive organs and mouth (White *et al*., 1986). Exposure to neurotoxic pesticides resulted in adverse effects on the nervous system of the nematode, *Caenorhabditis elegans* (Meyer and Williams, 2014). PAHs negatively affected locomotion, feeding behavior, brood size, growth and life span, ultimately leading to cell death in previous studies (Ruan *et al*., 2009; Meyer and Williams, 2014). The nervous system of nematodes can account for nearly one-third of the total number of cells in the body (White *et al*., 1986). PAH contamination could have negatively affected the nervous system of nematodes in this study, resulting in increased sensitivity and death.

In this study, *Ethmolaimus* sp. and species from the family Leptolaimidae were dominant in the oiled treatments. Some species of meiofauna exhibit greater resilience to oiling than others (Austen and McEvoy, 1997; Carman *et al*., 2000). Nematodes that remain dominant with oiling are highly resilient (Danovaro *et al*., 2000; Millward *et al*., 2004; Mahmoudi *et al*., 2005).

Species from the family Leptolaimidae increased with oiling and were therefore opportunistic (Millward *et al*., 2004; Mahmoudi *et al*., 2005). Oiling can increase robust and opportunistic species, causing them to be dominant (Thompson *et al*., 2007). *Ethmolaimus* sp. and the species from the family Leptolaimidae were the most resilient species in this study. Oiling reduced competition and favoured species resistant to oil (Carman *et al*., 1997, 2000; Millward *et al*., 2004). The change in abundance of dominant species, due to oiling, alters nematode community structure (Carman *et al*., 2000; Mahmoudi *et al*., 2005; Beyrem *et al*., 2010).

In the oiled treatments, species from the family Leptolaimidae exhibited the highest juvenile abundance. Oil stimulated species from the family Leptolaimidae to reproduce. High juvenile abundance in oiled sediment indicates a highly tolerant and resilient species (Mahmoudi *et al*., 2005). Juvenile abundance of *Monhystera* sp. was highest in the control and decreased significantly in the oiled treatment. The ability to reproduce affects community structure by changing species dominance (Beyrem *et al*., 2010).

6.5.2 The addition of fertiliser

In this study, the addition of fertiliser to oiled treatments significantly increased nematode abundance and species richness, as demonstrated in other studies (Widbom and Elmgren, 1988; Eisentraeger *et al*., 2002). The addition of fertiliser increased nematode abundance of *Ethmolaimus* sp. and opportunistic species from the family Leptolaimidae. Studies have shown that N-addition increases the abundance of dominant and opportunistic species (Murray *et al*., 2006; Sánchez-moreno *et al*., 2006). Fertiliser additions increased the decomposition rate of oil in soil by stimulating bio-degrading bacterial activity which in turn elevated N and P concentrations in the interstitial water (MacNaughton *et al*., 1999; Rohling *et al*., 2002; Xu *et al*., 2005).

Three species, *Rhabditis* sp., *Koerneria* sp. and *Rotylenchus* sp. survived oiling with the addition of fertiliser. This suggests that fertiliser improved conditions for these nematodes. Juveniles of *Rhabditis* sp. and *Rotylenchus* sp. were absent in the oiled treatment but present with the addition of fertiliser. This suggests that these species were able to reproduce because of the addition of fertiliser, consistent with a previous study (Widbom and Elmgren, 1988). In the fertilised oiled treatment, the species from the family Leptolaimidae exhibited the highest juvenile abundance suggesting that this is an opportunistic species (Thompson *et al*., 2007).

In the fertilised oiled treatment, juvenile abundances of five species were similar. Similar abundance of species is characterized as 'evening out' and was previously reported for nematodes in fertilised oiled treatments in a field mesocosm study (Schratzberger *et al*., 2003). Lubricating oil decreased the abundance of dominant nematode species in oiled treatments resulting in a decrease in competition and an evening out of species compared to those in the controls (Beyrem *et al*., 2010). In the fertilised oiled treatment, the decrease in juvenile abundance of *Ethmolaimus* sp., contributed to the increase of oil-sensitive species through reduced competition. Furthermore, the addition of fertiliser to oiled sediment increased the resilient microalgal and bacterial community that provide a food supply to oil-resistant nematodes (Montserrat *et al*., 2008; Sundbäck *et al*., 2010).

