

Ecological Implications of Allelopathic Interferences
with reference to *Phragmites australis*

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Summary

The effects of plant invasions on ecosystem structure and function are well studied but the pathways and mechanisms that underlie these effects remain poorly understood. In depth investigation of invasion mechanisms is vital to understanding why invasive plants impact only certain systems, and why only some invaders have disproportionately large impacts on the invaded community. There are many mechanisms such as lack of natural enemies or control mechanisms, the individual characteristics of the invader and invaded communities, direct and indirect resource competition, evolution or hybridisation, altered ecosystems processes, and allelopathy that may explain the invasion processes of plant species. Among these possible influences on invasion, allelopathy has received increased attention and study with the rise in understanding of its implications and potential disproportionate influence. However, identifying allelopathy and consequent phytotoxic effects as an important mechanism of plant invasion is a difficult task due to the potential for an individual plant to have many component chemicals with multiple modes of action, interactive effects, and synergistic interactions. For allelopathy to be implicated as a mechanism that facilitates invasion, multiple aspects of the plant species allelopathic properties must be examined. This research investigated allelopathy as a mechanism of the invasion process in *Phragmites australis* by a series of ecologically realistic experiments in the laboratory, greenhouse and field.

The first set of experiments were designed to explore phytotoxicity of *P. australis* on germination and growth of other plant species by using aqueous extracts of different organs. These studies showed that leaf and rhizome extracts exhibited significant inhibition on germination, and growth parameters ($P \leq 0.001$). Dose-

response studies confirmed LC₅₀ (4.68% and 11.25%) of *Lactuca sativa* for leaf and rhizome extracts respectively. Root growth of *Juncus pallidus* and *Rumex conglomeratus* were inhibited by 75% and 30% respectively in leaf leachate incorporated soil. Chlorophyll content and maximum quantum yield (F_v/F_m) were significantly reduced with leaf and rhizome leachate. *P. australis* organs were ranked in order of allelopathic potentiality: leaf > rhizome > root > stem.

The second group of experiments investigated phytotoxicity induced by *P. australis* on physiological and phenotypic parameters of the recipient plants with identification of the major phytotoxins in the donor plant. Bioassays using aqueous extracts of different organs and root exudates of *P. australis* were carried out in laboratory and greenhouse with *L. sativa* as the model test plant. The observed reduced liquid imbibition and altered resource mobilization in seeds of *L. sativa*, in particular an insufficient carbohydrate supply, demonstrated that the onset of germination might be negatively affected by phytotoxicity induced by *P. australis*. Oxidative stress through reactive oxygen species (ROS) production induced by phytochemicals from *P. australis* could potentially cause the observed germination and seedling growth reductions. In addition, the osmotic effects of the aqueous extracts demonstrated that the results were partially induced by it. Overall, the relative strength of inhibition on measured physiological parameters was highest in leaf extract, followed by rhizome, root, stem and inflorescence. Root exudates of *P. australis* had negative impacts by reducing germination and growth of test plants. HPLC analysis revealed gallic acid, a potent phytotoxin, as a major compound within the plant. The concentration levels of gallic acid were highest in leaves followed by inflorescence, rhizome, root and stem.

The third group of experiments examined the dynamics of physico-chemical characteristics and phytotoxicity through residue decomposition of *P. australis* with and

without soil under different conditions and density over time. Physico-chemical variables (water-soluble phenolics, dissolved organic carbon, specific ultraviolet absorbance, pH, electrical conductivity, osmotic potential and some anions namely, PO_4^{3-} , Cl^- , NO_2^- , NO_3^- and SO_4^{2-}) of extracts were more consistent and showed a normal range of variation in aerobic conditions compared to anaerobic conditions which were more variable. 'Residue alone' and 'residue with soil' extracts exhibited significant inhibition on germination and growth of *Poa labillardierei* and *L. sativa* initially but the effects reduced over time in aerobic condition whereas in anaerobic conditions the effect increased the inhibition sharply and remained almost stable ($P \leq 0.001$). Water-soluble phenolics were a significant predictor of the inhibitory effects on germination and growth of tested species compared to other variables in the extracts. Long-term decomposed residues exhibited significant effects on germination and growth of *Melaleuca ericifolia* ($P \leq 0.01$) depending on residue density in soil. The results demonstrated that decomposition condition and soil incorporation coupled with residue density play a crucial role over time in the dynamics of physico-chemical variables and associated phytotoxicity.

The fourth series of experiments explored the allelopathic interference of *P. australis* on plant communities by assessing the chemical characteristics of soil and water of invaded communities in the field, and its phytotoxicity assessment in the laboratory. The chemical characteristics of soil and water were monitored in four seasons taking into consideration the phenological cycle of *P. australis*. A series of bioassays were conducted in relation to assessment of phytotoxicity on different plant species in the laboratory. Significant chemical changes to *in situ* soil and water were observed in *P. australis* invaded areas compared with control. Soil-water and whole plant-leachate significantly inhibited germination and α -amylase activity of the test

species *L. sativa* at higher concentrations. The adventitious root formation of *Phaseolus aureus* was suppressed by plant-leachate, soil-water and soil-surface water of *P. australis* infested field. Seasonal impact on allelopathic interference of *P. australis* in terms of germination and growth of *L. sativa*, *M. ericifolia*, and *P. labillardierei* showed a distinct variation with no clear trend. Soil sterilization experiments indicated that soil biota play an important role in reducing the phytotoxicity in natural soil.

The fifth group of experiments were set to differentiate the effects between allelopathy and resource competition. The difficulty of distinguishing allelopathy from resource competition among plants has hindered investigations of the role of phytotoxic allelochemicals in plant communities. Considering the complexity, a series of ecological realistic experiments were conducted in the greenhouse and laboratory addressing the biological response of exposed plants in relation to density-dependent phytotoxicity. Experimental plant (*M. ericifolia*, *R. conglomeratus*, and *L. sativa*) were grown at varying densities with the allelopathic plant, *P. australis* and varying concentrations of aqueous leachate and extracts of *P. australis* litter to investigate the potential interacting influences of allelopathy and resource competition on plant growth-density relationships. Phytotoxicity decreased with increasing plant density, and positive effects on plant traits including maximum individual plant biomass occurs at an intermediate density. These results were attributed to dilution of phytotoxins, i.e. the sharing of the available phytotoxin among plants at high densities. The results demonstrated either decreasing phytotoxicity with increasing plant density or a reversal in slope of the growth-density relationship as an indication of the allelopathic interference of *P. australis* rather than resource competition.

The last series of experiments explored the allelopathic interference of *P. australis* through root exudates on the native *M. ericifolia*. This study was carried out to

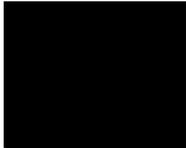
clarify the underlying invasion mechanisms as well as to determine potential management options. Germination and growth effects of *P. australis* on *M. ericifolia* were studied in the greenhouse using potting mix either with or without activated carbon and a combination of single and repeated cutting of *P. australis*. *Phragmites australis* had significant negative effects on germination and growth of *M. ericifolia* by inhibiting germination percentage, maximum root length and plant height, biomass, stem diameter, the number of growth points and leaf physiology. Activated carbon counteracted negative phytotoxic effects of *P. australis* on *M. ericifolia* modestly. The cutting of *P. australis* shoots significantly reduced the suppressive effects on *M. ericifolia* compared to the addition of activated carbon to soil. Furthermore, significant changes in the substrate such as pH, electrical conductivity, osmotic potential, phenolics and dehydrogenase activity were identified among cutting treatments with little variation between activated carbon treatments. The results demonstrated that allelopathy through root exudates of *P. australis* had relatively low contribution in suppression of *M. ericifolia* in comparison to other competitive effects. Management combining repeated cutting of *P. australis* shoots with AC treatments may assist partly in restoration of native ecosystems invaded by *P. australis*.

In conclusion, *P. australis* had significant phytotoxic potential on germination and growth of other plant species. Leaves were the most significant inhibitor compared with other organs of *P. australis*. Aqueous extracts of *P. australis* significantly influenced the physiological activities of the test plant species namely, liquid imbibition, resource mobilization, and oxidative condition with a partial induction by osmotic influences. In addition, gallic acid, an important phytotoxin, as major compound within *P. australis* was identified through HPLC with concentrations ordered from highest to lowest in leaf > inflorescence > rhizome > root > stem. Decomposition

experiments revealed longer stability and persistence of water-soluble phenolics in anaerobic compared with aerobic conditions. Moreover, this study demonstrated that the phytotoxic potential of soils in *P. australis* invaded wetlands is greatly increased as most wetlands experience anaerobic condition. The field evidence of phytotoxic potential by *P. australis* is further explained by the experiments that demonstrated the occurrences and implication of phytotoxicity in terms of inhibition of α -amylase in germination process, and adventitious rooting. Again, the density-dependent experiments distinguished the allelopathic effects by *P. australis* from resource competition stating that allelopathic interferences were more prominent rather than resource completion in suppressing the neighbouring plant species depending on the context. Finally, the greenhouse studies demonstrated that allelopathy through root exudates of *P. australis* had relatively low contribution in suppression of *M. ericifolia* in comparison to other competitive effects. Management combining repeated cutting of *P. australis* shoots with AC treatments may assist partly in restoration of native ecosystems invaded by *P. australis*. Overall, the studies carried out here, highlight the potential impacts of allelochemicals on plant recruitment in wetlands invaded with *P. australis*. This study may contribute to the understanding of ecological consequences of phytotoxins and may partially explain the invasion process of *P. australis* in wetlands. This synthesis may provide a logical understanding towards the invasion mechanisms of *P. australis* through allelopathy and contribute to the overall knowledge and management of the species and the ecosystems it occupies.

General declaration

I, Md. Nazim Uddin, declare that the PhD thesis entitled '**Ecological Implications of Allelopathic Interferences with reference to *Phragmites australis***' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature: 

Date: 24/07/2019

Declaration of authenticity

PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

| Item/Chapter No. | Paper Title | Publication status | Publication details |
|------------------|--|--------------------|---|
| Two | Phytotoxic evaluation of <i>Phragmites australis</i> : an investigation of aqueous extracts of different organs. | Published | July, 2012, in <i>Marine and Freshwater Research</i> ; Impact Factor: 1.982 |
| Three | Phytotoxicity induced by <i>Phragmites australis</i> : An assessment of phenotypic and physiological parameters involved in germination process and growth of receptor plant | Published | August 2014, in <i>Journal of Plant Interactions</i> ; Impact Factor: 0.897 |
| Four | Is phytotoxicity of <i>Phragmites australis</i> residue influenced by decomposition condition, time, and density? | Published | October 2014, in <i>Marine and Freshwater Research</i> ; Impact Factor: 1.982 |
| Five | Chemistry of a <i>Phragmites australis</i> dominated wetland and its phytotoxicity may suggest field evidence of allelopathy | Under review | <i>Journal of Ecology</i> ; Impact Factor: 5.431 |
| Six | Assessment of root and litter mediated allelopathic interference of <i>Phragmites australis</i> through density-dependent approach | Under revision | <i>Australian Journal of Botany</i> ; Impact Factor: 1.204 |
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Signature: 

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List of Publications and Awards

Peer reviewed publications

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3. **Uddin, M. N.**, Caridi, D., and Robinson, R. W. (2014). Phytotoxicity induced by *Phragmites australis*: An assessment of phenotypic and physiological parameters involved in germination process and growth of receptor plant. *Journal of Plant Interactions*, 9(1) 338-353.
4. **Uddin, M. N.**, Caridi, D., Robinson, R. W. and Harun, A. Y. A. (2014). Is phytotoxicity of *Phragmites australis* residue influenced by decomposition condition, time, and density? *Marine and Freshwater Research* 65, 505-516.
5. **Uddin, M. N.**, Robinson, R. W. and Harun, A. Y. A. (2014). Suppression of native *Melaleuca ericifolia* by the invasive *Phragmites australis* through allelopathic root exudates. *American Journal of Botany*, 101 (3) 479-486.
6. Harun, A. Y. A., Robinson, Randall W., Johnson, J. and **Uddin, Md N.**, (2014). Allelopathic potential of *Chrysanthemoides monilifera* subsp. monilifera (boneseed): a novel weapon in the invasion processes. *South African Journal of Botany*, 93: 157-166.

Published Abstracts

1. **Uddin, M. N.**, Robinson, R. W., Caridi, D., and Harun, A. Y. A. Assessment of root and litter mediated allelopathic interference of *Phragmites australis* using density-dependent approach, In Proceedings of *5th Joint Conference of New Zealand Ecological Society and Ecological Society of Australia*, held on 24-29 November 2013, Auckland, New Zealand.
2. Harun, A. Y. A, Robinson, R. W., Johnson, J., and **Uddin, M. N.** Allelopathy of Bonseed (*Chrysanthemoides monilifera subsp. monilifera*): a biochemical weapon of invasion, In Proceedings of *5th Joint Conference of New Zealand Ecological Society and Ecological Society of Australia*, held on 24-29 November 2013, Auckland, New Zealand.
3. **Uddin, M. N.**, Caridi, D., Robinson, R. W., and Harun, A. Y. A. Suppression of native *Melaleuca ericifolia* by the invasive *Phragmites australis* through allelopathic root exudates, In Proceedings of *INTECOL 2013*, held on 18-23 August 2013, ICC ExCel, London, UK.
4. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2012, Phytotoxicity of *Phragmites australis* through residue decomposition, In Proceedings of *Annual Conference Ecological Society of Australia (ESA)*, held on 03-07 December 2012, The Sibel-Albert Park, Melbourne, Victoria, Australia.
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6. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2012, Phytotoxicity of Secondary Metabolites Produced by *Phragmites australis* in South-eastern Australia, In

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7. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2011, Allelochemicals Inhibition of *Phragmites australis* against Neighboring Species, In Proceedings of **Annual Conference Ecological Society of Australia (ESA)**, held on 21-25 November 2011, West Point, Hobart, Tasmania, Australia.
8. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2011, Allelopathic Interactions of *Phragmites australis* in Ecosystem Processes, In Proceedings of **Biodiversity Across the Borders - Vulnerability and Resilience**, held on 09 June 2011, Centre for Environmental Management, University of Ballarat, Victoria, Australia.
9. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2011, Allelopathy as a Possible Mechanism of Invasion of *Phragmites australis* in Wetland Ecosystems, In Proceedings of **Postgraduate Research Conference**, held on 20 July 2011, Footscray Park Campus, Victoria University, Melbourne, Australia.

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1. **Uddin, M. N.**, Robinson, R. W., Caridi, D., and Harun, A. Y. A. Assessment of root and litter mediated allelopathic interference of *Phragmites australis* using density-dependent approach, In Proceedings of **5th Joint Conference of New Zealand Ecological Society and Ecological Society of Australia**, held on 24-29 November 2013, Auckland, New Zealand.
2. **Uddin, M. N.**, Caridi, D., Robinson, R. W., and Harun, A. Y. A. Suppression of native *Melaleuca ericifolia* by the invasive *Phragmites australis* through allelopathic root exudates, In Proceedings of **INTECOL 2013**, held on 18-23 August 2013, ICC ExCel, London, UK.

3. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2012, Phytotoxicity of *Phragmites australis* through residue decomposition, In Proceedings of *Annual Conference Ecological Society of Australia (ESA)*, held on 03-07 December 2012, The Sibel-Albert Park, Melbourne, Victoria, Australia.
4. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2012, Phytotoxicity of Secondary Metabolites Produced by *Phragmites australis* in South-eastern Australia, In Proceedings of *9th INTECOL International Conference*, held on 03-08 June 2012, Orlando, Florida, USA.
5. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2011, Allelochemicals Inhibition of *Phragmites australis* against Neighboring Species, In Proceedings of *Annual Conference Ecological Society of Australia (ESA)*, held on 21-25 November 2011, West Point, Hobart, Tasmania, Australia.

Poster presentation at conferences

1. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2011, Allelopathy as a Possible Mechanism of Invasion of *Phragmites australis* in Wetland Ecosystems, In Proceedings of *Postgraduate Research Conference*, held on 20 July 2011, Footscray Park Campus, Victoria University, Melbourne, Australia
2. **Uddin, M. N.**, Robinson, R. W. and Caridi, D., 2011, Allelopathic Interactions of *Phragmites australis* in Ecosystem Processes, In Proceedings of *Biodiversity Across the Borders- Vulnerability and Resilience*, held on 09 June 2011, Centre for Environmental Management, University of Ballarat, Victoria, Australia

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Chapter One

Introduction

Biological invasion and mechanisms

Worldwide, plant communities have been changing rapidly in response to human alterations of the landscape, global climate change, and biological invasions. At present, an integrated theory that explains plant community composition (Lortie *et al.*, 2004; Stohlgren *et al.*, 2005) or provides mechanisms that structure plant succession (Wiser *et al.*, 1998; Meiners *et al.*, 2001; Meiners *et al.*, 2004) is particularly needed. While invasive plants are considered a major threat to native ecosystems (Mack *et al.*, 2000), the study of biological invasion has contributed substantially to an improved synthetic understanding of evolutionary theory, community assembly, plant competition, plant–herbivore and plant–microbe interactions, and functioning of ecosystems (Callaway and Maron, 2006). However, despite significant advances in our understanding of invasion processes, we still fail to fully understand and, therefore, predict differences in success rates of invasive plant species. Invasion mechanisms are not similar for all plant species and only a small subset of thousands of invasive plants in the ecosystems is known to plant community ecologists. This basic lack of understanding of mechanisms determining differences in invasiveness is an impediment to developing predictions and risk assessments for potential impact or spread of future invaders.

When previous generations referred to biological invasion they considered these happenings natural phenomena or simply referred to them as range expansions of species into new areas. These previous concepts regarding invasions were challenged by Charles Elton, the modern founder of the science of biological invasions, who wrote that “biological invasions are so frequent now-a-days in every continent and island, and even in the oceans, that we need to understand what is causing them and try to arrive at some general viewpoint about the whole business” (Elton, 2000). The prediction by Elton regarding the outcome of global invasion processes and related homogenization of

regional floras and faunas was in stark contrast to Lyell and Deshayes (1830) who did not consider the human-mediated influence on invasions nor did they consider human influence a serious concern that would contribute to 'natural' processes of biological invasion (Lodge, 1993; Wilkinson, 2004).

Interest in biological invasions has rapidly increased in recent decades and today biological invasions are at the forefront of ecological investigations and a major concern in understanding ecological processes and conservation. Particularly, dramatic consequences of invasions have been reported from island ecosystems where endemic species have suffered severely, with many extinctions directly related to the introduction of 'alien' organisms (Sax *et al.*, 2002; Sax and Gaines, 2008). It is, however, wetlands (marshes, lakes, rivers) and estuary ecosystems worldwide that are among the most affected by introduced organisms (Ruiz *et al.*, 1997; Williamson, 1999). Because of these accelerating invasion rates, science has become increasingly interested in understanding the underlying mechanisms of biological invasions as a way to better predict invasion processes and to more fully appreciate their long-term impacts. High on the list of most serious threats to environments are those invasions associated with plants, commonly known as pest plants, weeds or just invasive. Indeed it is Australia that leads the way with the classification and formal listing of plants based on their risk to the environment or human activities, e.g. Weeds of National Significance (Parsons and Cuthbertson, 2001).

Invasion processes

Plant invasions have been described as occurring through a three-phase process: introduction, colonization, and naturalization/or invasion (Figs. 1a–c) (Groves, 1986; Cousens and Mortimer, 1995; Richardson *et al.*, 2000). Additional refinements to these processes are sometimes considered, such as extrinsic and intrinsic factors (Fig. 1d)

(Radosevich *et al.*, 2003). Richardson *et al.* (2000) challenged the un-occupied niche concept that was generally accepted but never proven by reporting that invasions can include a special class of plants that have the ability to enter and occupy already fully inhabited plant communities without further assistance from humans or the environment.

The invasion processes that determine the stability of plant populations during migration to the invaded area are scale dependent and range from individual plants to meta-populations (Radosevich *et al.*, 2003) (Table 1). Fundamentally, it is the transport of propagules either through 'natural' or 'human-assisted' means and various types of disturbances that remove environmental barriers that allow successful migration of alien plants into a new region (Radosevich *et al.*, 2003). However, successful introduction of a plant to a 'new' area depends on the recruitment of individuals in that new location. Recruitment involves the successful survival of newly arrived propagules, their ability to germinate and mature, and the successful reproduction of these individual plants to successive generations.

Full colonization depends on the reproductive and dispersal abilities of a founding population (Cousens and Mortimer, 1995). During the colonization phase, population growth is generally described by geometric and exponential population growth curves (Fig. 1b). While in this phase, a plant species might remain unnoticed. Once the new plant species becomes visible, control efforts regarding the protection of its spread become a priority for land managers. Favourable environmental conditions, including unrestricted resources, allow the plant population to maintain its high growth rate, by extending the function of the intrinsic biological characteristics restricted by its previous growing environment (Radosevich *et al.*, 2003). As a result, this colonization phase of an invasive species is often referred to as its intrinsic rate of increase. Thus,

colonization is thought to depend more on biological functions than environmental ones, despite the importance of both during this stage.

The plant species is considered fully naturalized in its introduced environment upon the establishment of new self-perpetuating inhabitants that sequentially propagate into a wide area, and the new species is merged into the resident population (Phillips *et al.*, 2010). This naturalization of invasive species may take years to decades from the first arrival to establishment. The largest part of this lag-time takes place during the early phase of exponential population growth of colonization.

In addition to these intrinsic phenomena, extrinsic factors also influence the rate of introduction by affecting the distribution and success of germinating seeds (Radosevich *et al.*, 2003). These extrinsic factors such as soil, climate, land use and condition of the environment greatly influence the likelihood of the introduction phase (Fig. 1d). The colonization and explosive growth phases are, however, closely associated with the intrinsic rate of increase for the invasive species. Therefore, intrinsic biology of the species has more potential in estimation of colonization rates and management options compared to extrinsic factors. Finally, both factors (intrinsic and extrinsic) might play an important role in defining the success and extension of the invasive species (Ortega and Pearson, 2005).

In recent time, 'invasion biology' has expanded away from the 'classical biology' concerning organisms within their natural distribution (Fig. 2). The traits of introduced species, their capacity to disperse, interactions with each other and with native species in receiving ecosystems have been explored in the field of invasion biology (Falk-Petersen *et al.*, 2006). Again, 'invasion biology' deals with the species composition, community structure, site resource availability, and disturbances of the original plant community which influence the susceptibility of those to plant invasion.

Now, it can be said that biological invasion poses a great threat to the local and global biodiversity worldwide (except Antarctica) and that creates a great concern among the plant scientific community.

Hypotheses for invasion mechanisms

There are many hypotheses in explaining the success of invasive species (Mack *et al.*, 2000), but few have survived rigorous investigations (Shea and Chesson, 2002). The following section briefly explains those hypotheses.

Natural enemies hypothesis: This is the oldest and most widely accepted hypothesis explaining the success of many invasive species liberated from their specialist herbivores and pathogens upon introduction to a new habitat (Darwin, 1859; Elton, 2000). An introduced species has an advantage due to lack of direct suppression by their specialist enemies and subsequently outcompetes natives in their new range (Klironomos, 2002; Callaway *et al.*, 2004).

Evolution of invasiveness hypothesis: This hypothesis posits that the invasive species experiences rapid genetic changes related to new selection pressures in the new environment (Blossey and Notzold, 1995; Stockwell *et al.*, 2003) while biotic and abiotic factors might act as important selective forces (Lee, 2002). Both presence and absence of new set of biotic organisms may influence the rapid evolution. In addition to this, the evolution of increased competitive ability (EICA) hypothesis states that species long liberated from their native specialist enemies might lose costly traits that gave resistance to those enemies (Blossey and Notzold, 1995). Release from those selective pressures results in the redirection of resources from those costly and now unnecessary traits to those which might have greater benefit in the new habitat. Thus, the EICA

expects that the invasive species has been evolving in ways that develop their performance in new communities.

Empty niche hypothesis: This hypothesis refers to the utilization of unused resources of the local population by the invasive species (MacArthur, 1970). Some invasive species may facilitate their successful growth due to easy access to the resources unexploited by local species (Levine and D'Antonio, 1999; Mack *et al.*, 2000). Again, this 'empty niche' hypothesis supports the 'fluctuating resource availability' hypothesis which states that the susceptibility to invasion of a community increases due to exploitation of the unused resources in that introduced community (Davis *et al.*, 2000). In such a way, some invasive plants certainly gain advantage of empty niches in the communities over the native communities.

Novel weapons hypothesis: This recent novel weapons hypothesis (NWH) states that some invaders may succeed in invasion because they possess novel biochemical weapons that function as unusually powerful allelopathic, defense, or antimicrobial agents to which naïve natives have not adapted (Callaway and Aschehoug, 2000; Bais *et al.*, 2003; Callaway and Ridenour, 2004). This hypothesis in particular postulates that exuded allelochemicals by some invasive species are relatively unsuccessful against well-adapted neighbours in their origin communities, whereas they exhibit comparatively higher suppressive effects to naïve plants in the introduced communities. Again, biogeographical difference in allelopathic effects has been demonstrated in case of different species such as *Centaurea diffusa* (Callaway and Aschehoug, 2000) and *Alliaria petiolata* (Prati and Bossdorf, 2004) which is well suited under this NWH theory (Vivanco *et al.*, 2004; Hierro *et al.*, 2005). The general consensus from those findings broaden the scientific view in invasion biology stating that exuded

allelochemicals from the invasive plants have stronger phytotoxic effects on naïve native communities compared with their original communities.

Disturbance hypothesis: The disturbance hypothesis claims that invasive species are adapted to disturbances, such as fire, grazing, soil disturbance, and nutrient addition, of types and intensities might be novel to natives (Baker, 1974). Disturbances increase the invasiveness of the introduced species in the new community (Hobbs and Huenneke, 1992). In addition to human-induced disturbances, natural disturbance is also responsible for the invasion by non-indigenous plant species (Witzell and Martín, 2008).

Species richness hypothesis: This hypothesis argues that species-rich communities are more unaffected to invasion than species-poor communities (MacArthur, 1970; Elton, 2000). In addition to this theory, the theoretical arguments state that poor inter-specific interactions and more 'empty niches' attributes have been shown in lower diversity communities (Drake, 1991). This hypothesis explains that reduced resource uptake in species-poor communities provides a corridor for more free resources availability, which makes them more susceptible for invasion than species-rich communities (Tilman *et al.*, 1996; Hooper and Vitousek, 1998; Tilman *et al.*, 2006). As an example, increasing water or nitrogen availability often facilitates invasion (Davis and Pelsor, 2001; Siemann and Rogers, 2003).

Propagule pressure hypothesis: This hypothesis explains that the magnitude and intensity of invasion depends upon the number, size, spatial and temporal distribution of individuals of an invasive species arriving into new community (Williamson and Fitter, 1996; Lonsdale, 1999; Simberloff, 2009).

Allelopathy

Among the above mentioned hypotheses, the Novel Weapons Hypothesis (NWH) is rarely studied in either terrestrial or aquatic ecosystem. The allelochemicals generally involved in allelopathy are carbon-based secondary metabolites with a wide range of chemical properties, and range from low molecular weight phenolic acids to the high molecular weight condensed tannins (Inderjit, 1996). These widespread chemicals available in plants play an important role in the soil–plant–environment interactions (Muscolo and Sidari, 2006). Allelochemicals actively released by plants or passively produced during the decomposition process of both above and below-ground plant residues affect abiotic and biotic processes in the ecosystem and thereby influence the invasion process (Inderjit, 1996; Uddin et al., 2012; Uddin et al., 2014b). Root exudates of many invasive plant species may play a direct role as phytotoxins in mediating chemical interference. In addition, root exudates are critical to the development of associations between some parasitic plants and their hosts. Finally, these chemicals may play an indirect role in resource competition by altering the soil chemistry (Weidenhamer and Callaway, 2010), soil processes and microbial populations (Niu *et al.*, 2007; Kong *et al.*, 2008). Many phenolics produced by dicotyledonous plants have the potential to form complexes with metallic micronutrients such as chelation and may increase metal availability, often through nutrient and/or metal chelation (Tharayil, 2009; Tharayil *et al.*, 2009; Inderjit *et al.*, 2011).

In addition to direct interactions, plant invasion may be partly due to allelopathy mechanisms that interfere with mutualisms between associated plant roots and their mycorrhizal fungi (Olsen *et al.*, 1971; Stinson *et al.*, 2006). Mycorrhizal fungi aid the host plant by the nutrient uptake from soil, especially phosphorous, and conversely, fungi utilise carbohydrates from the plant. The reduction of fungal populations in the

natural field through the allelopathic effect may negatively influence the growth of the other plants (Siqueira *et al.*, 1991). Additionally, the normal function of mycorrhizal fungi may be adversely affected by the release of allelochemicals of invasive species (Olsen *et al.* 1971). Different concentrations of allelochemicals may have variable effects on the growth of mycorrhizae and root colonizations in the field, thus explaining the failures of establishment of the other associated plant species with allelopathic plant species (Rose *et al.*, 1983). Allelopathy relating to mycorrhizae is an indirect mechanism by which one invasive plant suppresses the another native one (Stinson *et al.*, 2006). The empirical knowledge regarding the association of allelochemicals with mycorrhizae is very limited, especially the species level impact.

The influence of allelochemicals may play a role by not only altering the behaviour of other plants (Callaway and Aschehoug, 2000), but also by changing the microbial dynamics (Hättenschwiler and Vitousek, 2000). Allelochemicals released from invasive plant species into the soil system and/or rhizosphere may affect soil nutrient dynamics by forming complexes with proteins and delaying organic matter decomposition and mineralization (Castells *et al.*, 2005) and by increasing rhizosphere soil microbial activity (Ehlers, 2011) and N₂ immobilization (Castells *et al.*, 2003), resulting in a decrease in inorganic N₂ available for plants uptake (Inderjit and Mallik, 1997). Consequently, allelochemicals may inhibit seed germination and root elongation of associated organisms (Inderjit and Dakshini, 1994b; Hussain *et al.*, 2011), affect photosynthesis (Djurdjević *et al.*, 2008; Hussain and Reigosa, 2011), respiration (Lorenzo *et al.*, 2011; Uddin *et al.*, 2014a), and upset water balance (Blum and Gerig, 2005). Individually and collectively, these influences of allelochemicals result in reduced plant growth and reproduction. In addition, ion transport, protein synthesis,

hormone activity and energy metabolism in affected organisms may be also influenced by allelochemicals (Muscolo *et al.*, 2001).

Environmental factors such as light (Mole *et al.*, 1988; Paez *et al.*, 2000), nutrient (Yates and Peckol, 1993), drought (Karageorgou *et al.*, 2002), temperature (Solecka *et al.*, 1999), and biological effects such as herbivores (Park and Blossey, 2008) influence the production and release of allelochemicals into the environment. These above-mentioned biotic and abiotic influences suggest that any stressed condition may cause an increase in phenolics production and/or their release. In addition to natural environmental variation, plants cope with a variety of human-induced environmental changes, the rate and magnitude of which have greatly increased during the last decades. Human-induced alterations such as CO₂, O₃, and UV in the abiotic environment have a significant impact on the production and accumulation of phenolic compounds in plants (Bidart-Bouzat and Imeh-Nathaniel, 2008) which may influence the biological invasion through allelopathy.

Allelopathy in wetlands

Wetlands appear to be seriously vulnerable to biological invasions. Although wetlands occupy a small portion ($\leq 6\%$) of the earth's land mass, 24% of the total invasive plant species are wetland plants (Mitsch *et al.*, 1994). Most of these invaders dominate the wetland ecosystem by forming monocultures that alter habitat structure, lower biodiversity, change nutrient cycling and productivity, and modify food webs (Zedler and Kercher, 2004). In general, wetlands act as landscape sinks where debris, sediments, water, and nutrients from watershed accumulate and this facilitates invasions by accelerating the growth of invasive plant species. These and other disturbances such as propagule influx, salt influx, and hydro-period alteration in wetlands are

advantageous for invasive species. In addition to these factors, recently allelopathy has been proposed as a significant contributor for some species as well (Gopal and Goel, 1993; Fageria and Baligar, 2003; Jarchow and Cook, 2009). Actually, it is likely that a single hypothesis or plant attribute does not explain wetland plant invasions, because invasion is the result of cumulative impacts associated with all characteristics of landscape sinks including allelopathy. While several hypotheses have been proposed to explain causes and consequences of invasions in wetlands, the main focus of this research was on allelopathy because so far very few studies have been done.

Allelopathy has been proven as an important mechanism for biological invasion for many plant species such as *Centaurea diffusa* and *Alliaria petiolata* in terrestrial ecosystems, but has been largely ignored in aquatic ecosystems, especially in wetlands. The issue of allelopathy is controversial, particularly with regard to aquatic plants (Neori *et al.*, 2000). Some authors speculate that allelochemicals secreted from plants might be diluted with water and therefore, have less of a phytotoxic effect. Additionally, the mechanism by which allelopathy exerts influence has been argued among scientists mainly due to the difficulties in proving allelopathic effects on the ecosystem level (Gross *et al.*, 2007). An additional problem in proving mechanisms is the lack of easy accessible chemical methods to track and identify the biochemically active allelopathic compounds in the associated sediment and water.

Physical, chemical, and biological processes occur concurrently in ecosystems and interfere with the allelopathic activities simultaneously and in potentially interactive and synergistic ways. Their effects on the target organisms are very hard to pinpoint by studying direct allelopathic activity. Legrand *et al.* (2003) reported that ways of demonstrating allelopathy lack the criteria proposed by Willis (1985). However, more recently an increasing number of reports of allelopathy confirm its existence and its

structuring effect on primary producers in aquatic ecosystems (Gopal and Goel, 1993; Inderjit and Dakshini, 1994a; Neori et al., 2000; Gallardo and Martin, 2002; Erhard and Gross, 2003; Gross, 2003; Erhard, 2006; Jarchow and Cook, 2009; Leão et al., 2009). The evaluation of allelopathy's ecological relevance is hampered by the interference of other competitive processes such as resource competition with allelopathy in the natural aquatic ecosystem (Weidenhamer *et al.*, 1989). Generally, the impact of allelopathic effects on the target organism is related to the production and content of allelochemicals in the donor plant and factors changing the allelochemical after release into the aquatic environment.

The ecological implications of the varying allelochemical compounds produced by plants are difficult to quantify and evaluate in field conditions due to numerous confounding factors. Low concentration, little persistence and the possibility of chemical alterations by soil microorganisms makes it difficult to determine their *in situ* presence and effects in soils (Mitrović *et al.*, 2012). In addition, the interactive nature of phenolic compounds and occurrence of multi stressors under field conditions further complicates the problem. Recently, interests in allelopathy studies have been progresses as numerous chemical, biological, and agricultural aspects have been attributed to them, thus much information has accumulated on them. Clearly, allelopathy is worthy of more rigorous biochemical and ecological research regarding biological invasion in wetlands. Linking with these understanding, this PhD research focused on allelopathic interferences with reference to *Phragmites australis*, one of the most widespread wetland plants on earth.

***Phragmites australis* as a potentially useful study species**

Phragmites australis is one of the most widespread plants on the earth and grows in aquatic, semi-aquatic, and even terrestrial ecosystems world-wide (Wilcox *et al.*, 2003; Swearingen and Saltonstall, 2010). The following general characteristics of *P. australis* are adapted from Haslam (1972), Hocking *et al.* (1983), Mal and Narine (2004) and Szczepanska and Szczepansky (1973). It is a tall, warm-season, perennial, emergent aquatic plant. The culms are erect, rigid, smooth, and have 10–25 cm hollow internode. They may be nearly 2.5 cm in diameter and from 2 to 6 m in height terminating in 30 cm panicles. The size of the culms of *P. australis* is inversely proportional to the planting density as well as the total plant biomass per unit soil volume is independent of the shoot density. Leaves arise from the culm and are mostly 25–50 cm long and 1 cm wide. *Phragmites australis* has an extensive rhizome network and may occasionally produce stolons as well. Rhizomes are perennial and have both horizontal and vertical components. Rhizomes may have extensive aerenchymatous tissue and be buried to a depth varying from 10–200 cm. Roots develop from the rhizome and other submerged parts of shoots and may penetrate to a depth of about 1 m.

The feathery, plume-like inflorescence is 13–40 cm long and composed of many long branches that point upwards. The spikelets of the inflorescence are arranged densely along the branches. The spikelets are surrounded by silky white hairs that are purplish at first, becoming yellowish-brown to dark brown at maturity. Seeds are brown, thin and delicate. A long, narrow seed coat composed of the lemma and glume is attached to each seed. The seed and coat together measure approximately 8 mm long. *Phragmites australis* is a species with very high phenotypic and genetic variability (Lambertini *et al.*, 2008; Lambertini *et al.*, 2012) that is augmented by its cosmopolitan distribution, clonal growth form and the large variation in chromosome numbers

(Clevering and Lissner, 1999). The variability (phenotypic and genotypic) among *P. australis* populations in Australia is poorly known (Hocking *et al.*, 1983) but in general the species includes several ploidy levels with octoploids (8x) predominant in Australia and Asia, whereas tetraploids (4x) are predominant in Europe and North America (McCormick *et al.*, 2010; Achenbach *et al.*, 2012). A morphological view of *P. australis* is shown in figure 3.

Occurrence and habitat

Phragmites australis grows in coastland, estuarine habitats, lakes, riparian zones, disturbed urban areas, water courses and a range of fresh to brackish wetlands. It can grow at water depth of 2 m or more (Björk, 1967), although it might be incapable of vegetative spread at water depths greater than 0.5 m (Shay and Shay, 1986) or 1 m (Haslam, 1970). It is especially common in fresh, alkaline and brackish (slightly saline) environments (Haslam, 1971; Haslam, 1972) although it can also thrive in highly acidic wetlands (Marks *et al.*, 1993).

Geographical distribution

Phragmites australis is said to have a cosmopolitan distribution and is generally thought to be the most widely distributed angiosperm (Clevering and Lissner, 1999; Lambertini *et al.*, 2012). Figure 4 demonstrates the distribution of *P. australis* in different parts of the world and Australia respectively. It is an extremely abundant species particularly in the temperate regions (Haslam, 1972). *Phragmites australis* is introduced and naturalized in New Zealand, and is widespread in Polynesia and the non-arid, temperate regions of Australia, Europe, USA, Africa, and Asia (Haslam, 1972; Hocking *et al.*, 1983; Rudrappa and Bais, 2008), being especially common in south-eastern Australia (Morris *et al.*, 2008). Although it has been considered a serious weed in the USA and

Australia (Tschardtke, 1999), conservationists are seriously concerned about die-back of the species in Europe (Haslam, 2010) .

Ecological impact

Despite *P. australis* being considered an ecologically and economically important species in Europe, there are major issues in areas where it is considered 'introduced'. Introduced genotypes in the USA have been displacing native genotypes and outcompeting other native species (Keller, 2000; Meyerson *et al.*, 2000). Keller (2000) reported that invasion of foreign *P. australis* genotypes may result a reduction in species diversity. A comparative study (invaded versus uninvaded) in a freshwater tidal marsh and wetlands dominated by *P. australis* suggest an inverse relationship with species richness (Farnsworth and Meyerson, 1999). Warren *et al.* (2001) and Chambers *et al.* (1999) reported that most of *P. australis*-dominated marshes have become near or complete monocultures that exhibit a reduction in biodiversity due to loss of many native plant species . In addition to loss of plant diversity due to *P. australis* expansion, animal species such as muskrat (*Ondatra zibethicus*) (Marks *et al.*, 1994), larval and juvenile fish (Able and Hagan, 2000), red-winged blackbird (*Agelaius phoeniceus* L.) (Bernstein and McLean, 1980), waterfowl and other wading birds (Benoit and Askins, 1999; Chambers *et al.*, 1999) have also been reduced.

Water flow in *P. australis*-dominated marshes may influence trophic connections. The energy transfer might be limited due to major hydrologic disturbance in tidal marsh to higher trophic levels of the adjacent estuary that result higher *P. australis* production and accumulation. Again, small creeks created by *P. australis* in open water flow marsh systems limit secondary production by confining movements of fish and crustaceans into feeding areas (Roman *et al.*, 1984; Roman *et al.*, 2002). Gratton and Denno (2005) found a transformed arthropod assemblage and trophic

structure in a coastal *Spartina alterniflora* marsh system in southern New Jersey, USA, that had been invaded by *P. australis*. The plant altered both the abundance of trophic groups such as detritivores, herbivores, and carnivores and their taxonomic composition. On the other hand, no clear differences were observed in use of natural versus restored marshes by the fish, *Fundulus heteroclitus* (Smith *et al.*, 2002) as well as in feeding activities or food sources for *Fundulus heteroclitus* in *Spartina patens* and *P. australis* marshes in open tidal flows (Fell *et al.*, 1998) due to allow the fish to forage. It is also documented that the slow decomposition of *P. australis* stems may limit the nutritive value to muskrats and other animals (Chambers *et al.*, 1999). In contrast, leaves of *P. australis* influence the secondary production due to their quick decomposition.

Mechanisms for *P. australis* invasion

Several explanations have been proposed to explain the recent *P. australis* expansion in the USA (Kulmatiski *et al.*, 2011). Primarily, soil and hydrologic disturbances (natural and human-induced), such as changes in salinity, sedimentation rates, and nutrient cycling or addition, have been suggested to explain the mechanism of devastating *P. australis* growth (Van der Toorn and Mook, 1982; Marks *et al.*, 1994; Meyerson *et al.*, 2000; Meyerson *et al.*, 2002; Silliman and Bertness, 2004; Chambers *et al.*, 2008). More recent investigations have shown strong evidence suggesting that the introduction of an aggressive non-native strain may be responsible for the rapid expansion, particularly throughout the north-eastern USA (Kristin, 2002; Saltonstall, 2003; Saltonstall *et al.*, 2005). Again, disturbance has been shown to disproportionately increase growth of the non-native *P. australis* strain in relation to the native strain (Minchinton and Bertness, 2003; Jodoin *et al.*, 2008; Park and Blossey, 2008). Other potential explanations for most recent expansion including allelopathic interaction (Rudrappa *et al.*, 2007),

resource competition (light, space, nutrient and others) and the potential for native and non-native strains to hybridize (Meyerson *et al.*, 2002; Meyerson *et al.*, 2010; Paul *et al.*, 2010) have been linked to its invasiveness. The importance of these mechanisms remains unresolved, especially in wetlands.

Most previous studies focused on the effects of *P. australis* on flora and fauna, rate of its expansion, site characteristics, methods of control and management, and other invasion mechanisms such as role of natural enemies (Park and Blossey, 2008), evolution of invasiveness (Kirk *et al.*, 2011; Kettenring and Mock, 2012), phenotypic plasticity to resources (Brisson *et al.*, 2010; Zhao *et al.*, 2010), in detail. Whereas the Novel Weapons Hypothesis, in which mechanisms evolved elsewhere have stronger impacts on a native population by *P. australis*, has been understudied. (Rudrappa *et al.*, 2007; Bains *et al.*, 2009).

Over the last 100 years, plant ecologists have not satisfactorily explained the successful invasion of *P. australis* (Meyerson *et al.*, 2002). Apart from all responsible factors, recently, there has been renewed focus on allelopathy and its contribution to the invasion mechanism of *P. australis*. Some preliminary studies have been conducted to test the effects of aqueous extracts of *P. australis* on germination and growth of various plant species (Drifmeyer and Zieman, 1979; Kulshreshtha, 1981; Singh *et al.*, 1993; Qian *et al.*, 2007; Li *et al.*, 2011) and their autotoxicity effects (Sharma *et al.*, 1990). However, there are few details on root-secreted allelochemicals of *P. australis* in the USA population (Rudrappa *et al.*, 2007; Rudrappa *et al.*, 2009). Three triterpenoids (β -amanacin, taraxerol, taraxeron) and a flavone (tricin) as well as methyl gallate, lyoniresinol, lyoniresinol-3 α -O- β -D-glucopyranoside have been identified from the aerial portions and rhizome of *P. australis* respectively (Ohmoto, 1969; Kaneta and Sugiyama, 1972; Choi *et al.*, 2009). In addition, several studies have identified

chemicals within *P. australis* that have antialgal, antifungal or antibacterial effects (Li and Hu, 2005; Hu and Hong, 2008). Again, some studies reported that chemicals produced by decomposition of below-ground organs of *P. australis* may be responsible for die-back of *P. australis* itself (Armstrong and Armstrong, 1999) and photo-degradation of secreted phytotoxins by *P. australis* may execute severe phytotoxicity to other plant species (Rudrappa *et al.*, 2009).

But most recently, identified phenolics gallic acid in *P. australis* root exudates showed inhibitory effects on germination and growth of various seeds by triggering a wave of reactive oxygen species (ROS) initiated at the root meristem, which leads to a Ca²⁺ signalling cascade triggering genome-wide changes in gene expression and ultimately the death of the root system (Rudrappa *et al.*, 2007). This can be interpreted as evidence of allelopathy of *P. australis*. Weidenhamer *et al.* (2013) contradicted the findings of Rudrappa *et al.* (2007) and Bains *et al.* (2009), stating that gallic acid might not be present in high concentration in rhizosphere soil as well as in leaf and rhizome and concluding that gallic acid might be not a primary explanation for the invasion of *P. australis*. This complexity directs ample opportunity to do more allelopathy research related to the *P. australis* invasion process. Again, the evidence is limited by the low number of associated plant species tested, the relatively high concentrations of the root exudates used, lack of consideration of the osmotic potential effects of the aqueous extracts used, no separation of allelopathy from resource competition, no study on the allelopathic effects of decomposition of *P. australis* litter under either aerobic and anaerobic conditions, and more specifically, the artificial nature of *in vitro* experiments.

To date, no studies have been done on the role and operation of allelopathy in the invasion of *P. australis* populations in Australia. Local studies are necessary because bio-geographical variation may influence both plant community response to

allelochemistry and the varying natural concentrations of the allelochemicals involved (Vivanco *et al.*, 2004; Hierro *et al.*, 2005; Thorpe *et al.*, 2009). Studies on Australian populations of *P. australis* include habitat assessment (Roberts, 2000), dynamics of biomass production and nutrient accumulation and cycling (Hocking, 1989b; Hocking, 1989a), competitive effects (Morris *et al.*, 2008; Davies *et al.*, 2010) of *P. australis* and the weed biology of *P. australis* (Hocking *et al.*, (1983). No studies have been done so far on invasion mechanisms of *P. australis* in Australia, despite its widespread occurrence, invasive characteristics, and displacement of the associated plant species (Hocking *et al.*, 1983; Hocking, 1989b; Wapshere, 1990; Davies *et al.*, 2010). Because of this lack of research and prompted by evidence that *P. australis* may not only have evolved greater competitive ability but also possesses biochemical weapons, this study focuses on allelopathy research of *P. australis*. A number of detailed published studies have been addressed under this project, including phytotoxicity tests by water extracts of *P. australis* organs (Uddin *et al.*, 2012), root exudation (Uddin *et al.*, 2014c), and residue decomposition (Uddin *et al.*, 2014b), which, apart from root exudation, have shown strong phytotoxic effects on germination, growth and physiology of other plant. In addition, the effect of the phenolic compound gallic acid, which has been identified in *P. australis* organs (Uddin *et al.*, 2014a), is critically addressed to resolve the conflict among a number of other authors (Rudrappa *et al.*, 2007; Bains *et al.*, 2009; Weidenhamer *et al.*, 2013). Based on this review, we established objectives to determine the true significance of allelopathy as a mechanism for *P. australis* invasion here in Australia.