In this study, the majority of nematode taxa were predatory (feed on bacteria and fungi). Other studies found higher proportions of predatory nematodes in sites heavily polluted with PAHs, compared to light or uncontaminated ones (Erstfeld and Snow-Ashbrook, 1999; Chen *et al*., 2009). Bacterivorous nematodes proliferate in soils contaminated with PAHs (Blakely *et al*., 2002; Chen *et al*., 2009) probably because of increased microbial activity and availability of food resources (Erstfeld and Snow-Ashbrook, 1999).

Desmodora sp. was present in the fertilised oiled treatment but absent in the unfertilised oiled and control treatments. This suggests that *Desmodora* sp. is tolerant to oiling with fertiliser or that it is a rare tolerant species.

Out of the 16 nematode taxa identified in this study, seven were oil-resistant, and nine oil-intolerant. Altered species composition and abundance in nematodes, induced by

oil, could have repercussions on other meiofauna communities and ultimately ecosystems. The recovery of communities after an oil spill is slow. Although microcosms do not completely mimic natural conditions, the responses of nematodes to oil incubation of four weeks, *in situ*, provides valuable insight into the negative effects of PAHs.

Conclusion

This study demonstrated the deleterious effects of oil on coastal macrophytes, *A. marina, B. gymnorrhiza, R. mucronata* and on nematode meiofauna.

Seedlings of the three mangrove species exhibited various adverse morphological responses to sub-lethal contamination. Sub-lethal and residual oil contaminations occur more frequently than large-scale accidental spills and pose a serious threat to mangroves (Kathiresan and Bingham, 2001). Sub-lethal oil contamination on propagules, internodes, leaves and sediment reduced growth in all species.

Avicennia marina and *R. mucronata* were more sensitive to oil applications to propagules, stems and leaves than *B. gymnorrhiza*. For example, propagule oiling caused necrosis, deformed leaves and stems in *A. marina* and *R. mucronata* but not in *B. gymnorrhiza.* Oil-induced responses were most intense in *A. marina* (see Table 2.5, Chapter 2). The pericarp did not provide any protection from the adverse effects of oil in *A. marina*. *Avicennia marina* responded to sediment oiling in the field by producing adventitious roots, as reported previously (Naidoo *et al*., 2010).

Rhizotrons provided a non-invasive technique of measuring and evaluating root growth. Partially oiled propagules of *A. marina* and *B. gymnorrhiza* produced shorter and thicker roots than untreated controls. *Avicennia marina* and *B. gymnorrhiza* seedlings responded to sub-lethal oil contamination by increasing root diameter, an adaptive response, not described previously for mangroves. Growth of *B. gymnorrhiza* was stimulated by partial oiling of the propagules. Oil stimulated lateral root growth in *R. mucronata* seedlings, an adaptive response not described previously.

There is a paucity of information on the uptake and accumulation of PAHs in mangroves and their contribution to oil-induced plant stress. PAHs with low molecular weight were suggested to be the cause of oil toxicity in previous studies on mangroves (Getter *et al*., 1985; Suprayogi and Murray, 1999). This study clearly demonstrated that PAHs with low and high molecular weight are absorbed by mangroves. In the oiled treatments, roots contained a greater number of PAHs and in higher quantities than leaves in all three species. *Avicennia marina* accumulated more PAHs in roots and

leaves compared to those of *B. gymnorrhiza* and *R. mucronata*. This probably explains the greater oil-sensitivity of this species.

In oiled treatments, the most common PAHs in roots of all species were fluorene and acenaphthene (two rings), phenanthrene and anthracene (three rings), pyrene and chrysene (four rings) and benzo[a]pyrene (five rings). In the leaves of *R. mucronata* and *B. gymnorrhiza* in oiled treatments, the common PAHs were naphthalene and acenapthene (two rings) and phenanthrene (three rings). Leaves of *A. marina* in oiled treatments contained almost all PAHs tested, more than those in roots. This demonstrates that in *A. marina* all PAHs tested were translocated from the roots to the shoots. In leaves of *B. gymnorrhiza* in oiled treatments, high molecular weight PAHs were absent, while there was a negligible amount of pyrene in leaves of *R. mucronata*. These results suggest that *B. gymnorrhiza* possibly restricts oil entry into the roots, thereby reducing PAH accumulation in the foliage, as reported previously (Ke *et al*., 2003; 2011a). This explains the minimal oil effects on *B. gymnorrhiza* compared to the other two species. This study demonstrated that *A. marina* has larger air spaces and thinner hypodermis and epidermis in roots, compared to *B. gymnorrhiza* or *R. mucronata* (Pi *et al*., 2009). These anatomical differences probably permitted greater oil penetration and accumulation within tissues in *A. marina*. Furthermore, oil was probably translocated through aerenchyma channels in roots and leaves of *A. marina* whereas water does not, as demonstrated previously in *Phragmites australis* (Armstrong *et al*., 2009). In our study, the combined effect of PAHs caused disorganization of cells and degradation of cell walls, plasma membranes and organelles in root and leaf tissues in the three species.