Implications for understanding the mechanism

The understanding of mechanisms responsible for invasion is important not only in invasion ecology, but also in several fundamental issues of basic ecology. The differential allelopathic interactions among plant species have profound implications for traditional plant community theory and therefore in plant biology. Plant communities are widely thought to be 'individualistic', which is composed mainly of species that have alike adaptations to a specific physical environment settings. This 'individualistic concept of ecology', a traditional and most accepted concept in plant association, downplays any persistent and dominant role of co-evolution in determining the structure of interactions, and this does not support the interaction between species through direct and indirect effects among competitors (Lortie *et al.*, 2004). However, invasive plants disturb the integration of recipient plant communities upon introduction, and their expansion is dependent on the degree of integration of that community (Shea and Chesson, 2002). Linking with such type of thought, Novel Weapon Hypothesis (NWH) suggests that natural selection might be evolved with interactions among plant species in communities and demonstrates that plant communities may simultaneously function both individualistically with independent species and as assemblages of different species which function interdependently. Besides this theoretical importance of competition and allelopathy, practical application of these studies in case of management of natural resources and control of plant and animal pest is growing in interest (Szczepański, 1977; Putnam, 1988). Since Szczepański (1977) suggested that allelopathy may play an important role in the biological control of aquatic weeds, much of the work on the allelopathic potential of aquatic plants is directed towards that goal (Putnam, 1988; Vyvyan, 2002).

Aims of the study

Although increasing evidence suggests allelopathy is partially responsible as a structuring element in the biotic community composition in wetlands, knowledge of allelopathic mechanisms in invasion processes is still incomplete, especially for *Phragmites australis*. More detailed studies in allelopathic interactions in wetland communities are essential to an evaluation of the ecological impacts associated by the invasive species. This thesis as a ‘**Thesis by Publication**’ contains eight chapters including six articles written according to the requirements of the specific journal. These either have been published (Chapters: **Two, Three, Four & Seven**) or soon will be accepted (Chapters: **Five & Six**) in international peer-reviewed journals address the following research questions:

Establish if *P. australis* is phytotoxic: This study examined phytotoxicity in *P. australis* to quantify the relative importance and contribution of different organs to suppression of other plant species. We hypothesised that phytotoxins of *P. australis* negatively affect germination, growth and physiological aspects of model and associated species.

Identify chemicals and mode of action: This part of the thesis was designed to investigate the mode of action of phytotoxicity, emphasizing physiological effects of aqueous extracts of different organs and root exudates of *P. australis* on the germination processes and subsequent growth of plants. In particular, this study investigated the phytotoxic effects of *P. australis* on seed metabolic activities, namely liquid imbibition and resource mobilization as well as induction of oxidative stress by reactive oxygen species (ROS) production and their consequences on seedlings. In addition, chemical compounds in different organs of *P. australis* were identified.

Investigate the persistence of phytotoxins and their phytotoxicity: This study investigated the concentrations and dynamics of allelochemicals, including other physicochemical variables of extracts during decomposition of *P. australis* residue in soil systems using aerobic and anaerobic conditions in the laboratory. This also examined the effects of the extracts on seed germination and growth as well root elongation of the associated and model plant species. Additionally, a long-term decomposition study was performed considering residue density in soil in greenhouse, and examined the phytotoxic effects on germination and growth of the associated species to confirm the presence and potential of phytotoxins.

Assess field evidence of allelopathy: This study was performed by two separate methods namely observational and experimental studies in the field and laboratory respectively. This study observed the field condition by measuring the chemical changes in the soil and water of invaded wetlands as well as phytotoxic tests in the laboratory. Thus, this integrated study might be helpful to provide a more logical explanation for the invasion of *P. australis* than would be justified by field study alone.

Distinguish allelopathy from resource competition: This study has been designed to determine the occurrence and magnitude of phytotoxic effects on model and associated plant species mediated by *P. australis* root and litter for a wide range of doses using a density-dependent approach.

Examine the true significance of allelopathic effects incorporating management strategies: This study was aimed at evaluating the allelopathic impact of *P. australis* root exudates on *Melaleuca ericifolia* germination, survival, establishment, and growth. Activated carbon combined with mechanical treatments (shoot cutting) of *P. australis* was used to examine how these could reduce the species' impact.

Additionally, the effectiveness of two possible methods was assessed for the ability to reduce the impacts and/or production of allelochemicals by *P. australis*.

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List of tables

Table 1. Ecological processes, patterns, and scales at different phases of plant invasion.

Adapted from Radosevich *et al.* (2003)

| Phases of invasion | Ecological process | Ecological pattern | Scale |
|-------------------------------|--------------------|---------------------|-----------------|
| Introduction | Dispersal | Species recruitment | Individual |
| | Immigration | | |
| | Survival | | |
| Colonization | Birth | Patch expansion | Population |
| | Death | | |
| | Immigration | | |
| | Emigration | | |
| Naturalization/or Invasion | Birth | Range expansion | Meta-population |
| | Death | | |
| | Immigration | | |
| | Emigration | | |

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Fig.1. The invasion processes (a) introduction, (b) colonization, and (c) naturalization/or invasion of plants according to Cousens and Mortimer (1995) and (d) the relationship of extrinsic and intrinsic factors on these phases of population growth (Radosevich *et al.*, 2003).

Fig. 2. Schematic overview over the domains and main elements of ‘Classical biology’ versus ‘Invasion biology’. Adapted from Falk-Petersen *et al.* (2006).

Fig. 3. Morphological appearance of *Phragmites australis*. (a) *P. australis* stand; (b) *P. australis* stem with leaf; (c) *P. australis* inflorescence; and (d) *P. australis* rhizome with root

Fig. 4. Abundance and distribution of *P. australis* in the world and Australia. Adapted from Discover Life (Accessed 5 November 2013).

Fig. 1.

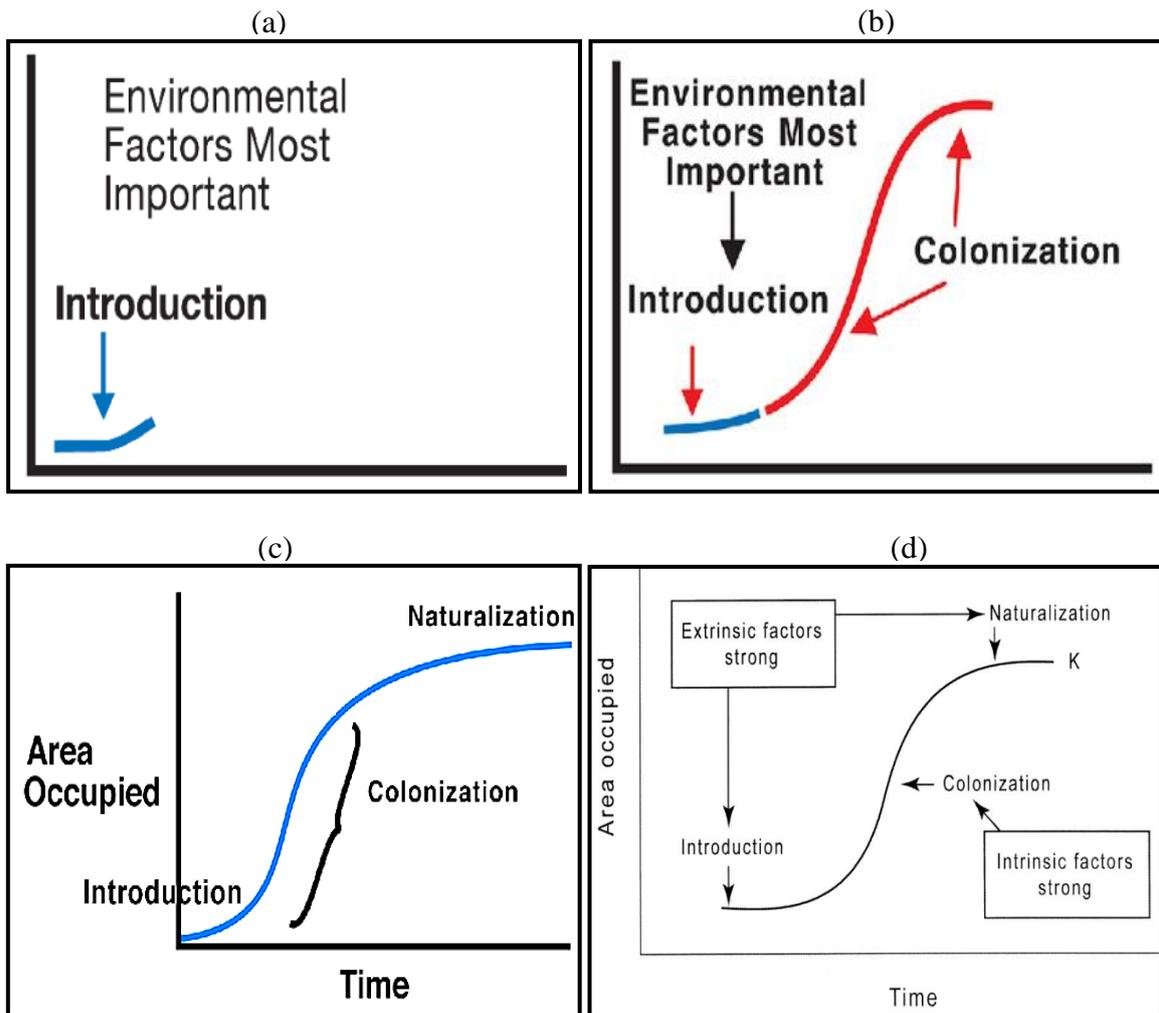


Fig. 2.

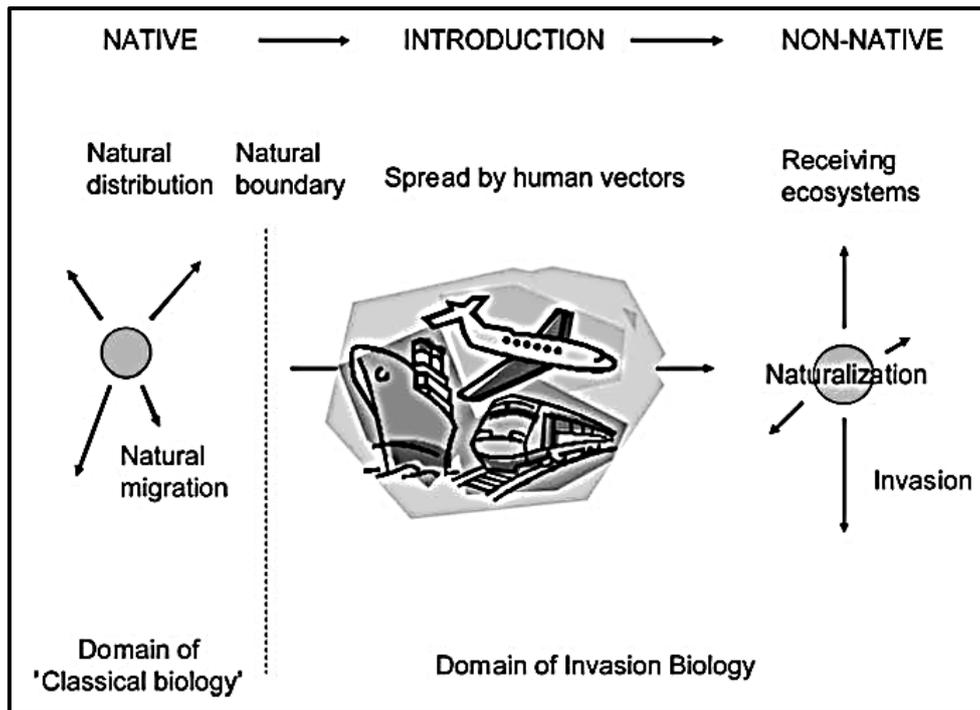


Fig. 3.



P. australis stand



P. australis stem with leaf

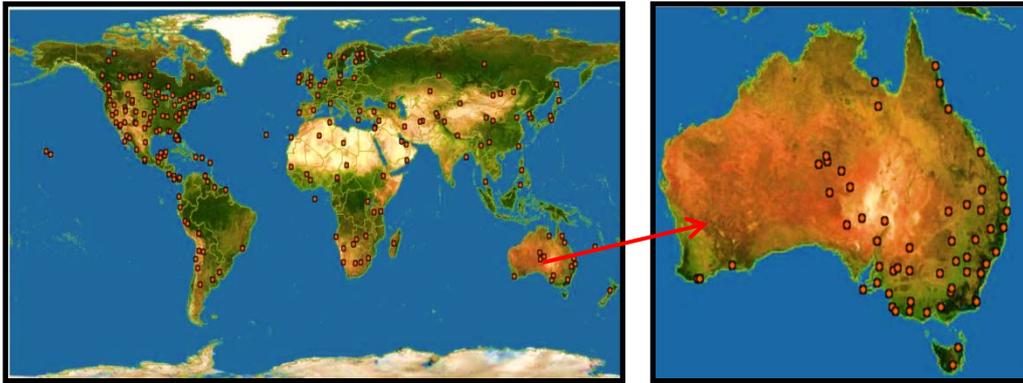


P. australis inflorescence



P. australis rhizome with root

Fig. 4.

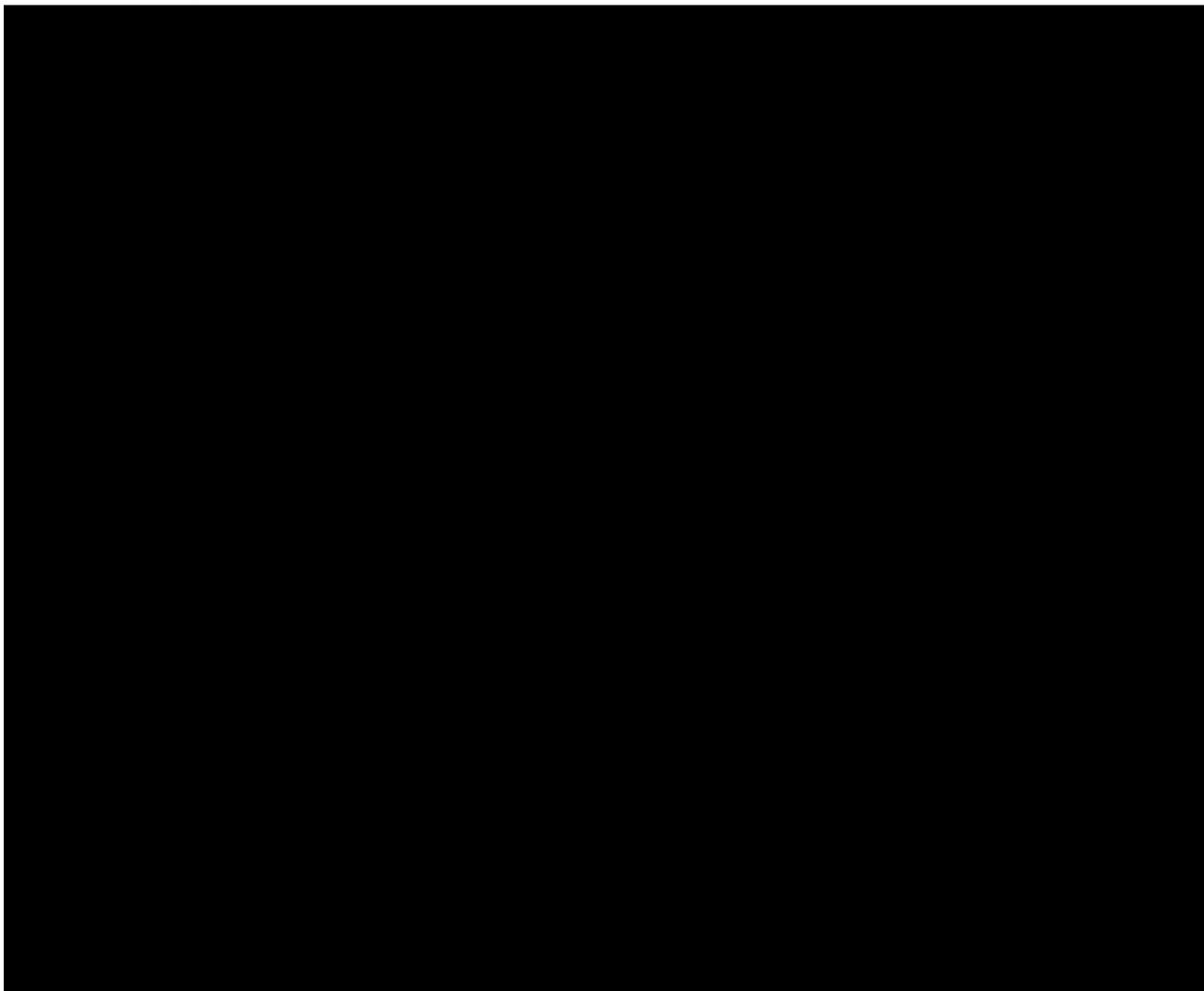


Chapter Two

Phytotoxic Evaluation of Phragmites australis: An Investigation of Aqueous Extracts of Different Organs

Introduction

This study examined allelochemical phytotoxicity of *P. australis* to quantify the relative importance of different organs in suppression of other plant species. We hypothesised that allelochemicals of *P. australis* negatively affect germination and growth of model test species and associated wetland species.



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PART B:
**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS
 INCORPORATED IN THESIS BY PUBLICATION**

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Md. Nazim Uddin

Signature: 

Date: 24/07/2014

Paper Title: Phytotoxic evaluation of *Phragmites australis*: an investigation of aqueous extracts of different organs

In the case of the above publication, the following authors contributed to the work as follows:

| Name | Contribution % | Nature of contribution |
|---------------------|----------------|--|
| Md. Nazim Uddin | 80 | Concept development; plant, soil and seed collection; conducting experiments and chemical analysis; data collection, statistical analysis and interpretation; and manuscript writing, editing and submitting for publication |
| Randall W. Robinson | 17 | Concept development; sample collection; and manuscript editing |
| Domenico Caridi | 3 | Manuscript editing |

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

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and will be held for at least five years from the date indicated below:

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| Signature 3 |  | 21/7/2014 |

Phytotoxic evaluation of *Phragmites australis*: an investigation of aqueous extracts of different organs

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Abstract. *Phragmites australis* is one of the most widespread and invasive plants on earth. Allelopathic interference has been considered as a possible way associated with its invasiveness in wetlands. A series of ecologically realistic experiments was conducted to explore allelochemical phytotoxicity of *Phragmites*. Germination bioassays using aqueous extracts of different organs (leaf, stem, root and rhizome) of *Phragmites* were tested with model seeds (*Lactuca sativa* and *Raphanus sativus*) and associated plant species (*Juncus pallidus* and *Rumex conglomeratus*). These studies showed that leaf and rhizome extracts exhibited strong inhibition on germination, biometric and physiological parameters (all $P \leq 0.001$). Dose-response studies confirmed LC₅₀ (4.68% and 11.25%) of *Lactuca* for leaf and rhizome extracts respectively. Root growth of *Juncus* and *Rumex* was inhibited by 75% and 30%, respectively, in leaf leachate-incorporated soil. Chlorophyll content and maximum quantum yield (F_v/F_m) were significantly reduced with leaf and rhizome leachates. The stability and quantity of water-soluble phenolics in anaerobic *versus* aerobic condition may influence phytotoxic effects to other species. *Phragmites* organs can be ranked in order of allelopathic potentiality as follows: leaf > rhizome > root > stem. The present study highlighted the potential impacts of allelochemicals on plant recruitment in wetlands invaded by *Phragmites*.

Additional keywords: Australia, cell integrity, ecosystems, litter decomposition, radicle length, soil systems

Introduction

Phragmites australis (hereafter called *Phragmites*) is found in oligohaline tidal wetlands, freshwater wetlands, ditches, and along roadsides world-wide (Swearingen and Saltonstall 2010). It is a perennial graminaceous plant, up to 3 m tall, that reproduces mainly through rhizomes and, at low frequency, through seeds (Coops and Van Der Velde 1995). *Phragmites* grows across a broad climatic and altitudinal range in Australia (Hocking 1989); being especially common in south-eastern Australia (Morris *et al.* 2008). Many river catchments in Victoria contain large populations of *Phragmites* (Jayawardana *et al.* 2006). Being one of the most widespread plants on earth, *Phragmites* is of considerable ecological and economic significance (Roberts 2000).

The distribution and relative abundance of *Phragmites* have increased dramatically over the past 150 years (Saltonstall 2003). Initially, it invades fresh and brackish wetlands and subsequently expands its range and abundance. Recent studies have shown that the expansion rate of *Phragmites* can be 1.5 m yr^{-1} (Silliman and Bertness 2004). Mixed wetland plant communities can, over time, be replaced by near monocultures of *Phragmites*, resulting in changed ecosystem processes with associated detrimental impacts on native wildlife (Meyerson *et al.* 2000). Changed ecosystem processes have been identified as the most important threat to global biodiversity (Jarchow and Cook 2009), ecosystem structure and function (Mack *et al.* 2000; Silliman and Bertness 2004). Some authors have proposed that the mechanisms behind the invasive characteristics of plant species, and by implication *Phragmites*, might be resource competition, allelopathy, altered ecosystem processes or other pathways

(Ehrenfeld *et al.* 2001; Levine *et al.* 2003). The phenomenon of allelopathy has been posited as one of the major drivers of the invasion process (Callaway 2002; Jarchow and Cook 2009) and an important factor in determining species distribution and abundance within plant communities (Kobayashi 2004). Allelochemicals may be released into natural environment by leaching of living plant parts, root exudates, volatilization, residue decomposition, microbial activities and certain agricultural practices (Inderjit 1996).

Relatively few studies have been carried out on allelopathy of *Phragmites* using ecologically realistic methods and concentrations of allelochemicals. Some preliminary studies have been conducted to test the effects of aqueous extracts of *Phragmites* on germination and growth of various plant species (Kulshreshtha 1981; Li *et al.* 2011; Qian *et al.* 2007; Singh *et al.* 1993) as well as autotoxicity effects (Sharma *et al.* 1990), however, there are few details on root-secreted allelochemicals of *Phragmites* (Rudrappa *et al.* 2007; Rudrappa *et al.* 2009). Several studies have identified chemicals within *Phragmites* that have antialgal, antifungal or antibacterial effects (Hu and Hong 2008; Li and Hu 2005). Some chemicals produced by decomposition of belowground organs of *Phragmites* may be responsible for die-back of *Phragmites* itself (Armstrong and Armstrong 1999; Ostendorp 1989). So far, no allelopathic studies of *Phragmites* have been carried out in Australia even though allelopathy is recognized as a major driving force for structuring and functioning in ecosystem in other parts of the world (Callaway and Walker 1997; Rice 1984). Additionally, no previous studies have examined the persistence of allelochemicals of *Phragmites* through decomposition in soil systems, their phytotoxicity and the relative importance of allelochemical concentrations from different organs on germination, growth and physiological aspects of associated plant species. We examined allelochemical phytotoxicity of *Phragmites* to

quantify the relative importance of different organs in suppression of other plant species as well as the presence and persistence of allelochemicals in soil. We hypothesised that allelochemicals of *Phragmites* negatively affect germination, growth, and physiological aspects of model test species and associated wetland species. There is potential for wide application of allelopathy research, especially in the light of the strong focus on wetland rehabilitation in Australia.

Materials and methods

Plant and soil materials

Whole, mature plants of *Phragmites* were collected in January 2011 from natural stands adjacent to Cherry lake (37°51'30"S, 144°50'5"E), a coastal wetland in Altona, Melbourne, Australia. Plant materials were collected from areas considered visually homogeneous with respect to shoot density and age. All samples were placed into sealable plastic bags for transportation to the laboratory. At the same time, soil samples at different zones of the rhizosphere and outside of the *Phragmites* population were collected and tightly sealed in plastic bags. In the laboratory, all plant samples were rinsed with pressurized water and then sorted into leaf and stem for aboveground organs, as well as root and rhizome for belowground organs. A portion of each organ was used for aqueous extracts of fresh plant and other portions were dried to estimate equivalent dry weight for aqueous extracts of dry plant. Large organic matter, pebbles and plant detritus were separated from the soil in the laboratory. These plant and soil samples were dried at 40°C in an oven until the weight was constant. Dry weights of each organ were measured separately. Oven-dried plant and soil samples were ground and sieved (0.5 mm) before storing in sealed plastic vials at room temperature, until chemical analyses and other experiments were conducted.

Seed collection

Seed capsules of non-native, *Rumex conglomerates* (hereafter called *Rumex*) and native, *Juncus pallidus* (hereafter called *Juncus*) were collected in May 2011 from Cherry Lake and stored in paper bag at room temperature for 1 week. Seeds were shaken from the capsules and sieved to remove empty capsules and other detritus. In the case of *Rumex*, the seeds were gently rubbed to remove enclosed flower parts and cleaned. Model seeds, *Lactuca sativa* (hereafter called lettuce) and *Raphanus sativus* (hereafter called radish) were purchased from a commercial source (DT Brown Seeds, South Windsor, NSW, Australia).

Collection and storage of aqueous extracts

To formulate extracts, 50 g fresh (chopped) and 25 g dry ground materials of each organ were submerged in 500 ml of distilled water and agitated for 24 h on an orbital shaker (Orbital Mixer, EOM5, Ratek Instruments Pty. Ltd, Vic., Melbourne, Australia) at room temperature. The extracts were filtered through cheese-cloth, centrifuged at 11952 g (10000 rpm) for 10 min at 4°C (Beckman Avanti 30 High Speed Compact Centrifuge, 364105, Beckman Coulter Inc., Brea, CA, USA) and sterilized (microfiltration with 0.22- µm pore filter). pH was determined with a pH meter (pocket digital pH meter, 99559, Dick-Smith Electronics, Australia) and electrical conductivity (EC) with a conductivity meter (TPS Digital conductivity meter, 2100, TPS Pty Ltd., Brisbane, Australia) of the extracts. Osmotic potential (OP) was calculated using the equation $OP = EC \times - 0.36$ according to McIntyre (1980). All extracts were subsequently stored at -20°C in freezer until bioassays were conducted.

Phenolics determination

Total phenolics of dry leaf, stem, root, rhizome and soil extracts were determined as described by Singleton and Rossi (1965), with gallic acid used as the standard.

Bioassay with aqueous extract of fresh plant organs on model seeds

Lettuce was used as a model seed because of its sensitivity and common use in bioassay. The seeds were surface sterilized with 1.5% (v/v) sodium hypochlorite for 1 min and subsequently washed (three times, 3 min per wash) with sterile distilled water. Each organ extract (5 mL) was placed into a sterile 9-cm petri dish underlain with two sterile filter papers (Whatman No. 1) with distilled water used as a control. Five replicates (each having 25 seeds) were used for each treatment. The petri dishes (Techno-Plas, Australia) were sealed with paraffin film (Pechiney, Plastic Packaging Company, Menasha, WI 54952, USA) then placed in polyethylene bags to prevent water loss by evaporation and to avoid contamination. The prepared dishes were arranged in a completely randomized design (CRD) and placed in a growth chamber (Westinghouse, Electrolux Home Products, Australia) set to 25°C in dark condition. Germination was deemed to have occurred when the radicle protruded beyond the seed coat by at least 1 mm. Germinated seeds in all petri dishes were counted daily at noon for 8 days, the point where cumulative germination levelled off in all treatment. Germination indices (total germination, speed of germination, coefficient of the rate of germination, speed of accumulated germination), physiological parameter (dehydrogenase enzyme activity) and biometric characteristics (length and weight of radicle and hypocotyl) were measured.

Bioassay with aqueous extract of dry plant organs on model seeds

Lettuce (25 seeds) and radish (20 seeds) were used as model seeds. Bioassay procedures and counting the germinated seeds were the same as for the first experiment, with six replicates. Physiological parameters, such as electrolyte leakage, dehydrogenase enzyme activity and lipid peroxidation of germinated seedlings were measured.

Bioassay with aqueous extract of dry plant organs on associated plant seeds

Rumex (25 seeds) and *Juncus* (50 seeds) were used as associated plant species. The bioassay procedures were similar to those of the first experiment, with very slight modification. The prepared dishes were placed in a growth chamber set to 25/15°C day/night temperature and a 12-h photoperiod with illumination of 84 $\mu\text{mol s}^{-1}\text{m}^{-2}$. The germination percentage and physiological parameters of plantlets were measured after 14 days.

Dose-response study with aqueous extract of dry plant organs on model seeds

A base concentration of 20% (20 g dried ground material in 100 ml distilled water) aqueous extracts of dry leaf and rhizome was used because our previous experiments showed higher inhibition using these extracts rather than stem and root extracts. The 20% base concentration was diluted with distilled water to obtain concentrations of 15%, 10%, 5%, 2.5% and 1.25%, and distilled water was used as a control. Lettuce was used as the model species following the same procedures as mentioned previously, except that only four replicates were used. On the basis of this experiment, LC₅₀ concentrations (concentration required to obtain 50% inhibition) and physiological parameters were determined.

Growth study of associated plant species in Phragmites leachate-incorporated soil

Glass dishes (12 cm by 6 cm) containing 150 g of processed soil were moistened uniformly with 50 mL leachate (5%) from each organ of *Phragmites* and distilled water was used as a control. Pregerminated seedlings (10 for *Rumex* and 15 for *Juncus*) with visible radicles not exceeding 1 mm in length were placed on the soil surface at equal distance, with three replicates. The dishes were wrapped with transparent plastic film and kept in a germination growth chamber as mentioned above. After 4 weeks, biometric parameters (root, shoot and leaf length, leaf area, plant height, and biomass) and physiological parameters (Chlorophyll *a*, Chlorophyll *b*, total chlorophyll, maximum quantum yield (F_v/F_m)) were measured.

Residue decomposition study

Two treatment series (residue alone and a mixture of residue with soil) and two types of decomposition (aerobic and anaerobic) were maintained in the laboratory. In the case of the first treatment, 4 g of dried and ground *Phragmites* residue (leaves and stems) was placed in a 500-mL glass jar with 100 ml of distilled water. For the second treatment, 4 g of each residue was placed in 100 g of soil and 50 mL of distilled water was added to make field condition. Microbial inoculum (10%) was obtained from a mixture of water and *Phragmites*-infested soil. The inoculum solution was added to each treatment and the pots were incubated in room temperature. Distilled water was added to the pots to compensate for the evaporative loss. Three replicates were set for each treatment and sampling was carried out each week of 5-week incubation period. Aqueous extracts were obtained by adding 100 mL of distilled water to the second treatment and shaking the water-soil mixture in orbital shaker for 1 h at room temperature. The mixture was

then centrifuged for 15 min at 3000 rpm at room temperature, sterilized (microfiltration with 0.22- μm pore filter) and the extracts were stored at -20°C until chemical analyses.

Measurement of germination indices, and biometric and physiological parameters

Germination indices were evaluated following the procedures of Allaie *et al.* (2006) and Rashid *et al.* (2010a). Digital slide callipers (Electronic digital calliper, N 287, Australia) were used to measure root and shoot length. Leaf-area measurement followed Wood and Jason (2000). The fresh weight of root and shoot were measured using a digital scale (A & D, HR200 lab analytical balance, Japan), following the separation of individual organs. Measurements of electrolyte leakage and dehydrogenase enzyme activity (in terms of formazan production) were conducted following the methods of Bogatek *et al.* (2006) and Sampietro *et al.* (2006) respectively. The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) concentration as described by Jambunathan (2010). Chlorophyll content was determined using the method of Inskeep and Bloom (1985). Chlorophyll fluorescence was measured on fully expanded leaves using a plant efficiency analyser (PEA, Hansatech, Norfolk, UK). The irradiance was about $3400 \mu\text{molm}^{-2}\text{s}^{-1}$ of red radiation at a wavelength 650 nm. The parameters of chlorophyll fluorescence namely, initial fluorescence (F_o), maximum fluorescence (F_m) and variable fluorescence F_v ($F_m - F_o$) were measured after 30-min dark adaptation.

Data analyses

All the experiments were conducted in a completely randomized design, with at least three replicates. Mean \pm standard error (s.e.) was computed by using raw data. Percentage data were arc-sin transformed to meet the requirements of the one-way ANOVA tests. Significance tests were performed after applying Dunnett tests at the

0.05 probability level. All data were analysed using Predictive Analytics Software (PASW) statistics 18.0 (IBM Corporation, NY, USA).

Results

Phenolics and other properties in extracts of plant organ and soil

Phragmites leaf showed the highest concentration of allelochemicals (as phenolics) followed by rhizome, root and stem (see Fig. S1, available as Supplementary Material on the web). Concentrations were 32.10 and 23.24 mg g⁻¹ in leaf and rhizome, respectively. *Phragmites*-infested soil had a higher concentration of phenolics (1.26 mg gm⁻¹) than did other soils (see Fig. S1). pH, EC and OP were 5.32 - 7.34, 0.36 - 2.33 mS cm⁻¹ and 0.12 - 0.83 bar for the fresh-plant extracts, whereas extracts of dried plant exhibited slightly different readings, being 5.1 - 7.1, 1.36 - 3.54 mS cm⁻¹ and 0.48 - 1.27 bar, respectively.

Phytotoxicity of fresh plant extracts on model seeds

Fresh aqueous extracts had significant effects on germination indices of lettuce seeds (Table 1). Leaf and rhizome extracts had greater phytotoxicity than did other organs. Speed of germination was strongly influenced by leaf extract (70% inhibition) compared to the control. The data showed strong inhibition on the length and weight of the radicle (Fig. 1a), and conversely, stimulation of hypocotyl length and weight (see Fig. S2, available as Supplementary Material on the web). Calculated ratios for length (radicle: hypocotyl) and weight (radicle: hypocotyl) showed a significant variation from control for leaf and rhizome extracts compared to the control (all $P \leq 0.001$). The leaf, rhizome and root extracts had a significant effect on the dehydrogenase enzyme activity (Fig. 1b).

Phytotoxicity of dry-plant extracts on model seeds

Germination inhibition

Phytotoxic effects on the germination indices of lettuce and radish are presented in Table 2. The various organs exhibited varying degrees of influence; leaf and rhizome extracts had strong inhibition on both species whereas stem and root extracts had low inhibition. Germination inhibition from leaf and rhizome was 34.23% and 32.20% for lettuce, and 12.75% and 15.25% for radish seeds respectively. Speed of germination was very low for both trial species grown in leaf and rhizome extracts. Speed of germination rates were 0.74 and 1.90, respectively, for lettuce and radish in leaf extract whereas rates were 11.86 and 8.23 in the control. Speed of accumulated germination and coefficient of the rate of germination were also significantly lower in all aqueous-extract treatments than in the control.

Biometric inhibition

The inhibitory effects of *Phragmites* alleochemicals were more pronounced on radicle length than on the hypocotyl (Fig. 2) of both test species. All organ extracts significantly reduced the radicle length of lettuce (54-89%) whereas the hypocotyl length did not reduce significantly. Leaf and rhizome extracts inhibited hypocotyl length whereas stem and root extracts stimulated hypocotyl growth. In the case of radish, the radicle length was reduced to 3-78% of that in the control. Of all the *Phragmites* organs, rhizome extract had a higher inhibition (78%) than did the other organs. Hypocotyl growth was inhibited only by rhizome extract (47%), and was stimulated by other organs. Radicle: hypocotyl (weight and length) ratios of both test species suggested that all organs of *Phragmites* had a significant inhibitory effect on seedling growth. All aqueous extracts significantly reduced the radicle fresh weight of

both species (see Table S1, available as Supplementary Material on the web). Leaf and rhizome extracts inhibited the radicle weight by 53% and 62% in lettuce, whereas the values were 19% and 74% in radish, respectively. Hypocotyl fresh weight of lettuce was negatively affected by all extracts, whereas only rhizome extract significantly lowered the hypocotyl weight in radish.

Physiological inhibition

Allelochemical inhibition caused a significant ion leakage from the plantlet of both test species, as measured by increased conductivity of the bathing solution. Results showed that leaf and rhizome extracts had a stronger effect than did other organ extracts (Fig. 3a). Extracts of different organs of *Phragmites* increased the lipid per oxidation of plantlets of lettuce and radish (Fig. 3b) with the increase of MDA concentration. Aqueous extracts significantly affected cell respiration of germinated seedlings of lettuce and radish as measured by formazan production in the plantlet, this being related to dehydrogenase enzyme activity. Leaf and rhizome extracts had the strongest effects on the formazan production of the germinated seedlings of both test species (Fig. 3c). However, the results showed that all aqueous extracts induced a decrease in formazan production in the plantlet.

Phytotoxicity of dry-plant extracts on associated plant seeds

Aqueous extracts had a significant negative effect on germination of both *Rumex* and *Juncus* species (Fig. 4). Leaf extract had 32% and 69% germination inhibition for *Rumex* and *Juncus*, respectively. Rhizome extract also had a strong inhibitory effect. However, all extracts inhibited root growth of both species. The leaf and root extracts had a greater root-length inhibition (54% and 62%, respectively) for *Rumex*, whereas these extracts caused 100% inhibition for *Juncus* (see Fig. S3, available as

Supplementary Material on the web). The shoot height (petiole and lamina) was not significantly reduced by extracts for *Rumex*, whereas culm length of *Juncus* was significantly reduced by all extracts (see Fig. S4, available as Supplementary Material on the web). Fresh-weight biomass per plant of *Juncus* in all treatments was significantly lower than control (see Fig. S5, available as Supplementary Material on the web). Root biomass of *Rumex* was significantly affected by all extracts, whereas shoot weight was not reduced significantly (see Fig. S5). Electrolyte leakage, lipid peroxidation and dehydrogenase enzyme activity for *Rumex* plantlets and electrolyte leakage for *Juncus* exhibited allelochemical inhibition in most cases (see Fig. S6, available as Supplementary Material on the web).

Dose-response effect on model seeds

Results showed that different extract concentrations derived from leaf and rhizome had differential effects on the germination and growth of lettuce seeds. The LC_{50} was calculated to be 4.68% and 11.25% for leaf and rhizome extracts, respectively (Fig. 5). In the leaf extract, no germination occurred beyond 15% concentration, whereas the rhizome extract showed a different germination percentage. Speed of germination, radicle weight and length were all negatively affected by increasing extract concentrations (see Fig. S7, available as Supplementary Material on the web).

Growth of associated plant species in leachate-incorporated soil

Phragmites leachate had a strong inhibitory effect on root and shoot length, as well as biomass of both associated plant species (Table 3). Leaves per plant, leaf length and leaf area per plant were significantly reduced in *Rumex* species (see Fig. S8, available as Supplementary Material on the web). In the case of *Juncus*, culm number per plant was significantly lower than in the control (see Fig. S8). Changes in the physiological

parameters of plant tissues were also observed in both plant species. Table 4 shows that the leachate-incorporated soil had significant negative effects on Chlorophyll *a*, Chlorophyll *b* and total chlorophyll for both species. Leaf and rhizome leachate showed more phytotoxic potential to reduce the chlorophyll content in both species. Total chlorophyll reduction was ~ 40% for both species with these treatments. Maximum quantum yield to determine plant stress condition was only carried out for *Rumex* only. Allelochemicals from *Phragmites* had an effect on the photo-system II (PSII) that negatively influenced photosynthesis of the test species. *Fv/Fm* values in chlorophyll fluorescence were significantly reduced in *Rumex* with leaf and rhizome treatments, compared with the control (Fig. 6). The overall results of this study showed that leaf and rhizome extracts were more phytotoxic than were other treatments.

Residue decomposition study

Water-soluble phenolics from the decomposition experiment are shown in figure 7. In the case of aerobic decomposition, the amount of soluble phenolics in the residue-alone treatment reduced slowly from its initial condition; however, the rate of reduction was slightly faster initially and slowing and then becoming almost constant. In the case of residue with soil decomposition, the results showed a sudden reduction in the first week and then remaining almost constant for the rest of observation period. More than 150 ppm and about 40 ppm of soluble phenolics were stable in residue alone and residue with soil decomposition respectively. In anaerobic decomposition, the water-soluble phenolics fluctuated in both treatments, but the total quantity remained almost the same. The quantity of water-soluble phenolics in anaerobic decomposition was always higher than that in the aerobic decomposition.

Discussion

Phenolics and other properties in extracts of plant organ and soil

Phenolics concentrations varied between different organs with highest concentration occurring in the leaf extracts, followed by rhizome extracts. When the phenolics of the leaf and rhizome extracts are transferred to the rhizosphere they clearly exhibit phytotoxicity on associated plant species. Significant amount of phenolics occur in *Phragmites* rhizosphere soil. These soil phenolics are stable and retain potential phytotoxic effects on recruitment and colonization of associated plant species. These results are supported by findings on other species (Inderjit and Weiner 2001; Xuan *et al.* 2005). Differing levels of pH, EC and OP suggest that chemical composition amongst aqueous extracts of plant organs may provide an indirect explanation for differential effects on plant species.

Germination bioassays on model seeds

Leaf and rhizome extracts showed clear inhibitory effects on germination, and biometric and physiological parameters of tested species for extracts of both fresh and dry plant. Aqueous extracts of dry ground organs showed greater potential for phytotoxicity than did those of fresh organs. This toxicity may be related to an accelerated release of free phytotoxins as a result of increased surface-area contact with water (Mason-Sedun and Jessop 1988). Germination indices are not generally regarded as sufficient to elucidate phytotoxicity of donor species because they lack information regarding physiological effects or mode of actions (Gross *et al.* 1996). However, the speed of germination is sometimes used as a key indicator for understanding the effect of phytotoxicity (Allaie *et al.* 2006). In our study, the total germination of test species was less inhibited than the speed of germination, a finding well aligned with previous

studies (Rashid *et al.* 2010a; Wardle *et al.* 1992). Lettuce appeared more sensitive than radish when all parameters were compared. Regarding biometric parameters, radicles were more inhibited than hypocotyls, in length and weight for both test species. Our results and those of Kulshreshtha (1981) indicate that *Phragmites* organs had significant negative effects on the growth of plant; however, the effects were species-specific. These findings also support those of other studies (Burgos and Talbert 2000; Javaid *et al.* 2006) where germination and root length were more inhibited than the shoot length and biomass in all tested species. Paradoxically, in some extracts (stem and root), hypocotyl length was stimulated rather than inhibited. Leaf and rhizome extracts exhibited more potential than other organs for inhibition, possibly related to a correlation between relative phytotoxicity and pH of the extracts (An *et al.* 1997).

Physiological parameters (lipid peroxidation, electrolyte leakage and dehydrogenase enzyme activity) were significantly affected in test species. Leaf and rhizome extracts significantly increased the MDA quantity in plantlet of lettuce, a process that supports the concept of loss of germination (Bogatek *et al.* 2006). In the case of radish, only the rhizome extract had significant effects on MDA. Increasing electrolyte leakage indicates the inability of seedlings to maintain coherent membranes leading to a negative effect on germination (Bogatek *et al.* 2006). Loss of membrane integrity, increased lipid peroxidation and electrolyte leakage in plant tissue, induced by allelochemical toxicity, are primary indicators of cell injury. Our results indicated that aqueous extracts disrupt the membrane integrity, resulting in solute leakage. These effects were directly caused by phytotoxicity because the leaf and rhizome extracts had higher phenolics than did other plant organs. Our results showed that leaf and rhizome extracts significantly decreased dehydrogenase enzyme activity in plantlets, which in turn affects ATP production in plant cells and consequently, growth of the plant

(Sampietro *et al.* 2006). The direct cause-effect relationship of phytotoxins on physiological parameters, which is very clear in our studies, is very similar to that found in *Helianthus* and *Cassia* species (Oracz *et al.* 2007; Singh *et al.* 2006).

Germination bioassay on associated plant species

Measuring the effects of allelochemicals on sensitive model species such as lettuce and radish, is primarily a predictive tool (Gallardo-Williams *et al.* 2002). Phytotoxicity evaluation using associated plant species may give more concrete suggestions with regard the allelopathic effect. Some studies regarding allelopathic potential have stated that *Phragmites* effectively inhibits the germination of associated plant species such as *Typha angustata* (Singh *et al.* 1993), *Solidago canadensis* (Li *et al.* 2011), *Spartina alternifolia* (Qian *et al.* 2007) or 'weeds' (Khan *et al.* 2011) and *Phragmites* itself (Gopal and Goel 1993). Our studies also showed that the aqueous extracts had a significant effect on the associated plant species, *Rumex* and *Juncus*, in relation to germination, and biometric and physiological parameters.

Growth of associated plant species in leachate- incorporated soil

Leachate-incorporated soil reduced the root and shoot length as well as biomass of both associated species. Root growth was more affected than were other biometric parameters because root membranes are the primary target of all phenolics (Hussain and Reigosa 2011). *Phragmites* phenolics disintegrate the structural protein in the roots of associated species and therefore 'topple' the competition (Bains *et al.* 2009). These effects lead to the reduction of mycorrhizal associations, water and nutrient uptake, depolarization and an efflux of ions. Ultimately, phytotoxic allelochemicals negatively affect overall plant growth, a fact found in several studies, including ours (Rashid *et al.* 2010a; Rashid *et al.* 2010b). More specifically, *Phragmites* induces rhizotoxicity

(Neori *et al.* 2000), a more extreme effect that causes a marked inhibition of root growth leading to necrosis and collapse (Qin *et al.* 2006). Allelochemicals can alter the physiological systems of plants even though the mode of action differs. In our studies, chlorophyll content and photosystem II were affected in the tested species. The decrease of chlorophyll content in plant tissues is a common phenomenon related to allelochemicals that cause cellular damage (Leather and Einhellig 1988). The effects may vary with test species and the compounds tested (Mersie and Singh 1988). Our studies showed that *Juncus* species were more susceptible than *Rumex*. Leaf and rhizome leachates of *Phragmites* had more phytotoxic potential than others. Photosystems, the functional and structural units for protein complexes involved in photosynthesis for plants, may be interrupted by allelochemicals. F_v/F_m tests whether or not plant stress affects the photosystem II in dark-adapted states as a measure of plant efficiency. Optimal values for F_v/F_m tests are ~ 0.83 (Johnson *et al.* 1993; Rashid *et al.* 2010a). The lower F_v/F_m values of *Rumex* in leaf and rhizome leachate-incorporated soil demonstrate strong allelopathic potential to affect the associated plant species. The findings in our study supported those of other studies (Marwood *et al.* 2003).

Dose-response effect

Our observations showed that LC₅₀ concentration of the leaf extracts was more toxic than that of the rhizomes. These values carry high significance and may serve as a benchmark for subsequent studies evaluating the effects of *Phragmites* and other plant allelochemicals of other plants (Batish *et al.* 2002). Whereas the extracts of different plant organs varied in their phytotoxicity against tested seeds, the actual exposure potential depends on biomass production in the field. The literature shows that biomass production of *Phragmites* is more than 200 t ha⁻¹ (Engloner 2009). *Phragmites*-dominated ecosystems produce a large volume of above and belowground biomass and

about two-thirds of the total biomass is produced in the rhizome system (Park and Blossey 2008). The worldwide distribution and the extremely large areas covered by reed stands might have a considerable effect on the accumulation of phytotoxins through decomposition in wetlands. Deposition of allelochemicals on this scale may actually create a 'leading edge' of plant death and damage. These allelochemicals may be attributed to various physiological changes induced by damage of cellular membrane, loss of dehydrogenase enzyme activity in plantlets and loss of chlorophyll in leaves of associated plants (Pandey 1996).