Of the three species, *R. mucronata* propagules are the largest, followed by *B. gymnorrhiza* and *A. marina*. Larger propagules in *R. mucronata* and *B. gymnorrhiza* could serve as additional sites of accumulation, thus minimizing the amount of oil entering the shoots. This could account for the greater tolerance of *R. mucronata* and *B. gymnorrhiza* to oil than *A. marina*. Additional research on oil accumulation in propagules of other species could provide more insight into the role of propagule size in restricting PAH accumulation in the shoots.

The concentrations of individual PAHs in roots and leaves varied amongst the three species but identities of the compounds were similar. This suggests that mechanisms

controlling the uptake of PAHs in mangroves could be similar. However, studies on PAH distribution in mangrove tissues are lacking. Kang *et al*. (2010) suggested that transpiration and the lipid content of cells are the main drivers of PAH accumulation and distribution in ryegrass roots by demonstrating that phenanthrene and pyrene primarily accumulated in cell walls and organelles. Distribution of absorbed oil components in tissues appears to be PAH-specific (Gauthier *et al*., 2014). In fish, naphthalene accumulated almost exclusively in the intestine and anthracene in the liver and kidneys (Advaiti *et al*., 2013). Pyrene, which has a higher lipophilicity, accumulated primarily in cellular walls and organelles in ryegrass roots (Kang *et al*., 2010). The mechanism of toxicity appears to be PAH-specific and species-specific (Gauthier *et al*., 2014). Although this study offers valuable insights into oil accumulation within root and leaf cells, further research is required to determine the sites of PAH-specific accumulation within tissues and their toxic effects. Studies on the toxicity of specific PAHs and the ability of tissues to detoxify or metabolise these compounds could be useful for phytoremediation.

In another series of experiments, the stain propidium iodide was used to detect dead cells in oiled root tips. The majority of cells in oiled root tips of all three species exhibited red fluorescence indicating dead and damaged cells, demonstrating that PAHs are cytotoxic. Transmission electron micrographs further demonstrated the extent of oil-damage in root tips and leaves and provided insights into the activities of oil within cells. Oil accumulated within cell walls, cytoplasm, vacuoles and organelles in the meristematic and conducting tissue of root tips. Oil infiltrated adjoining cells through ruptured walls, plasma membranes and plasmodesmata and caused the deterioration of these structures. Phloem cells in all species were completely disorganised while xylem tissue exhibited minimal damage. The results suggested that *A. marina* was more susceptible to oil injury than the other two species and support a previous study (Naidoo *et al.*, 2010) that adventitious root development was a response to oil-damaged phloem tissue. In leaves of all oiled seedlings, chloroplasts were disorganised with degraded grana and lamellae as a result of oil accumulation. This is the first report to demonstrate the precise location of oil within root and leaf cells.

This study also demonstrated the effects of 50% seawater, in combination with oil, on growth. Salinity is one of the principle environmental factors limiting the development of mangrove forests (Koch and Snedaker, 1997). In this study, 50% seawater in

combination with oil resulted in reduced growth in all species. This study demonstrated that the adverse effects of 50% seawater were exacerbated by oil.

Oiling reduced salt secretion in *A. marina* and led to the accumulation of Na^+ in leaves. Sodium accumulation in leaves significantly increased Na^+/K^+ ratios in oiled treatments. This study demonstrated that oil-damage to root and leaf cells probably led to accumulation of Na^+. *Avicennia marina* accumulates more Na^+ and Cl^- than other mangroves (Naidoo, 1985; Medina, 1999). When roots are damaged by oil, ultrafiltration and salt exclusion are impaired and salt accumulated has to be removed by salt glands. PAHs could have damaged the plasma membrane in salt glands as an ion-selective barrier and matrix for important enzymes, as reported previously for cell membranes in the mussel, *Mytilus edulis* (Antoun, 2011) and trout (Schirmer *et al.*, 1998). The results of this study suggest that if the integrity of the membranes of salt gland cells is compromised by oil, the capacity of *A. marina* to secrete salt decreases. Further investigation into oil accumulation in the collecting, stalk and secretory cells of the salt glands will give more insight into the mechanisms of PAH-induced damage. Excess trace metals, Cu and Zn, are secreted with other solutes such as Na and Cl, in *A. marina* leaves (Naidoo *et al*., 2014), *A. germinans* and *Aegiceras corniculatum* (MacFarlane and Burchett, 1999; MacFarlane *et al*., 2007). Further research is required to determine whether PAHs are secreted with other solutes.