Fate of phenolics in residue decomposition study

Some specific allelochemicals, such as phenyl acetic, and salicylic and benzoic acids are highly inhibitory to the growth of lettuce at concentrations between 25 and 50 ppm (Chou and Patrick 1976) and all allelochemicals have allelopathical potential at a concentration of *ca.* 100 ppm (Rashid *et al.* 2010a). Phytotoxins, (e.g. acetic, propanoic, *n*- and *iso*-butyric and *n*-caproic acids and sulphides) derived from decaying materials of *Phragmites* and organic matter deposited in sediment from eutrophic conditions in lakes might amplify the growth, morphological and anatomical symptoms of die-back of *Phragmites* itself, as well as associated plant species, at a concentration of ~ 1mM (Armstrong and Armstrong 2001). Soluble phenolics in our study may retain their phytotoxicity after decomposition, a fact compatible with other studies (Bains *et al.* 2009). The constant accumulation of phenolics in the field and their effects may strongly influence plant-plant interactions, especially in wetlands (Jarchow and Cook 2009). Several studies have identified that death and decay of underground organs may produce a higher than normal concentration of organic acid than normal and that these may be toxic to the associated plant and *Phragmites* itself (Armstrong *et al.* 1996; Čížková *et al.* 1999). *Phragmites* death can arise through eutrophic conditions that can

lead to reduced organic-layer depth through accelerated decomposition by phytoplanktonic blooms (Armstrong and Armstrong 1999). It has been observed that die-back of *Phragmites* can be attributed to phytotoxins from organic matter decomposition, especially from reeds and algal blooms (Ostendorp 1989). Our results predict that the same phenomenon might happen in each *Phragmites*-dominated wetland.

The results presented here have demonstrated that extracts and leachates of leaves and rhizomes of the wetland plant *Phragmites* have more potential to produce phytotoxic chemicals than those of other organs of this species. The allelopathic potentiality can be ordered as leaf > rhizome > root > stem. These findings lend strong support to the allelopathic potential of *Phragmites*. Germination inhibition and reduction of growth, with consequent lowering of chlorophyll content and maximum quantum yield provide direct evidence of phytotoxicity and the persistence of allelochemicals in soil. We posit that the LC₅₀ response is a key parameter for measuring phytotoxicity in allelopathic studies of *Phragmites* and, potentially, of other species. The persistence and fate of phenolics in the decomposition processes showed that the water-soluble phenolics sharply reduced in the first 2-weeks and remained at a constant level thereafter. The study suggests that decomposing and decomposed litter may have allelopathic potential under natural conditions especially in anaerobic conditions. The additive or synergistic inhibitory effects of allelopathic compounds may have detrimental effects even at low concentrations. On the basis of the observed results of our studies, it can be concluded that allelochemicals from leaves and rhizomes could play an important role on the recruitment of associated plant species through an inhibition of germination, growth and physiological processes. We have shown the importance of studying the allelopathic potential before intensive chemical isolation,

identification and specification, a proposal that is supported by other authors (Rashid *et al.* 2010a; Thijs *et al.* 1994). The present studies were laboratory based, and it is an important methodology to understand a general aspect of allelopathy by eliminating all possible alternative interferences, and may act as a first step to investigate the allelopathic potential of a plant. However, semi-natural (greenhouse) and natural (field) evidence regarding allelopathy of *Phragmites* is essential to understanding the full ecological process (Hegazy *et al.* 2001; Inderjit *et al.* 2001). In addition to experimental methodologies, chemical identification and selection of growth parameters are other important aspects in allelopathy study that should be addressed in further research. To gain a better understanding of the mechanisms responsible for *Phragmites*-dominated wetland ecosystems, there is a need for multi-directional allelopathic research.

Ecological implications

Some studies have shown that the expansion of *Phragmites* in wetlands is increasing rapidly, control is difficult or practically impossible and that these invasions cause local extinction of native plant species (Silliman and Bertness 2004). Conversely, die back of *Phragmites* in European countries is equally problematic (Brix 1999; van der Putten 1997). Both syndromes, expansion and contraction, are happening in Australia and could be explained by allelopathy. The results of the present study may be informative for land managers, ecologists and scientists focused on the restoration of wetland ecosystems. The goal of wetland restoration is the return, to some extent, of their initial structure and function. In light of our observations, it appears that *Phragmites* exhibits its allelopathic potentiality in many ways, especially through the accumulation and persistence of allelochemicals in soil systems. The results of the present study provide land managers with a mechanistic understanding of the *Phragmites* invasion process that strongly implies that successful wetland restoration may involve limiting the

invasion and expansion of *Phragmites*. The present study may also influence sustainable management practices in wetlands through the knowledge of allelochemical activities in wetland ecosystems. Through understanding the potential impacts of allelochemicals on recruitment of associated plants in wetlands that contain *Phragmites*, the present study goes some way to explaining the long-term effects, particularly the floristic simplifications, on wetlands resulting from the invasion of *Phragmites*.

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Figure Captions

Fig. 1. Effect of fresh-plant extracts on (a) radicle length (mm) and weight (mg) and (b) formazan production (% control) of lettuce seeds. Values are mean \pm s. e. ($n = 5$). *** $P \leq 0.001$; * $P \leq 0.05$; and ns, non-significant (after applying the Dunnett test)

Fig. 2. Effect of dry-plant extracts on (a) radicle and (b) hypocotyl length (mm) of lettuce (light-grey square) and radish (dark-grey square) seeds. Values are mean \pm s. e. ($n = 6$). *** $P \leq 0.001$; ** $P \leq 0.01$; and ns, non-significant (after applying the Dunnett test)

Fig. 3. Effect of dry-plant extracts on (a) electrolyte leakage (micro-simens cm^{-1}), (b) lipid peroxidation (nmol MDA gm^{-1}) and (c) formazan production (% control) of lettuce (light-grey square) and radish (dark-grey square) plantlets. Values are mean \pm s.e. ($n = 6$). *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; and ns, non-significant (after applying the Dunnett test)

Fig. 4. Effect of dry-plant extracts on germination of *Rumex* and *Juncus*. Values are mean \pm s. e. ($n = 6$). *** $P \leq 0.001$; ** $P \leq 0.01$; and * $P \leq 0.05$ (after applying the Dunnett test)

Fig. 5. Dose-response curve between different concentrations of leaf and rhizome extracts of *Phragmites* and germination percentage of lettuce. Values are mean \pm s. e. ($n=4$). Horizontal line indicates LC_{50} concentration.

Fig. 6. Changes in quantum efficiency of open PSII reaction centres in dark-adapted (F_v/F_m) leaf of *Rumex*. Values are mean \pm s. e. ($n=3$). *** $P \leq 0.001$; ** $P \leq 0.01$; and ns, non-significant (after applying the Dunnett test)

Fig. 7. Dynamics of water-soluble phenolics (ppm) (gallic acid equivalent) in aerobic and anaerobic decompositions. Values are mean \pm s. e. ($n=3$).

Table Captions

Table 1. Effect of fresh-plant extracts of *Phragmites* on germination indices of lettuce seeds. Values are mean \pm s. e. ($n = 5$). *** $P \leq 0.001$; * $P \leq 0.05$; and ns, non-significant (after applying the Dunnett test)

Table 2. Effect of dry-plant extracts of *Phragmites* on germination indices of lettuce and radish seeds. Values are mean \pm s. e. ($n = 5$). *** $P \leq 0.001$; * $P \leq 0.05$; and ns, non-significant (after applying the Dunnett test)

Table 3. Effect of *Phragmites* leachate on root and shoot length (mm) and fresh biomass (mg plant^{-1}) of *Rumex* and *Juncus*. Values are mean \pm s. e. ($n = 3$). *** $P \leq 0.001$; * $P \leq 0.05$; and ns, non-significant (after applying the Dunnett test)

Table 4. Chlorophyll content (mg gm^{-1}) of associated plant species, *Rumex* and *Juncus*, in *Phragmites* leachate-incorporated soil. Values are mean \pm s. e. ($n = 3$). ** $P \leq 0.01$; * $P \leq 0.05$; and ns, non-significant (after applying the Dunnett test)

Fig. 1.

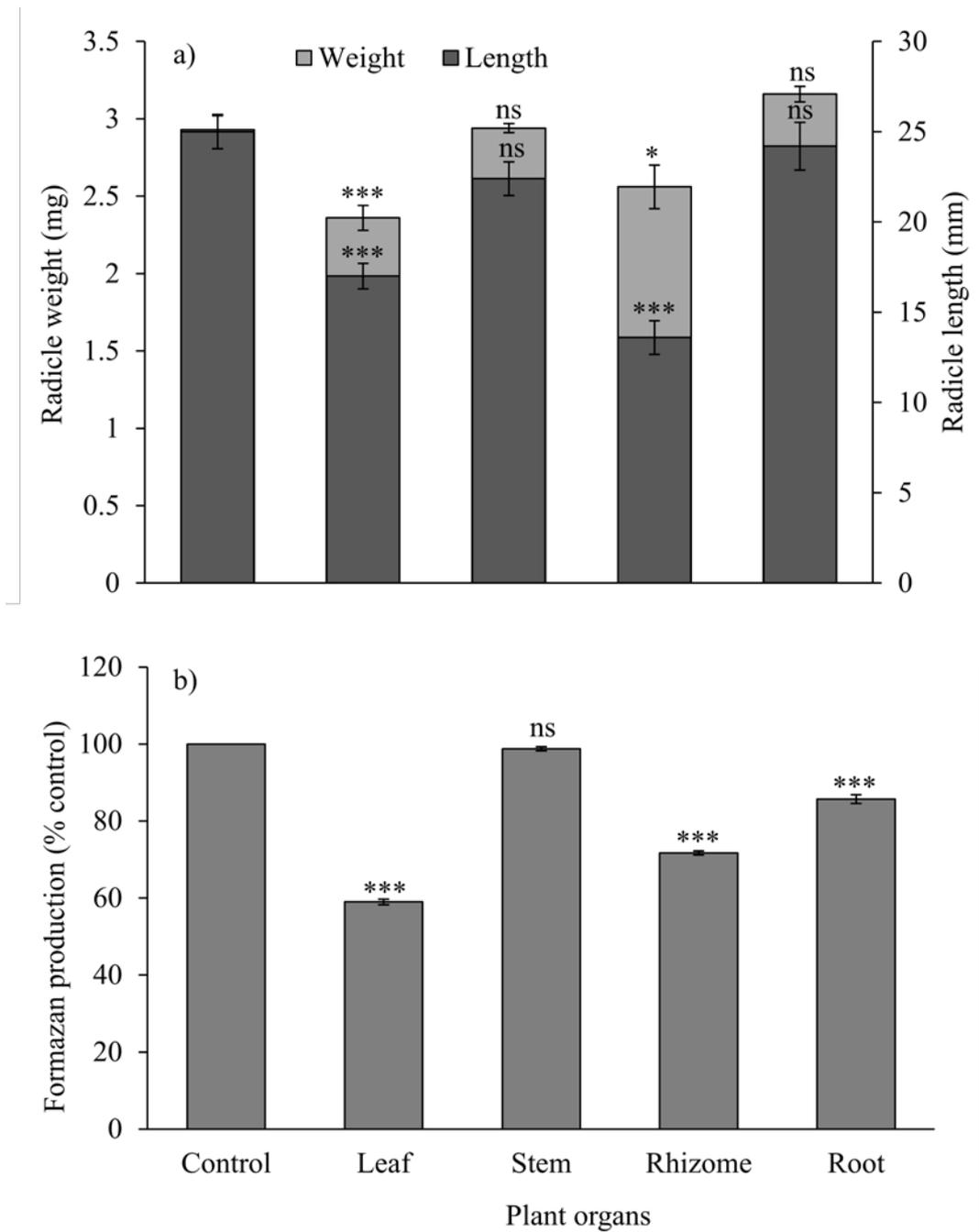


Fig. 2.

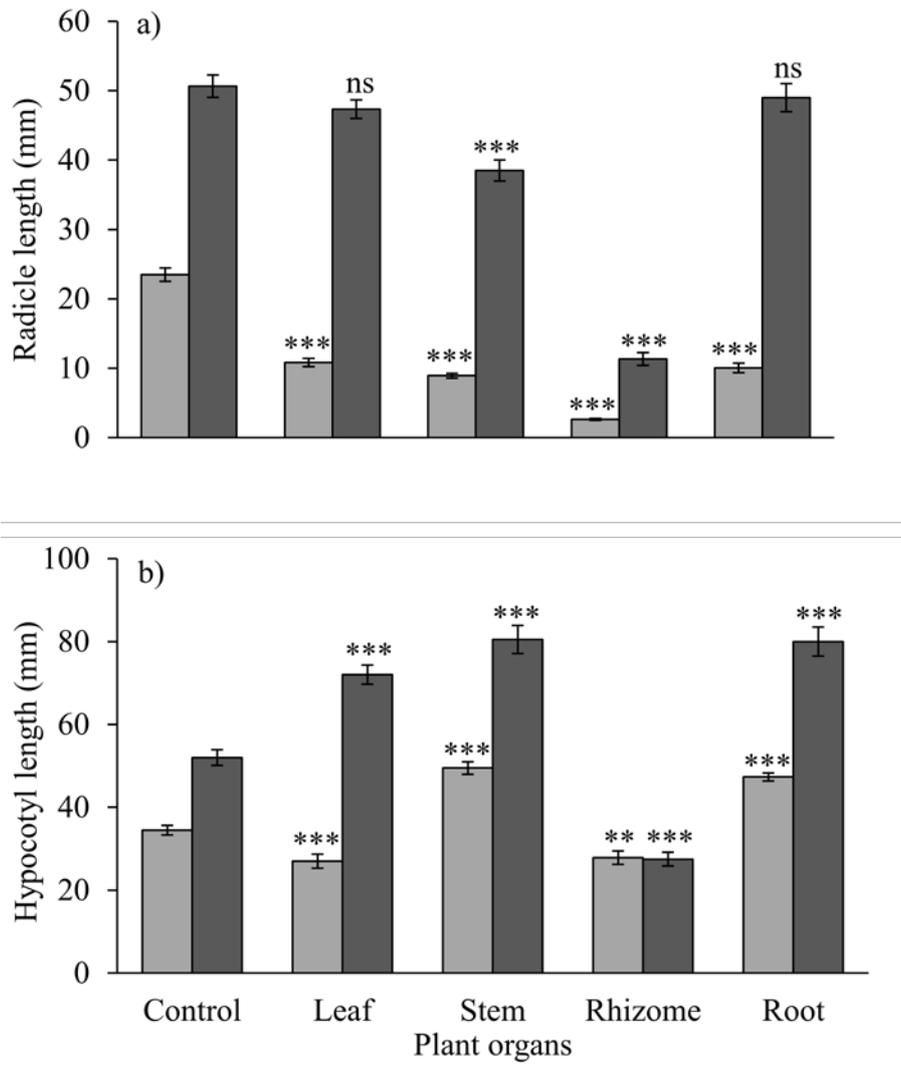


Fig. 3

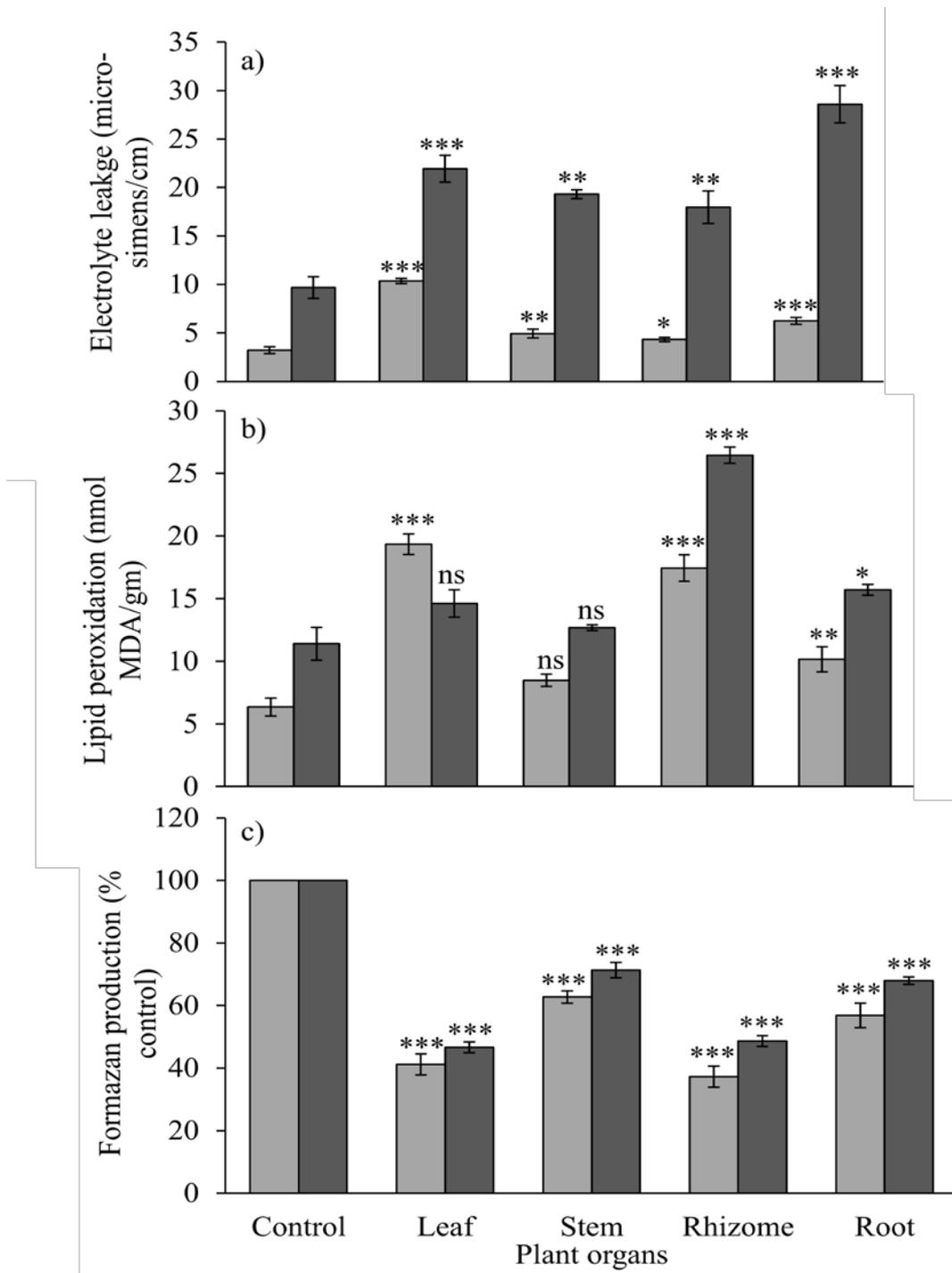


Fig. 4.

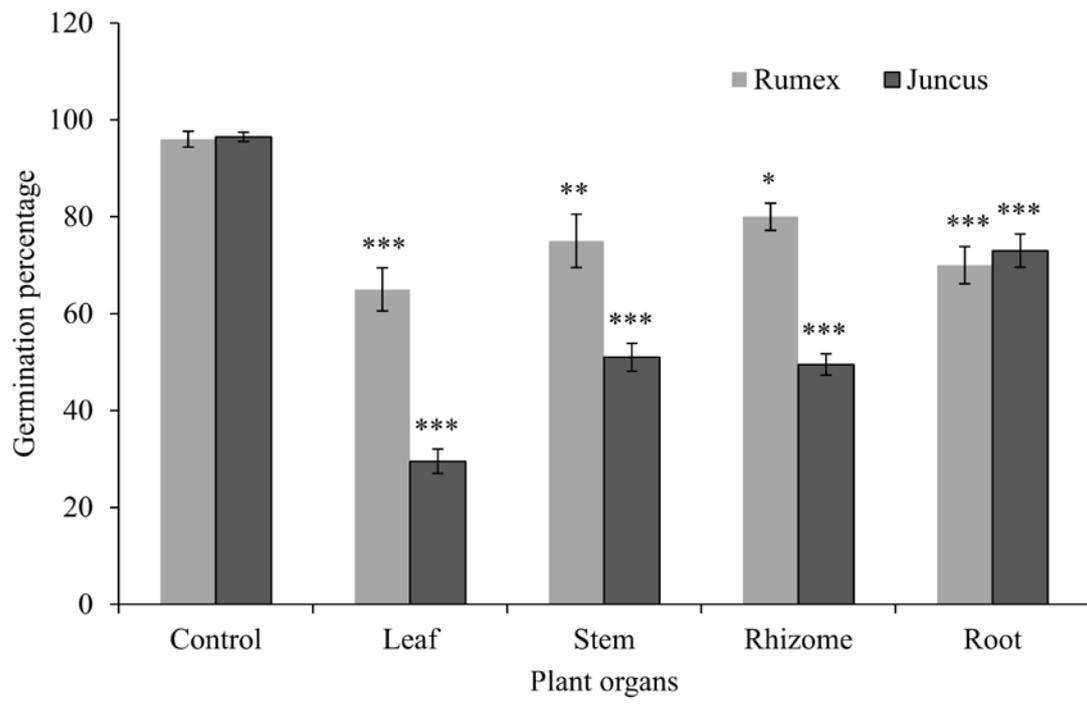


Fig. 5.

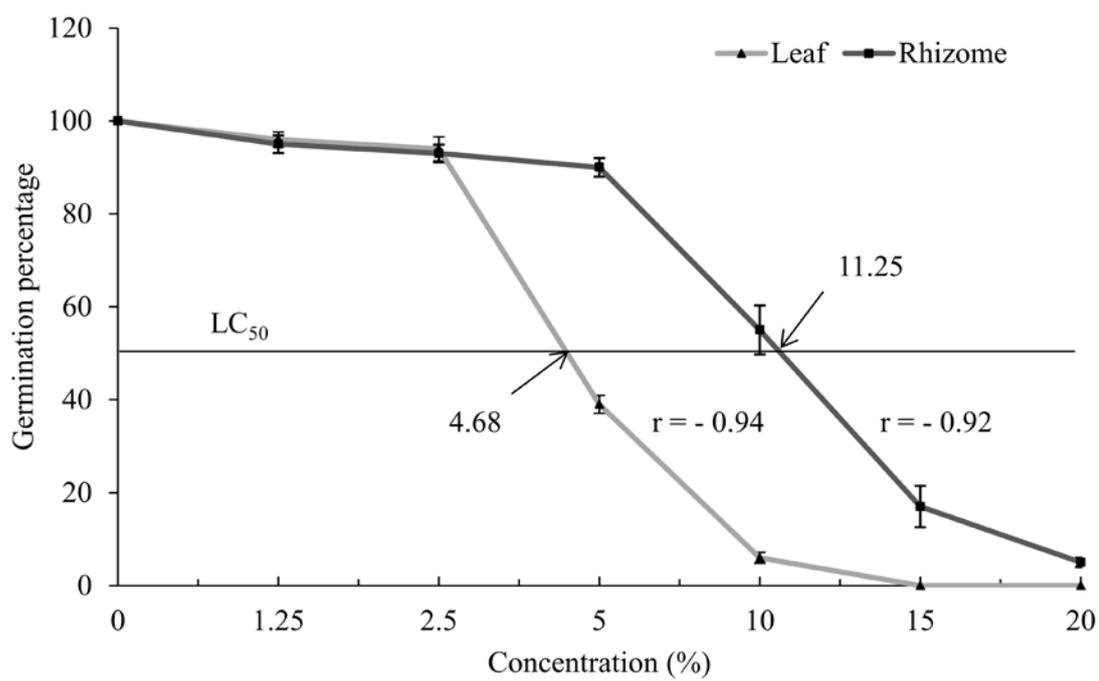


Fig. 6.

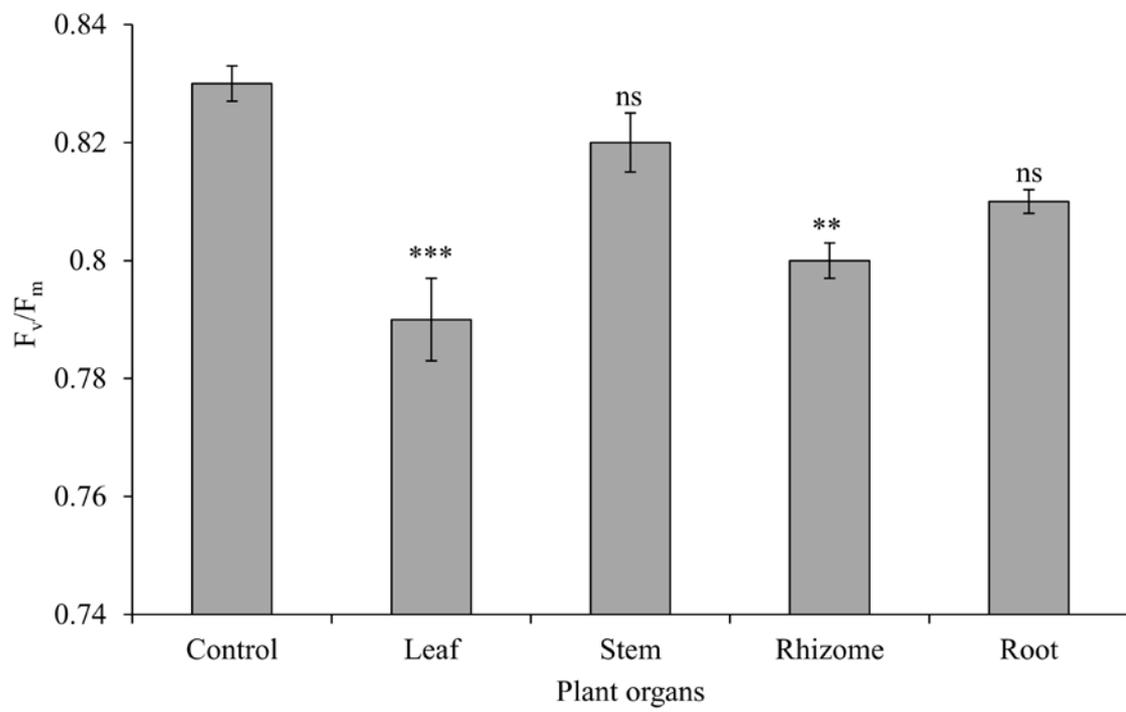


Fig. 7.

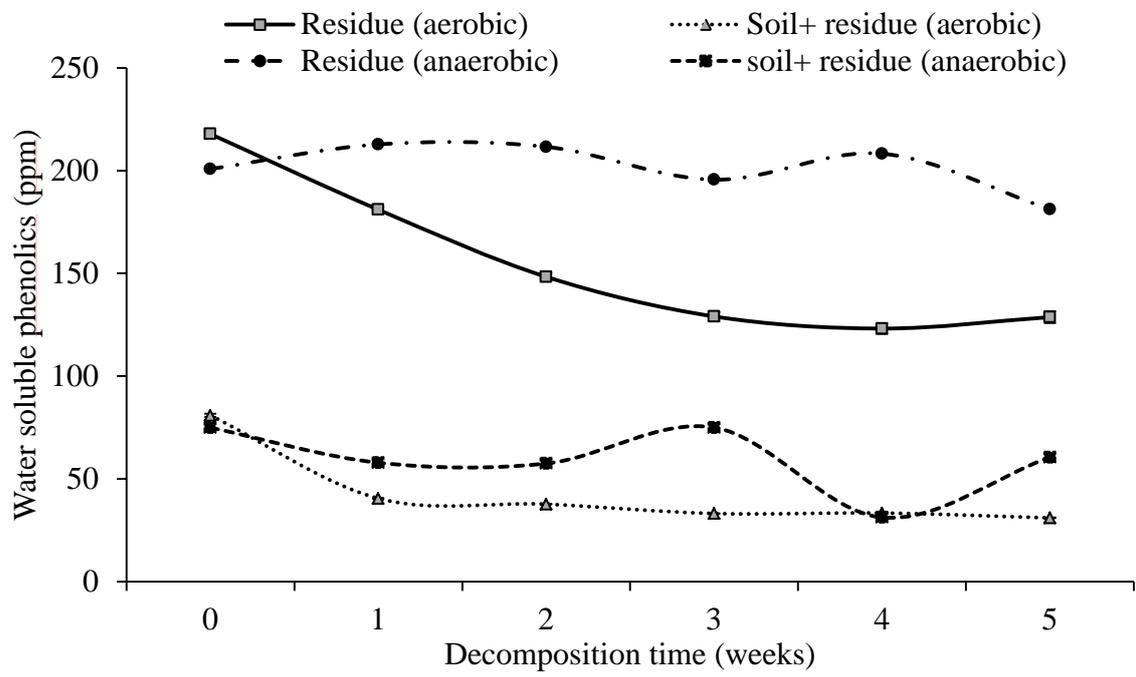


Table 1.

| Treatment | Total germination (%) | Speed of germination | Speed of accumulated germination | Coefficient of the rate of germination |
|-----------|----------------------------|----------------------------|----------------------------------|--|
| Control | 99.2 ± 1.78 | 11.04 ± 0.39 | 56.11 ± 3.57 | 81.34 ± 9.43 |
| Leaf | 91.2 ± 3.34 [*] | 3.20 ± 0.32 ^{***} | 28.72 ± 2.42 ^{***} | 40.78 ± 3.55 ^{***} |
| Stem | 94.4 ± 4.56 ^{ns} | 10.98 ± 0.50 ^{ns} | 55.12 ± 2.61 ^{ns} | 88.32 ± 4.99 ^{ns} |
| Rhizome | 88.8 ± 5.21 ^{***} | 9.32 ± 0.81 ^{***} | 48.31 ± 3.22 ^{***} | 74.03 ± 6.08 ^{ns} |
| Root | 92.8 ± 3.34 | 10.71 ± 0.35 | 57.07 ± 1.42 ^{ns} | 88.70 ± 4.17 ^{ns} |

Table 2.

| Treatment | Total germination (%) | | Speed of germination | | Speed of accumulated germination | | Coefficient of the rate of germination | |
|-----------|-----------------------------|-----------------------------|----------------------------|----------------------------|----------------------------------|-----------------------------|--|-----------------------------|
| | Lettuce | Radish | Lettuce | Radish | Lettuce | Radish | Lettuce | Radish |
| Control | 99.33 ± 1.63 | 98.33 ± 2.58 | 11.86 ± 0.50 | 8.23 ± 1.17 | 65.13 ± 1.67 | 47.96 ± 4.91 | 93.97 ± 5.14 | 75.30 ± 15.11 |
| Leaf | 65.33 ± 9.00 ^{***} | 66.67 ± 9.83 ^{***} | 0.74 ± 0.29 ^{***} | 1.90 ± 0.85 ^{***} | 11.74 ± 4.22 ^{***} | 17.31 ± 4.09 ^{***} | 21.13 ± 4.74 ^{***} | 34.93 ± 6.95 ^{***} |
| Stem | 89.33 ± 5.47 ^{***} | 86.67 ± 9.83 ^{ns} | 9.87 ± 0.51 ^{***} | 5.83 ± 1.22 ^{***} | 56.34 ± 2.52 ^{***} | 38.17 ± 6.15 ^{***} | 81.19 ± 8.52 ^{***} | 64.62 ± 10.45 ^{ns} |
| Rhizome | 86.67 ± 6.02 ^{***} | 83.33 ± 11.25 [*] | 2.99 ± 0.51 ^{***} | 3.48 ± 0.89 ^{***} | 33.18 ± 4.15 ^{***} | 28.92 ± 5.87 ^{***} | 41.58 ± 4.81 ^{***} | 44.43 ± 5.29 ^{***} |
| Root | 92.67 ± 6.89 [*] | 90.00 ± 5.48 ^{ns} | 4.13 ± 0.85 ^{***} | 6.58 ± 0.89 [*] | 41.69 ± 6.29 ^{***} | 41.53 ± 3.94 ^{ns} | 50.51 ± 4.48 ^{***} | 70.41 ± 6.11 ^{ns} |

Table 3.

| | Root | | Shoot | | Biomass | |
|-----------|-----------------|----------------|----------------------------|----------------|----------------------------|--------------------------|
| Treatment | <i>Rumex</i> | <i>Juncus</i> | <i>Rumex</i> | <i>Juncus</i> | <i>Rumex</i> | <i>Juncus</i> |
| Control | 31.85 ± 3.14 | 14.68 ± 0.24 | 13.22 ± 1.39 | 16.03 ± 1.47 | 96.17 ± 5.42 | .51 ± 0.04 |
| Leaf | 22.00 ± 1.15** | 3.68 ± 0.18*** | 7.66 ± 0.63* | 5.36 ± 0.05*** | 29.24 ± 2.23*** | .25 ± 0.05*** |
| Stem | 22.46 ± 0.58** | 8.44 ± 0.32*** | 11.23 ± 1.20 ^{ns} | 11.19 ± 0.65** | 58.73 ± 6.36** | .38 ± 0.02 ^{ns} |
| Rhizome | 18.45 ± 0.68*** | 4.26 ± 0.53*** | 8.21 ± 0.57* | 7.60 ± 0.77*** | 73.63 ± 6.42 ^{ns} | .36 ± 0.05 ^{ns} |
| Root | 24.55 ± 0.84** | 6.67 ± 0.29*** | 9.84 ± 1.08 ^{ns} | 8.55 ± 0.70*** | 68.37 ± 8.61* | .37 ± 0.01 ^{ns} |

Table 4.

| Treatment | Chlorophyll <i>a</i> | | Chlorophyll <i>b</i> | | Total Chlorophyll | |
|-----------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| | <i>Rumex</i> | <i>Juncus</i> | <i>Rumex</i> | <i>Juncus</i> | <i>Rumex</i> | <i>Juncus</i> |
| Control | 1.49 ± .14 | 1.57 ± .12 | 0.67 ± 0.06 | 0.73 ± 0.08 | 2.16 ± 0.20 | 2.29 ± 0.20 |
| Leaf | 0.90 ± 0.07 ^{**} | 1.08 ± 0.14 [*] | 0.41 ± 0.04 ^{**} | 0.45 ± 0.04 [*] | 1.31 ± 0.10 ^{**} | 1.53 ± 0.18 [*] |
| Stem | 0.96 ± 0.09 ^{**} | 1.32 ± 0.03 ^{ns} | 0.43 ± 0.04 ^{**} | 0.78 ± 0.02 ^{ns} | 1.39 ± 0.13 ^{**} | 2.10 ± 0.02 ^{ns} |
| Rhizome | 0.97 ± 0.07 ^{**} | 0.95 ± 0.14 [*] | 0.44 ± 0.02 ^{**} | 0.48 ± 0.08 ^{ns} | 1.41 ± 0.10 ^{**} | 1.43 ± 0.21 ^{**} |
| Root | 1.01 ± .05 [*] | 1.25 ± .15 ^{ns} | 0.45 ± 0.03 [*] | 0.88 ± 0.08 ^{ns} | 1.46 ± 0.08 [*] | 2.13 ± 0.08 ^{ns} |

Supplementary material

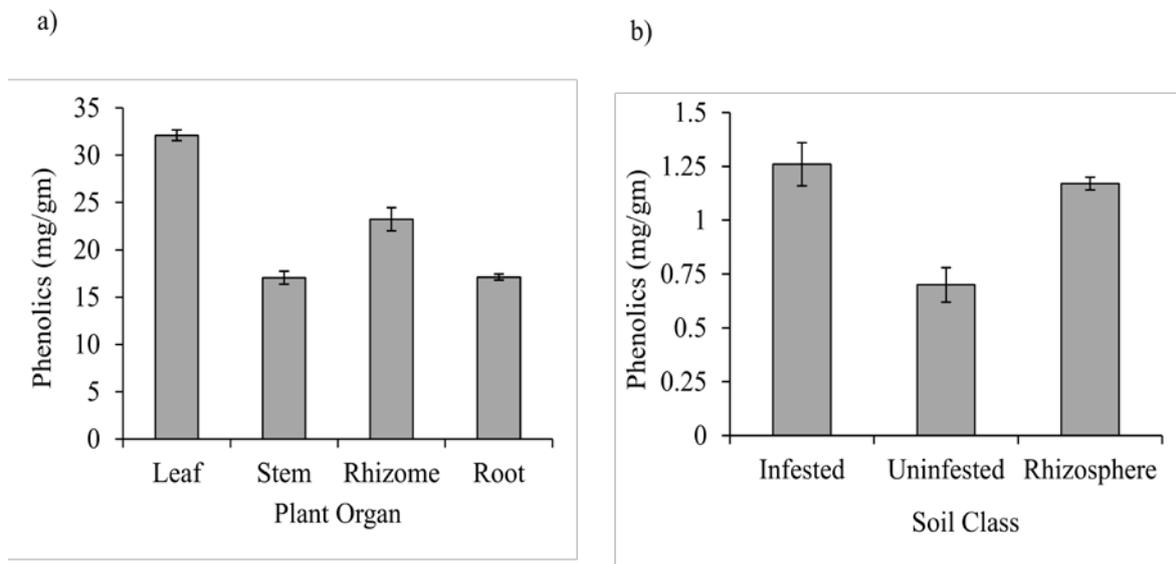
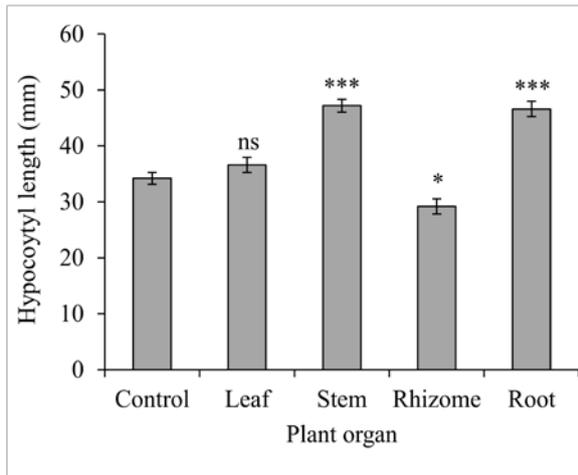


Fig. A1. Phenolics (mg/g) in a) plant organs of *Phragmites* and b) soil. Values are mean \pm standard error ($n = 3$).

a)



b)

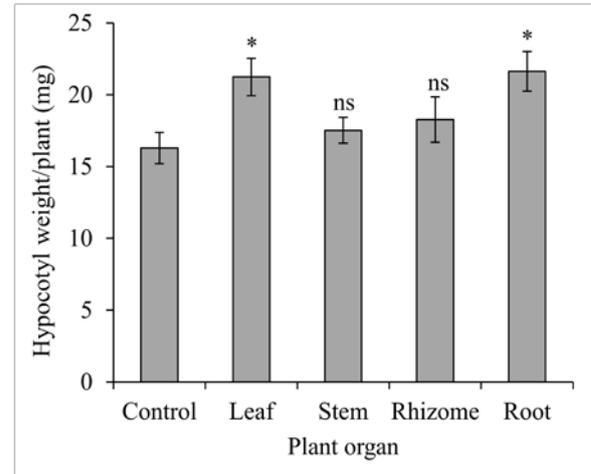


Fig. A2. Effect of fresh plant extracts on hypocotyl a) length (mm) and b) fresh weight (mg) of lettuce seeds. Values are mean \pm standard error ($n = 5$). ***, * and ns indicate significant difference from control at $P \leq .001$, $P \leq .05$ and non-significant respectively after applying the Dunnett test.

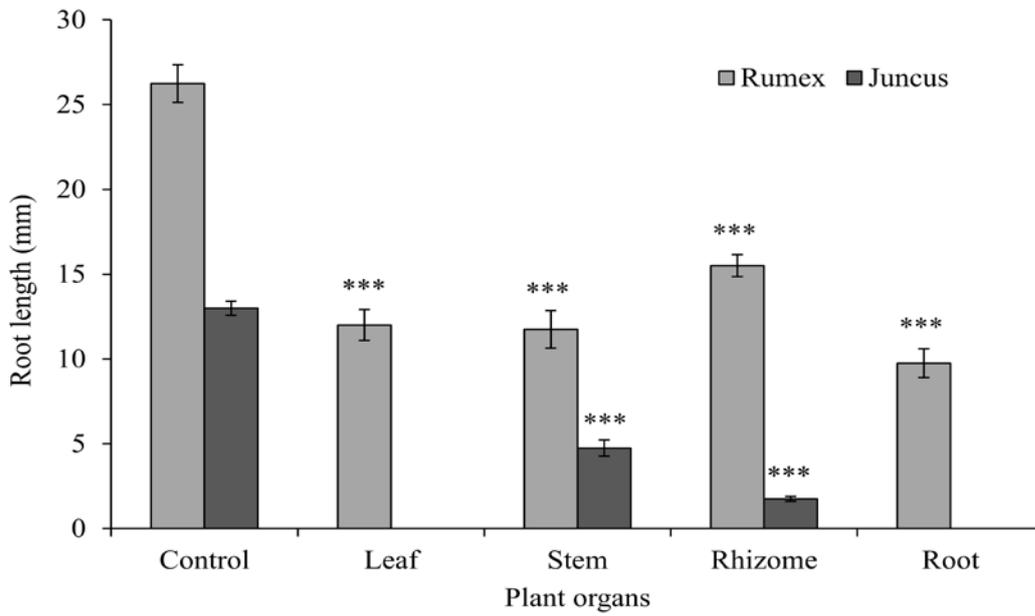


Fig. A3. Effect of dry plant extracts on root length of *Rumex* and *Juncus*. Values are mean \pm standard error ($n = 4$). *** indicate significant difference from control at $P \leq .001$ after applying the Dunnett test.

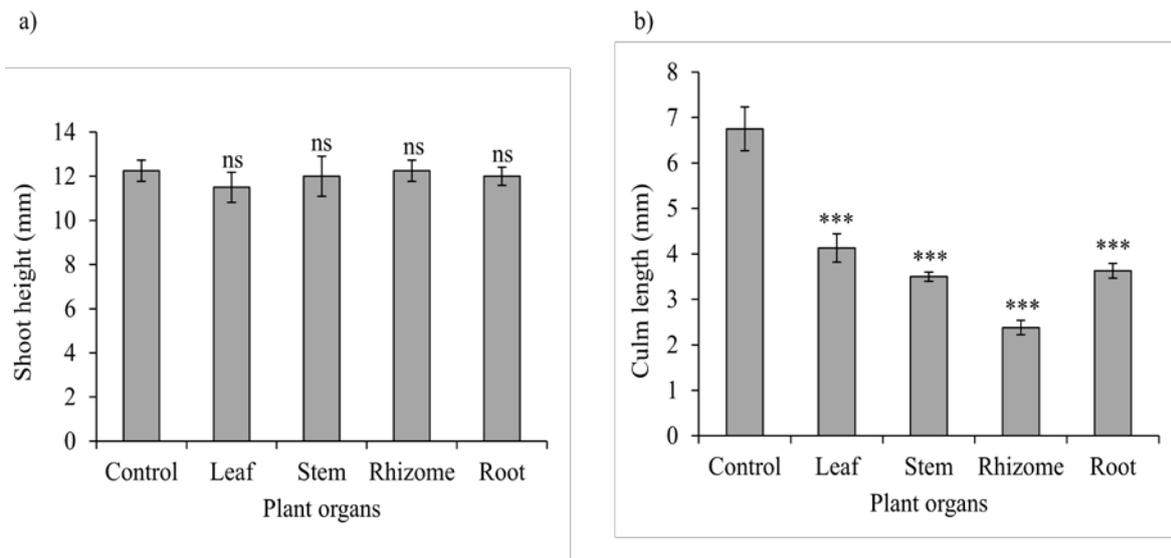


Fig. A4. Effect of dry plant extracts on a) shoot height (mm) of *Rumex* and b) culm length (mm) of *Juncus*. Values are mean \pm standard error ($n = 3$). *** and ns indicate significant difference from control at $P \leq .001$ and non-significant after applying the Dunnett test.

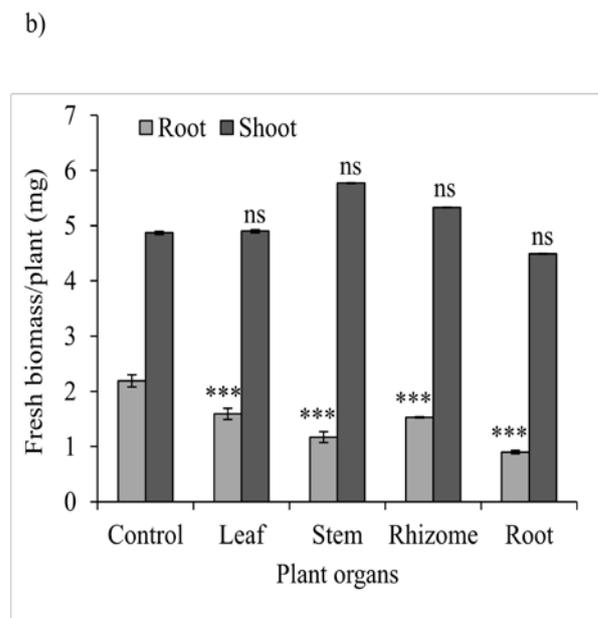
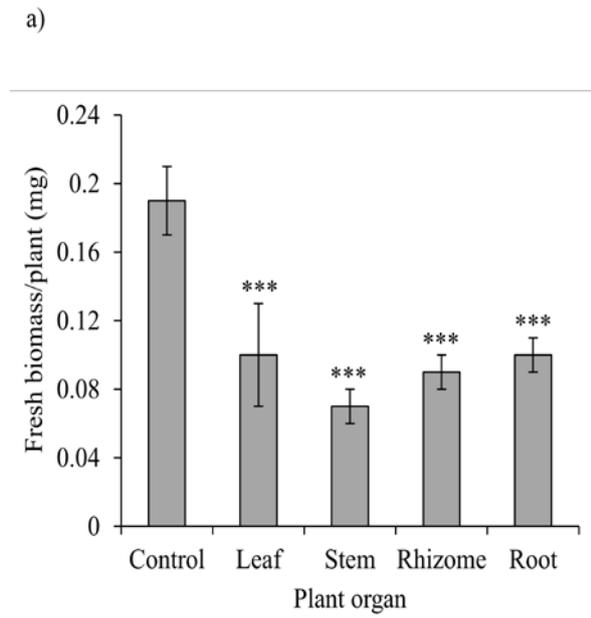
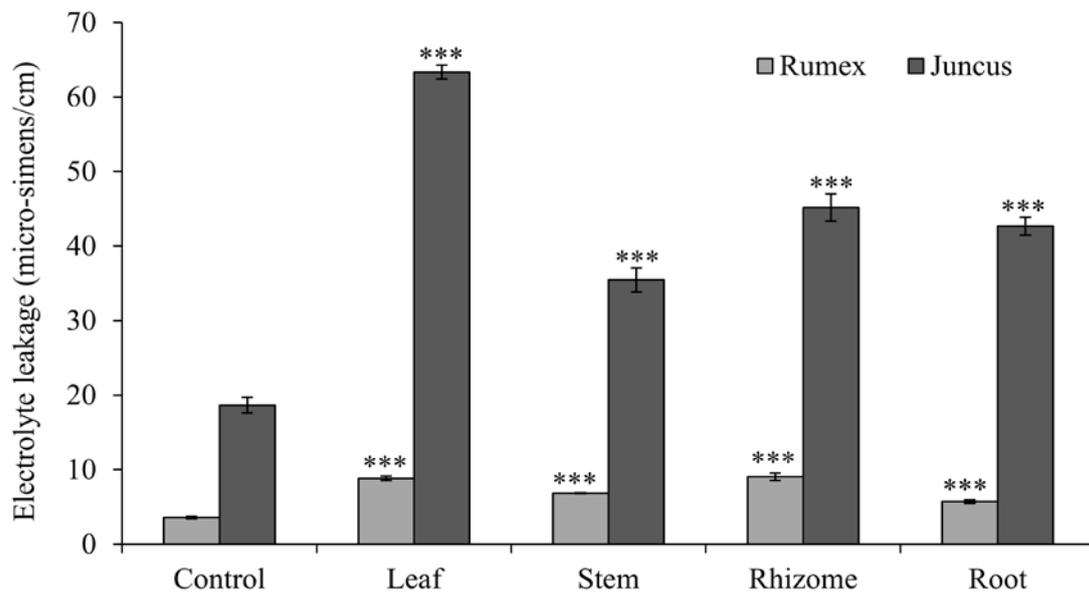
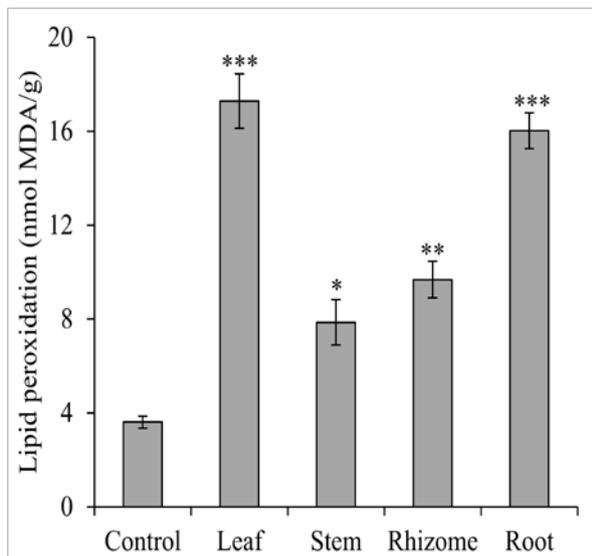