Oil also decreased growth by reducing the capacity of seedlings to acquire resources (e.g. gas exchange and nutrients) through damage to cells and tissue. *Bruguiera gymnorrhiza* was the most oil-tolerant species followed by *R. mucronata* and *A. marina*. Many studies previously suggested that oil damaged cell walls and membranes in conducting tissue leading to reduced growth and mortality (Gilfillan *et al.*, 1989; Ye and Tam, 2007; Zhang *et al.*, 2007a). This study clearly demonstrated (using chemical testing and electron microscopy) that PAH accumulation and oil-damage to cell walls and membranes reduced growth and caused mortality. Further research is required to determine PAH-specific effects on root and leaf cells.

In microcosm experiments, natural free-living populations of nematodes from the Isipingo estuary were adversely affected by oil contamination. Oiling decreased nematode abundance and species richness. Out of the 16 different taxa identified in this study, seven were oil-resistant and nine were oil-intolerant. Oiling eliminated five

genera. Oiling reduced competition and favoured resilient, resistant species as well as the abundance of opportunistic species. Oiling resulted in changes in nematode abundance, species richness and dominant species, thereby altering community composition and structure, as reported previously (Mahmoudi *et al*., 2005; Beyrem *et al*., 2010).

The addition of fertiliser to oiled treatments significantly increased nematode abundance and species richness. The addition of fertiliser to oiled treatments is known to accelerate bio-degradation of PAHs in sediments and increase nematode survival (Schratzberger *et al*., 2003; Lima *et al*., 2012). Fertiliser addition also increased reproduction in dominant species and improved conditions for oil-sensitive species, ensuring their survival. Fertiliser addition is a cheaper, less toxic alternative to chemical cleaners and is effective in areas impacted by oiling without posing a threat to the environment (Moreira *et al*., 2011).

In this study, the majority of nematode taxa were predatory (feeding on bacteria), probably because PAHs increase microbial activity in sediments thereby increasing available food resources (Erstfeld and Snow-Ashbrook, 1999). Further investigation into the microbes in mangrove soils will be beneficial in bioremediation.

This study has implications for interpreting the ecological consequences of hydrocarbon contamination to benthic communities. Altered species composition and community structure by oil could significantly influence interactions between nematodes and other benthic organisms (Carman *et al*., 1997; Schmid-Araya *et al*., 2002). Many macrofauna depend on nematodes as a food source (Gee, 1989; Coull *et al*., 1995). Responses of free living nematodes to oil (increase, decrease or elimination) could lead to food limitation that ultimately affects and alters entire communities and ecosystems. Although microcosms are useful in demonstrating broad changes to nematode assemblages, a more detailed examination of the benthic nematode ecology in South African mangroves is needed, under field conditions, to better understand the impacts of oil contamination.

Mangroves are unique and significant ecosystems of high ecological and conservation value (Holguin *et al.,* 2001; Feller *et al*., 2010). There is a great need to better understand the effects of oil pollution on mangrove flora and fauna. Mangrove forests

are disappearing in many parts of the world (Kathiresan and Bingham, 2001; Polidoro *et al*., 2010; Bayen, 2012). This study provided important information on the effects of oil on above and below ground components in South African mangroves. This is the first study to use rhizotrons in oil contamination experiments on mangroves. Our results showed that mangroves can survive the physical and chemical effects of oil contamination by developing morphological adaptations. *Avicennia marina* and *B. gymnorrhiza* increased their root diameters in response to sub-lethal oil contamination. *Avicennia marina* produced adventitious roots while *R. mucronata* produced numerous lateral roots. Valuable insight into the accumulation and activities of oil within roots and leaves of mangroves was demonstrated in this work. Salinity effects were exacerbated by oil. Valuable information on the effects of oil and added fertiliser on nematode assemblages in the mangrove sediments of a South African estuary was gained. New nematode species (currently under investigation) contribute to an increase in mangrove benthic taxonomical studies. The results of this study contributed significantly to our understanding of marine oil pollution effects in mangroves of South Africa.

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