Fig. A5. Effect of dry plant extracts on fresh weight biomass (mg) of a) *Juncus* and b) *Rumex*. Values are mean \pm standard error ($n = 3$). *** and ns indicate significant difference from control at $P \leq .001$ and non-significant after applying the Dunnett test.

a)



b)



c)

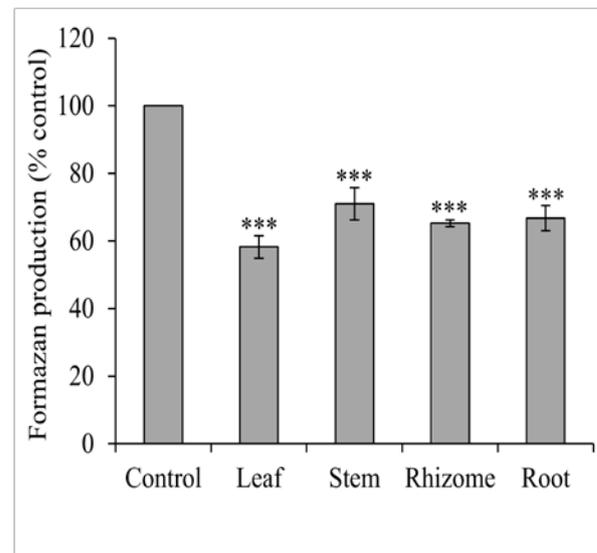


Fig. A6. Effect of dry plant extracts on physiological parameters such as a) electrolyte leakage for *Rumex* and *Juncus*, b) lipid peroxidation and c) formazan production of *Rumex*. Values are mean \pm standard error ($n=3$). ***, ** and * indicate significant difference from control at $P \leq .001$, $P \leq .01$ and $P \leq .05$ respectively after applying the Dunnett test.

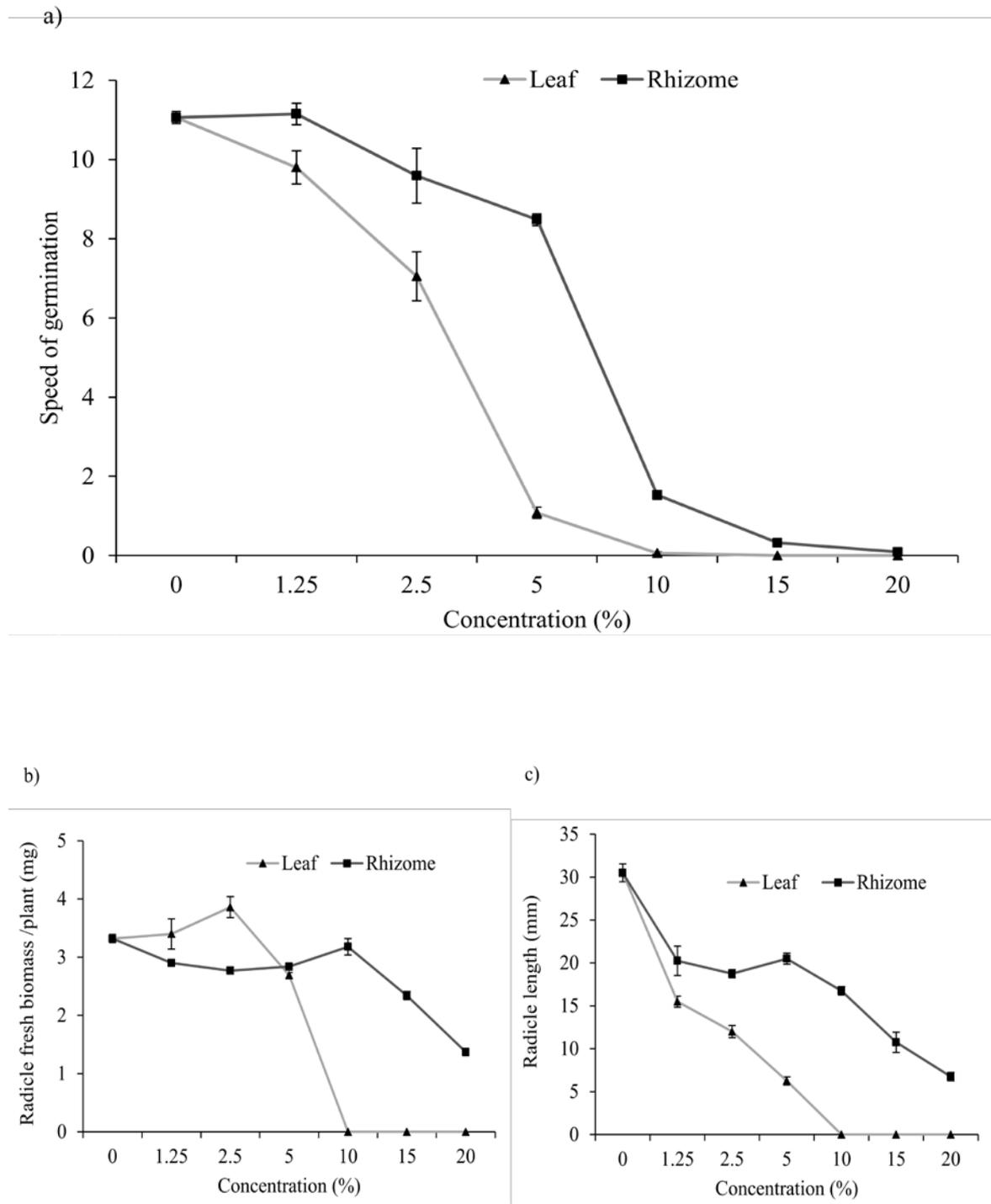


Fig. A7. Effect of different concentrations of leaf and rhizome extracts on a) speed of germination, b) radicle fresh weight (mg) and c) radical length (mm) of lettuce seeds. Values are mean \pm standard error ($n = 4$).

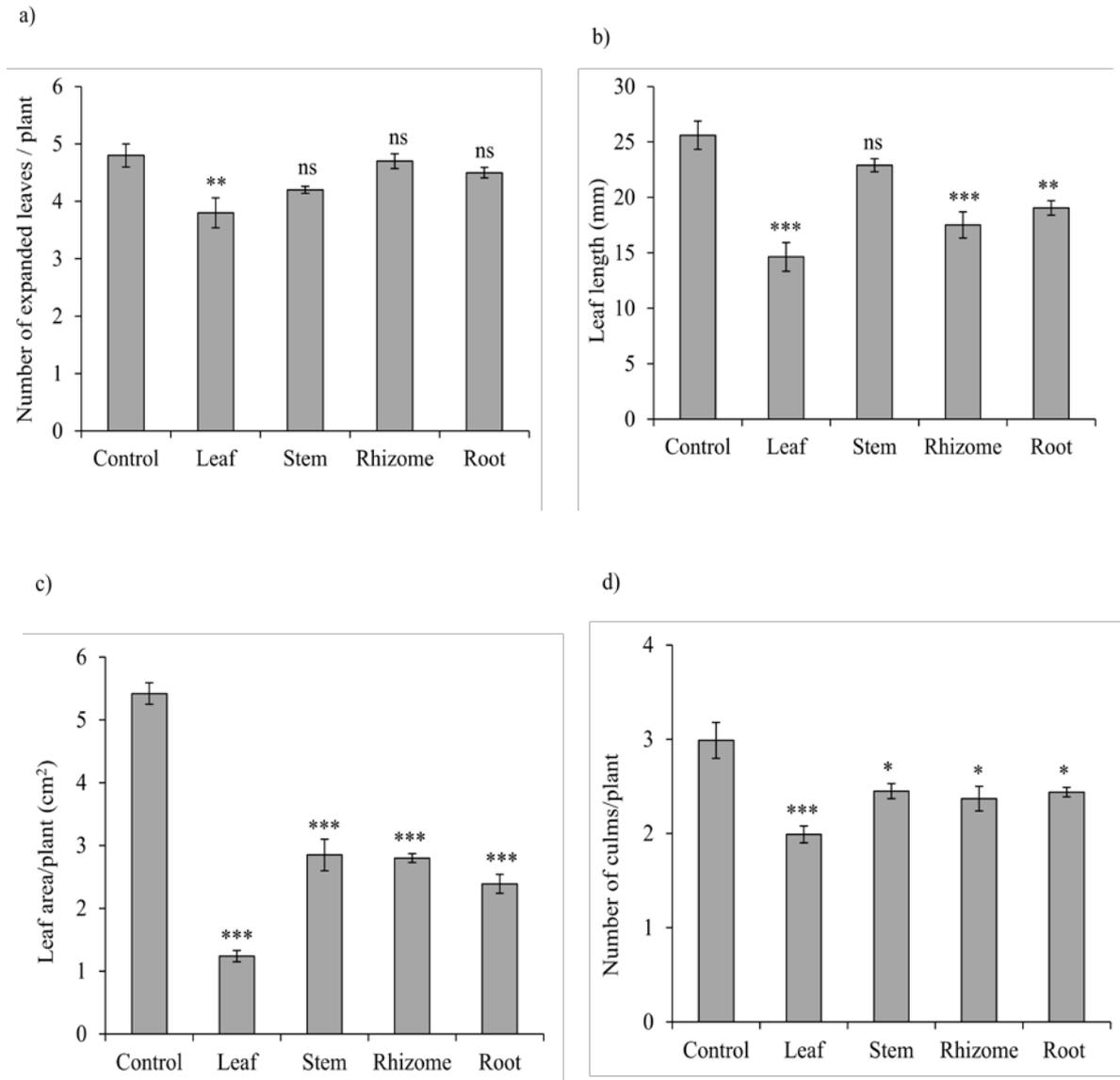


Fig. A8. Effect of *Phragmites* leachate on a) leaf area/plant, b) number of expanded leaves/plant, c) leaf length (mm) of *Rumex* and d) number of culms/plant of *Juncus* in soil system. Values are mean \pm standard error ($n = 3$). ***, **, * and ns indicate significant difference from control at $P \leq .001$, $P \leq .01$, $P \leq .05$ and non-significant after applying the Dunnett test.

Table A1. Effect of dry plant extracts of *Phragmites* on fresh radicle and hypocotyl weight (mg) of lettuce and radish. Values are mean \pm standard error ($n = 6$). ^{***}, ^{**} and ^{ns} indicate significant difference from control at $P \leq .001$, $P \leq .01$ and non-significant respectively after applying the Dunnett test.

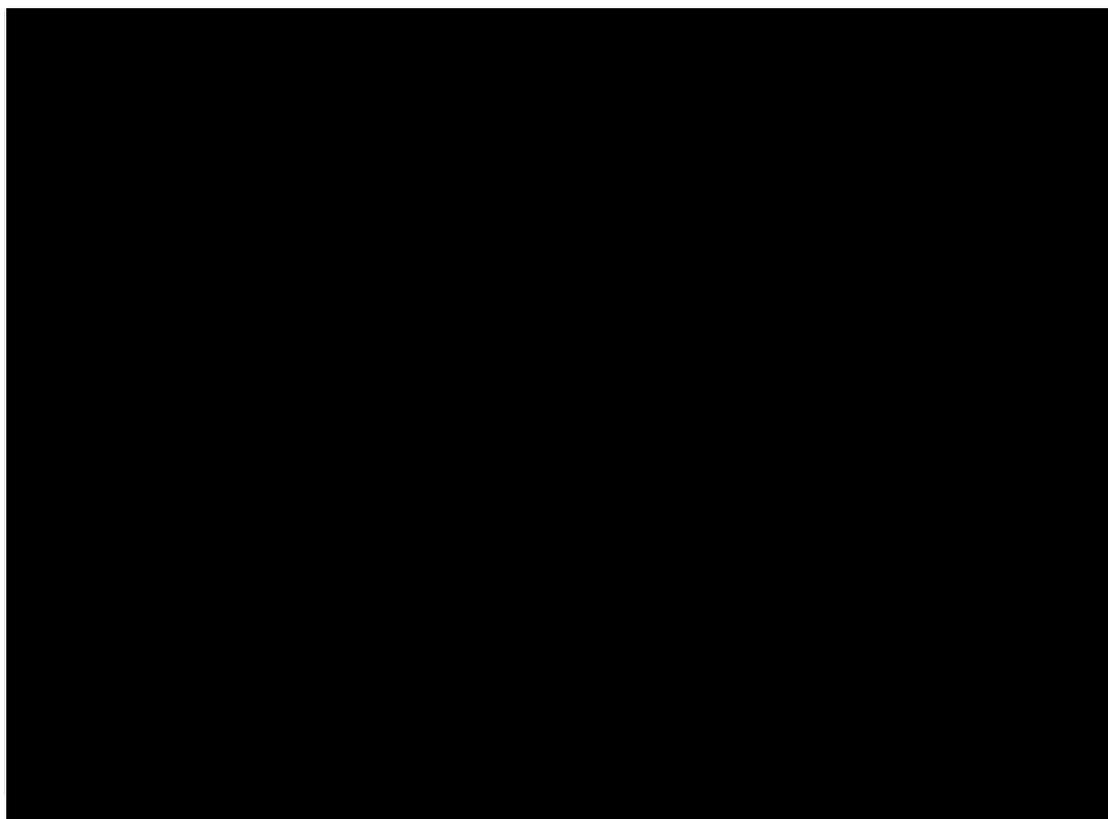
| Treatment | Radicle | | Hypocotyl | | Radicle:Hypocotyl | |
|-----------|-------------------------------|--------------------------------|--------------------------------|----------------------------------|-------------------------------|-------------------------------|
| | Lettuce | Radish | Lettuce | Radish | Lettuce | Radish |
| Control | 2.24 \pm .07 | 29.56 \pm 1.29 | 12.96 \pm .12 | 95.31 \pm 2.64 | .17 \pm .005 | .31 \pm .013 |
| Leaf | 1.05 \pm .09 ^{***} | 23.91 \pm 2.14 ^{ns} | 8.70 \pm .86 ^{***} | 111.08 \pm 2.92 ^{**} | .12 \pm .012 ^{***} | .22 \pm .019 ^{***} |
| Stem | 1.38 \pm .05 ^{***} | 23.94 \pm 2.64 ^{ns} | 15.09 \pm .50 ^{**} | 112.17 \pm 2.73 ^{**} | .09 \pm .004 ^{***} | .21 \pm .020 ^{***} |
| Rhizome | .85 \pm .05 ^{***} | 7.68 \pm .94 ^{***} | 9.92 \pm .80 ^{**} | 66.39 \pm 3.02 ^{***} | .09 \pm .005 ^{***} | .12 \pm .013 ^{***} |
| Root | 1.95 \pm .09 ^{**} | 26.98 \pm 1.50 ^{ns} | 17.76 \pm .36 ^{***} | 115.82 \pm 4.27 ^{***} | .11 \pm .004 ^{***} | .23 \pm .010 ^{**} |

Chapter Three

Phytotoxicity Induced by Phragmites australis: An Assessment of Phenotypic and Physiological Parameters Involved in Germination Process and Growth of Receptor Plant

Introduction

Our study was designed to investigate the phytotoxicity emphasizing physiological effects of aqueous extracts of different organs and root exudates of *P. australis*. In addition, we endeavoured to identify chemical compounds in different organs of *P. australis*.



Published as: Uddin, Md nazim , Robinson, Randall William and Caridi, Domenico (2014) Phytotoxicity induced by Phragmites australis: an assessment of phenotypic and physiological parameters involved in germination process and growth of receptor plant. Journal of Plant Interactions, 9 (1), . pp. 338-353.

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PART B:
**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS
 INCORPORATED IN THESIS BY PUBLICATION**

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Md. Nazim Uddin

Signature: 

Date: 24/07/2014

Paper Title: Phytotoxicity induced by *Phragmites australis*: An assessment of phenotypic and physiological parameters involved in germination process and growth of receptor plant

In the case of the above publication, the following authors contributed to the work as follows:

| Name | Contribution % | Nature of contribution |
|---------------------|----------------|--|
| Md. Nazim Uddin | 80 | Concept development; conducting experiments, physiological parameter analysis, and HPLC analysis; data collection, statistical analysis and interpretation; and manuscript writing, editing and submitting for publication |
| Randall W. Robinson | 17 | Concept development and manuscript editing |
| Domenico Caridi | 3 | Manuscript editing |

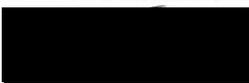
DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
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Phytotoxicity induced by *Phragmites australis*: An assessment of phenotypic and physiological parameters involved in germination process and growth of receptor plant

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This study investigated the possible phytotoxicity induced by *Phragmites australis* on phenotypic and physiological parameters of recipient plants with identification of major inhibitor in donor plant. This was achieved using aqueous extracts of different organs and root exudates of *P. australis* in laboratory and greenhouse experiments with *Lactuca sativa* as the model test plant. The observed reduced liquid imbibition and altered resource mobilization in seeds of *L. sativa*, in particular an insufficient carbohydrate supply, demonstrated that the onset of germination might be negatively affected by phytotoxicity. Dose-response studies pointed out that oxidative stress through reactive oxygen species production could potentially cause the observed germination and seedling growth reductions. The osmotic effects by mannitol solution on germination as well as growth and physiology at a level of - 0.57 and - 0.45 bar respectively demonstrated that the results from aqueous plant extracts were partially induced by osmotic potential on and above those levels. Overall, relative strength of inhibition on measured parameters was the highest in leaf extract, followed by rhizome,

root, stem and inflorescence. Root exudates of *P. australis* had also negative impacts by reducing germination and growth of plant. High-performance liquid chromatograph (HPLC) analysis revealed gallic acid, a potent phytotoxin, as a major compound with an order of leaf > inflorescence > rhizome > root > stem.

Keywords: Activated carbon; gallic acid; oxidative damage; *Phragmites australis*; physiological processes; phytotoxicity

Introduction

Ecological disruption due to the invasion of plants is of great concern to conservationists and land managers worldwide. Many studies have been conducted to identify the mechanisms of plant invasiveness such as life history characteristics, physiological properties, changes in genetics, resource competition and in more recent years allelopathy (Pisula and Meiners 2010). Allelopathic potential is an important attribute to the success of an invasive species in natural ecosystems, particularly when the species produces novel biochemical weapons (Callaway and Ridenour 2004). Interactions associated with allelochemicals produced by invasive or native plant species have the potential to impact seed germination, seedling growth, development and establishment of neighbouring plant species, as well as of the same species, in both natural and agricultural systems (Dorning and Cipollini 2006, Lara-Nuñez et al. 2006).

Most allelopathy studies are limited to seed germination and growth interference bioassays using aqueous extracts of invasive plants in the laboratory with very few of these studies carried out under more natural conditions (Inderjit et al. 2001, Rice 1984). In field conditions, several mechanisms of interference might be operative in sequence and simultaneously so it makes it difficult to separate the general interference effects from those of allelopathy. Furthermore, various authors speculate that seed germination may not be the primary site for allelopathic interactions (Lorenzo et al. 2010, Stowe

1979). Relatively, little is known about the physiological and biochemical effects induced by allelochemical phytotoxicity in seed germination and seedling growth (Lorenzo et al. 2011, Weir et al. 2004) though that contributes fitness and survival of plants in competition with other species. In particular, phytotoxicity studies examining seedling growth response with physiological effects have been identified as useful in understanding the mechanisms of actions (Inderjit and Dakshini 1995).

Phragmites australis, a ubiquitous wetland plant, has been considered one of the most allelopathic and invasive species in the world (Uddin et al. 2012). However, the origin of the species is still unclear (Plut et al. 2011). It is a perennial graminaceous plant, to 3 m tall, which reproduces mainly through rhizomes and, at low frequency, through seeds. It grows in all temperate zones of the world, especially North America, most of the countries in Europe, some part of Canada and Australia (Hocking et al. 1983, Kulmatiski et al. 2011), being especially common in south-eastern Australia (Morris et al. 2008). The distribution and abundance of *P. australis* has expanded over the last 150 years and in most areas forms dense monocultures (Saltonstall et al. 2005). Due to impacts of *P. australis* invasions, habitats have been diminished or altered significantly for other flora and fauna causing loss of biodiversity and ecosystem functions (Mack et al. 2000). Several studies have identified chemicals within *P. australis* organs which have antialgal, antifungal or antibacterial effects (Li and Hu 2005). Some chemicals produced by decomposition of belowground organs of *P. australis* may be responsible for die-back of *P. australis* itself (Armstrong and Armstrong 1999) and photo-degradation of secreted phytotoxins by *P. australis* execute severe phytotoxicity to other plant species (Rudrappa et al. 2009). Previous studies have shown that water extracts of *P. australis* organs have strong phytotoxic effects on germination and growth of other plant species (Rudrappa et al. 2007; Uddin

et al. 2012). Identified phenolics such as gallic acid in *P. australis* root exudates showed inhibitory effects on germination and growth of various seeds (Rudrappa et al. 2007, Uddin, Caridi and Robinson 2012) but the physiological and biochemical effects of the extracts have been lacking. Generally, phytotoxic activity in nature depends on the release of a mixture of phytotoxins and joint effects of them rather than an individual chemical (Inderjit and Duke 2003, Reigosa et al. 1999). Hence, plant extracts with phytotoxins showing synergistic, additive or antagonistic mixture effects were shown to have higher and longer lasting effects on target species than individual chemical (Inderjit et al. 2005, Koul and Walia 2009).

It is known that the standard germination process consists of several key phases that include imbibition, catabolism, anabolism, and finally radicle protrusion. Undisturbed reserve mobilization is an important requirement for germination during the catabolic process just after liquid imbibition (Kupidłowska et al. 2006). Rudrappa et al. (2007) found that root secreted allelochemicals from *P. australis* deployed a reactive oxygen species (ROS) that is responsible for stress conditions on the affected plant. However, most of the studies focused on individual organ that makes them difficult and assessment of phytotoxicity of that relative to other organs. Against this background, our study was designed to investigate the possible phytotoxicity emphasizing physiological effects of aqueous extracts of different organs of *P. australis* to find out the relative importance of phytotoxicity and root exudates of *P. australis* on the germination processes and subsequent growth of plants. In particular, we investigated phytotoxic effects of *P. australis* on seed metabolic activities namely liquid imbibition and resource mobilization as well as induction of oxidative stress by ROS production and their consequences on seedlings. In addition, we identified chemical compound in different organs of *P. australis*.

Materials and methods

Plant materials and extraction

Whole, mature plants of *P. australis* were collected in January 2011 from natural stands adjacent to Cherry lake (37° 51' 30"S, 144° 50' 5"E), a coastal wetland in Altona, Melbourne, Australia. Individual organs (leaf, stem, rhizome and root) and inflorescences (collected later in April 2011) were dried at 40°C in an oven until the weight was constant. Oven-dried plant samples were ground and sieved (0.5 mm) for experiments. To formulate extracts, 20 g dry ground material of each organ was submerged in 100 mL distilled water (20 % extract) and agitated for 24 h on an orbital shaker (Orbital Mixer, EOM5, Ratek Instruments Pty. Ltd, Vic-3155, Australia) at room temperature. The extracts were filtered through cheese-cloth, centrifuged at 10,000 rpm for 10 min (Beckman Avanti 30 High Speed Compact Centrifuge, 364105, Beckman Coulter Inc., USA) and sterilized (microfiltration with 0.22 µm pore filter). The treatment with extracts rather than individual chemical in our experiments was due to satisfactory level of efficacy and become more ecologically realistic. pH of the extracts was determined with a pH meter (Pocket digital pH meter, 99559, Dick-Smith electronics, Australia) and osmotic potential (OP) was calculated in bar using the equation ($OP = \text{Electrical Conductivity} * - 0.36$) according to McIntyre (1980). During experiment set-up, the 20 % base concentration was diluted with distilled water to obtain concentrations of 15, 10, 5, 2.5, and 1.25 %. All extracts were buffered with 0.01M sodium phosphate buffer and pH values of the extracts including distilled water (control) were adjusted to 6.5 with 1N NaOH and 1N HCl. We used a wide range of doses to ensure that they would encompass the lowest dose for an observable effect, as well as the highest dose for maximal effect for all organs (Belz et al. 2007). The OP of all extract concentrations in different organs was in the range of - 0.048 to - 1.27 bar. In

bioassays such as that carried out here, OPs in this range could affect germination and growth (Laterra and Bazzalo 1999), so we also evaluated the OPs effects on the above mentioned parameters of the test species .

Choice of target species

Lactuca sativa var. all the year round (lettuce), commercially available, was used because of its sensitivity and common use in phytochemical bioassays. Lettuce is commonly used in studies aimed to find out the physiological effects of the plant extracts. The use of known susceptible species is useful for screening and being a 'known' entity allows for easy comparison to other studies. In addition, lettuce is easily grown, minimizing the risk of observed growth differences being due to factors other than the treatments applied in the experiments. Though our previous studies showed phytotoxic effects of *P. australis* on associated species such as *Rumex conglomeratus* and *Juncus pallidus*, it also showed high variability among the measured parameters. Associated species may not be a reliable first line test species in phytotoxicity studies due to the inherent genetic variability of wild populations.

Laboratory experiments

Effects of extracts on seed imbibition

100 mg of seeds were soaked in aqueous extracts of each organ at 5% concentration (OP ranged from - 0.18 to - 0.35 bar) or in distilled water (control) and the increase in fresh weight was determined at 8, 16, 24 and 32 h of imbibition. Treatments were arranged in a complete randomized design (CRD) with three replicates at 25°C in the dark in a growth chamber (Westinghouse, Electrolux home products, Australia).

Effects of extracts on seed reserve mobilization

100 mg of seeds were soaked in either aqueous extracts of each organ at 5 % concentration or in distilled water (control) and maintained at 25°C in the dark in a growth chamber with three replicates. Seeds after imbibition of 8 and 32 h were collected and immediately processed for carbohydrate analysis as a measurement of reserve mobilization.

Effects of extracts on germination and seedling growth

Dose-response studies were conducted using seven extract concentrations (0, 1.25, 2.5, 5.0, 10, 15 and 20 %) of each organ in Petri dishes (Techno-Plas, Australia). Each organ extract (5 mL) was placed into a sterile 9 cm Petri dish containing two sterile sheets of filter paper (Whatman No. 1). At least, three replicates [each having 25 surface sterilized seeds with 1.5% (v/v) sodium hypochlorite for 1 min and subsequent washed] were used for each treatment. Petri dishes were sealed with parafilm (Pechiney, Plastic Packaging Company, Menasha, WI 54952) then placed in polyethylene bags to prevent water loss by evaporation and to avoid contamination by fungi and bacteria. The prepared dishes were arranged in a Completely Randomized Design (CRD) and placed in a growth chamber set to 25°C in dark condition. Germination was deemed to have occurred when the radicle protruded beyond the seed coat by at least 1 mm. Germinated seeds in all Petri dishes were counted daily at noon for 8 days, the point where cumulative germination levelled off in all treatments. Germination indices (total germination, speed of germination, coefficient of the rate of germination, speed of accumulated germination), physiological and biochemical parameters [hydrogen peroxide, electrolyte leakage, lipid peroxidation (LP) and dehydrogenase enzyme activity] and biometric characteristics (length and weight of radicle and hypocotyl) were measured at the end of the experiment.

Effects of osmotic potential on germination and growth

Thirteen concentrations of mannitol were evaluated with the corresponding OP: 0, - 0.11, - 0.17, - 0.22, - 0.27, - 0.37, - 0.45, - 0.57, - 0.67, - 0.77, - 0.87, - 0.97 and - 1.30 bar. Mannitol was used due to its inert osmotic potential (Wardle et al. 1992). 5 mL of each solution was added to the Petri dishes and followed the same procedures for germination study as well as measurement of biometric and physiological parameters as mentioned previously.

Greenhouse experiments

Spring buds of *P. australis* with rhizome attached were collected in September 2011 from Cherry Lake (see above). Each living rhizome was cut to contain exactly one active node. Rhizomes were then replanted as soon as possible (within 6 h) in 7 L plastic pots lined with plastic watertight bags filled with 4 L potting mix. Sixteen replicates were used, half of which contained activated carbon (AC) (40 mL/L substrate) to reduce the allelopathic effect as AC has a high affinity to organic compounds (Hille and Den Ouden 2005). To all of the treatments 1 g/L of mixed pelletised fertilizer (Pivot fertilizer-900; N-P-K: 16-8-9) was added bimonthly. Pots were kept in a natural lit greenhouse at $23 \pm 3^{\circ}\text{C}$ and $12 \pm 2^{\circ}\text{C}$ day/night temperature as well as watered regularly with an auto irrigation system to keep soil moist at a level of $55 \pm 5\%$. The lettuce seeds (50) were sown in each pot during the vegetative stage of *P. australis*. After 10 days, plants were counted and harvested for biometric (root-shoot length and weight) and physiological parameters (electrolyte leakage and chlorophyll) measurement. A separate experiment was conducted to test the direct effect of activated carbon only on rhizomes of *P. australis* (one rhizome per pot) and lettuce (fifty seeds per pot) There were five replicates per treatment (with and without carbon) for a total of 20 pots.

Allelochemicals identification with HPLC

400 mg of dried and ground material of each organ passing through 0.5 mm was placed in a 15 ml eppendorf tube and extracted with 10 ml 50% acidified methanol (HCl was added to obtain a final concentration of 1.2 M). Samples were homogenized using roller mixer in cold room at 4⁰C for 24 h. Then, centrifuged at 10,000 rpm for 10 min at 4⁰C and the supernatants were collected. The extracts were filtered through a 0.2 µm Phenomenex RC membrane and then analyzed by HPLC. All standards (arbutin, gallic acid, 5-hydroxymethyl furfural, catechin, gentistic acid, chlorogenic acid, epicatechin, coumaric acid, ferulic acid, rutin, and phloridzin) and solvents were purchased from Sigma-Aldrich, Australia. The standard stock solutions were prepared by dissolving standards in milli-Q water to 100 µg/mL. For the calibration curves, four additional concentrations (20, 40, 60, and 80 µg/ mL) were prepared by the dilution of the stock solutions with water. A HPLC system (Shimadzu, Tokyo, Japan) equipped with a Kinetex PEP 5 µm 100 Å LC Column 250 x 4.6 mm was used. The mobile phase consisted of 0.1 % phosphoric acid in water (mobile phase A) and 0.1% phosphoric acid in acetonitrile (mobile phase B) at flow rate 1 mL/min with a gradient elution program and a 65 min run time. The gradient elution profile was: 0 to 30 min 90:10 (A:B), 30.01 to 40 min with linear decrease of A and increase of B to 50:50 (A:B), 40.01 to 45 min 10:90 (A:B), 45 to 55 with linear increase of A and decrease of B to 50:50 (A:B) and then back to initial condition. There was a 5 min equilibration time between runs to allow the operating system to stabilize. The injection volume was 20 µL and the temperature was ambient. The analysis was monitored with a photodiode array (PDA) detector @ 220 and 271 nm. All the chemical analyses were done in three replicates and the results were calculated using standard curve. Confirmation of gallic acid was made by retention time as well as spiking the extracts with the standard of gallic acid in

different concentrations on the basis of extracts peak absorbance. In addition, ultraviolet (UV) spectrums of the unknown peak of the extracts were compared to that of gallic acid as well.

Measurement of physiological and biochemical parameters

Carbohydrate concentration

The total amount of all sugars [total non-structural carbohydrate (TNC), water soluble carbohydrate (WSC)] was measured using a phenol sulphuric acid method modified from Kabeya et al. (2003). Sulphuric acid (0.4 N) and water extractions were made for TNC and WSC determination respectively. Each sample (~6 mg) was placed in a 100 ml round-bottom flask with 50 ml acid for TNC and water for WSC and then refluxed for 1 h in a boiling water bath. The hot solution was filtered through Whatman No. 42 filter paper. The carbohydrate content of the cooled filtrate was determined spectrophotometrically at 485 nm using the above mentioned method and glucose solution as a calibration standard at a concentration of 0 to 50 mg/L.

Hydrogen peroxide (H₂O₂) content

H₂O₂ levels were measured according to Velikova et al.(2000). Plantlets (100 mg) were homogenized with 5 mL of 0.1 % trichloroacetic acid (TCA) and centrifuged at 12000 rpm for 15 min. Then 0.5 mL supernatant was mixed with 0.5 mL of a 10 mM phosphate buffer (pH 7.0) and 1 mL of 1M potassium iodide (KI). The absorbance was measured at 390 nm and the amount of H₂O₂ was calculated using the extinction coefficient 0.28 $\mu\text{M}^{-1} \text{cm}^{-1}$ (Singh et al. 2006).

Electrolyte leakage

100 mg of germinated seeds (control and treatment) were placed in 15 cm³ distilled water at room temperature in darkness. Electrical conductivity (EC) in the medium was

measured with an EC meter after 2 h of incubation. Results were expressed as % of total leakage from seedlings boiled for 20 min (Bogatek et al. 2006).

Lipid peroxidation

Lipid peroxidation (LP) was measured in terms of malondialdehyde (MDA) content. MDA level was used as an index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight (f. wt) (Jambunathan 2010). About 200 mg of control and treated plantlets were homogenized with 4 mL of 0.1 % TCA, and then centrifuged at 15,000 rpm for 15 min. 1 mL supernatant was mixed with 2 mL of 20 % TCA and 2 mL of 0.5 % thiobarbituric acid (TBA). The mixture was heated at 95°C in a fume hood and later cooled with running tap water. The absorbance was read at 532 and 600 nm using a spectrophotometer. A_{600} is the non-specific absorbance and subtracted from the values for A_{532} . The MDA amount was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Dehydrogenase enzyme activity

Lettuce plantlets (100 mg fresh weight) were washed, blotter-dried, and soaked in 5 mL of 2 g L^{-1} 2,3,5- triphenyl tetrazolium chloride (TTC) at 37°C for 4 h in the dark. Thereafter, 0.5 mL of 1 M sulphuric acid was added to stop the reaction. The plantlets were removed, washed with distilled water, blotter dried, and ground by mortar and pestle with 7 mL ethyl acetate. The extract was centrifuged at 20,000 rpm for 15 min (Sengar and Srivastava 1995). The resultant volume was increased to 7 mL with ethyl acetate due to loss by evaporation, and the absorbance was read at 485 nm with a spectrophotometer to measure formazan production as a measurement of dehydrogenase enzyme activity comparing with percentage of control.

Chlorophyll

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll were determined by placing about 15 mg of fresh leaf in 7 ml of N-N dimethylformamide (DMF) for 24 h in the dark at 4°C for extraction (Moran and Porath 1980). The absorbance of the extracts was measured spectrophotometrically at 664 and 647 nm and chlorophyll was determined following the equation proposed by Inskeep and Bloom (Inskeep and Bloom 1985).

Statistical analysis

All the experiments were conducted in a completely randomized design with at least three replicates. Data were analysed by one way ANOVA using IBM SPSS statistics 20.0. Variance homogeneity was tested using Levenes's test and data were arcsine-square root transformed only for percentage of germination and natural log for carbohydrate using SPSS to compensate for variation within population. Significance tests were performed after applying 2 sided Dunnett test at the 0.05 probability level. Furthermore, results were subjected to linear regression analysis and the correlation coefficient values, evaluating the dependence of each measured variables on the concentration of the extracts, were calculated using Microsoft Excel 2010 according to Singh et al. (2002). Coefficient values from regressions also were used to compare the relative strength of plant extracts on measured parameters (Pisula and Meiners 2010). ANOVA table of regression analysis gives the F statistic for testing the claim of whether a significant relationship exists. LC₅₀ was measured by graphical estimation as the concentration of the extracts causing 50% mortality of the test seeds. An attempt has been made to differentiate the phytotoxic effects from osmotic effects by plant extracts using osmotically adjusted control values in developing calibration curves containing

identical osmotic potential to the plant extracts used in this study. After that the various adjustments were made, and t-tests were used to perform the analysis.

Results

Seed imbibition

Water uptake by seeds of lettuce during the first 8 h after treatment was rapid in all extracts but no significant variations were observed at 8 and 16 h (Fig. 1). After 8 h, rate of imbibition was slowed and control seeds gained more than 100 % weight, this figure being lower in all extracts. By 24 h, leaf and rhizome extracts showed a significant difference compared to control. A more significant effect was observed at 32 h when leaf, rhizome, and root extracts showed a reduced imbibition compared to the control by 10, 9 and 6 % respectively, while there was no significant effect observed in inflorescence and stem extracts (Fig. 1).

Carbohydrate metabolism

Leaf and rhizome extracts significantly affected the total carbohydrates both after 8 and 32 h, while the other organ extracts affected carbohydrate metabolism only at 32 h (Fig. 2). The extracts had profound effects on preventing the loss of TNC and the build-up of WSC. After 32 h, 31 % of TNC had been metabolized in the control whereas leaf and rhizome extracts treatments showed 6 and 3-fold lower carbohydrate metabolization respectively. Leaf and rhizome extracts also significantly increased WSC by 23 % compared to the control, while the increase was only 8 % both in leaf and rhizome treatments.

Germination, growth and physiology

The dose and the nature of different extracts of *P. australis* significantly affected germination, growth and bio-physiological parameters of lettuce seeds (Table 1). The

ANOVA table shows that the effect of each organ extract on each measured parameters significantly differed among concentrations. Furthermore, different extract concentrations had differential effects on the germination and growth of lettuce seeds (Fig. 3). The LC₅₀ concentrations for germination of lettuce seeds were 4.7, 8.5 and 11.3 % for leaf, root and rhizome extracts respectively (Fig. 3a). In the case of inflorescence and stem extracts, the response in germination was insufficient to estimate LC₅₀ values. In the leaf extract, no germination occurred at 15 and 20 % concentration, whereas other extracts showed higher germination percentages at these concentrations. The percentage germination of lettuce seeds was reduced at all concentrations except for the inflorescence extract. Decreased germination was coupled with increasing concentration, exhibiting a strong inverse relationship (Fig. 3a). The inhibition threshold varied among organs but was less than 1.25 % for leaf, rhizome and root extracts compared to germination percentage of control. In addition, speed of germination was inversely related to all extract concentrations (Fig. 3b). Leaf extract significantly reduced the speed of germination, ranging from 0.06 at 10 % to 12.5 at control followed by rhizome, root, and stem extracts and finally the inflorescence extract. The subsequent growth of seedlings, measured in terms of radicle and hypocotyl length and weight, was significantly reduced at every concentration of leaf and rhizome extracts whereas other organ extracts showed contrasting effects (Fig. 3c and Table 1). In general, the inhibitory effect was greater on radicle growth (Fig. 3c) than on hypocotyl growth (data not shown). Radicle length was reduced by 49 and 34 % in response to the lowest concentration (1.25%) of leaf and rhizome extracts, respectively.

The H₂O₂ content of plantlets increased in response to aqueous extracts of *P. australis* compared to the control and the increase was concentration dependent (Fig. 4a). A significant effect was observed in the leaf extract, followed by rhizome, root,

inflorescence and stem extracts. The increase was significant even at 1.25 % concentration of the leaf extract or higher for other organs extracts. At 10 % concentration of leaf and rhizome extract, there was nearly a 10 and 6-fold increase in H₂O₂ content compared to the control respectively. The dehydrogenase activity in treated plantlets was investigated using TTC reduction measurement. Lettuce plantlets treated with different extracts had significantly reduced TTC to formazan compared to the control. Both leaf and root extracts decreased the dehydrogenase enzyme activity even at 1.25 % concentration and all extracts significantly reduced enzyme activity at 2.5 % or higher (Fig. 4b). The highest inhibition (80 %) was observed in the 10 % leaf extract followed by rhizome (76 %) and root (69 %) extracts. There was an inverse correlation between H₂O₂ production and enzyme activity ($r = - 0.69$). There was distinct decline in dehydrogenase enzyme activity with increasing concentration of *P. australis* extracts; in particular, the leaf extract exhibited a higher inhibition than extracts from other organs.

All aqueous extracts caused significant cell membrane injury measured by ion leakage from plantlets of lettuce as indicated by accumulative electrical conductivity in the bathing solution with increasing concentration (Fig. 5a). The root extract treatments showed highest ion leakage (23 %) followed by leaf (16 %) and rhizome (14 %) extracts at 10 % concentration. The aqueous extracts significantly increased the MDA content in plantlets of lettuce with increasing concentration with the exception of the 1.25 % concentration ($P < .05$) (Fig. 5b). MDA was most abundant in plantlets exposed to leaf extracts (4-fold) compared to the control at 10 % concentration followed by rhizome, root, stem and inflorescence extracts. Increased MDA contents corresponded to the LP that caused membrane damage. There was also a strong positive correlation between ROS as H₂O₂ and LP ($r = 0.88$). In the leaf extract, the rise of MDA content with

increasing concentration was observed to be steep. Enhanced LP and electrolyte leakage resulting in loss of membrane integrity were among the key factors to determine cellular injury. LP was related to decreasing membrane permeability and our results showed a strong positive correlation between LP and cell membrane injury ($r = 0.62$). In some cases, data were not available for high concentration of leaf, rhizome and root extracts due to no germination and small amount of plant sample.

OP effects

The OP of all organ extracts ranged from - 0.05 to - 1.27 bar at concentration of 1.25 to 20% with highest values in root and lowest values in inflorescence extracts (Table. S1¹). There were significant differences in OP among plant organs for the same extract concentration. Among those, the extracts at 10% had an OP lower than - 0.506 bar except leaf (- 0.62 bar) and root (- 0.63 bar) extracts. Our experimental results showed that there was no significant effect on germination percentage until - 0.45 bar but effects existed on seedling development at - 0.45 bar and plant physiology at - 0.27 bar and afterwards (Table 2). OPs affected growth and physiology more than germination. Additional increase of OP from - 0.77 to - 1.30 bar inhibited germination significantly and even no germination (Table 2). On comparing these results with the results of aqueous plant extracts it may be seen that the observed inhibition of leaf and rhizome extracts was more severe than the osmotic effects alone, and therefore the inhibitory effects were most likely due to phytotoxins present in the extracts (Table. 3). In addition, the inhibition of germination and growth in some cases, such as inflorescence, stem and root extracts, might have been the results only for OPs but those extracts had some physiological effects through phytotoxicity (Table. 3).

Root exudates-mediated effects in greenhouse

P. australis root exudates had strong negative effects on germination, growth and physiological parameters of lettuce in the greenhouse (Fig. 6 and Fig. 7). When grown with *P. australis*, germination percentage and root length and weight were inhibited by 30, 39 and 82 % respectively (Fig. 6). Activated carbon treatment significantly increased germination, root length and total biomass although this was not the case for all parameters measured, i.e. shoot length, electrolyte leakage and total chlorophyll (Fig. 6 and 7). There was a significant negative effect on membrane integrity of lettuce roots grown with *P. australis* but no significant effect on total chlorophyll (Fig. 7). The overall effect of activated carbon on lettuce in competition with *P. australis* was positive but all measurements were not significant (Fig. 6 and 7). Activated carbon had no significant direct effect on total biomass of *P. australis* and lettuce when they were grown alone (all $P > 0.5$).

Allelochemicals identification

HPLC analysis revealed the presence of gallic acid with a detectable level among 11 polyphenolics in all organs of *P. australis*. The HPLC chromatograms of all standards and extracts have been evaluated to confirm the presence of gallic acid on the basis of retention time, spiking and UV spectrum (Figures S1- S3¹). The concentration in dry weight (DW) basis was maximum in leaf extracts (0.441 μ g/mg) followed by inflorescence (0.075 μ g/mg), rhizome (0.041 μ g/mg), root (0.039 μ g/mg) and minimum in stem (0.029 μ g/mg). The spiked chromatogram of gallic acid with all extracts confirmed the evidence of the compound (Figs. 1 & 2, available as Supplementary Material). Some observed peaks before and after gallic acid might be some unidentified compounds from organs and involved in combined phytotoxic activity.

Discussion

The observed seed imbibition results suggest that phenolics concentration may play an important role in water uptake as leaf, rhizome and root extracts of *P. australis* contain higher amounts of total phenolics and specific chemical, gallic acid than stem and inflorescence extracts (Uddin, Caridi and Robinson 2012). Some studies report that extracts of alfalfa, kudzu and black mustard had inhibitory effects on seed imbibition (Chon et al. 2004, Rashid et al. 2010a, Tawaha and Turk 2003). Seed imbibition, the first phase of germination, is an important step to germination and if water uptake does not reach a critical level, seeds will not germinate. Delays in reaching this critical moisture content and subsequent transition to the second phase of germination can significantly disrupt metabolism and initiation of germination (Bewley 1997). Decreased carbohydrate metabolism in the second phase of the germination process may affect plants growing under stress conditions and specifically may affect total carbohydrate degradation and soluble sugar accumulation (Dubey and Singh 1999). The results obtained from our studies on reserve mobilization are in close agreement with the findings of Lara-Núñez *et. al.* (2009) showing that lechate of *Sicyos deppei* caused delay of starch degradation and sucrose hydrolysis which is related to the mobilization of reserve carbohydrates. Thus, the phytotoxic effects on carbohydrate mobilization might negatively influence cellular respiration (Kraus and Lambers 2001) and therefore the overall germination process (Fritz and Braun 2006).

Dose-response relationships are the most effective way to study the potential effects of phytotoxins (Belz, Velini and Duke 2007). In general, a gradual decline was observed in germination, radicle length and weight with increasing concentration, indicating dose-response effects. Our observations showed that the LC₅₀ concentration of the leaf extract was lower than for the other organs extracts indicating that leaf

contributes strong phytotoxicity than others. LC_{50} , a useful tool predicting the effects of a potential toxin in the ecosystem, may serve as a benchmark for subsequent studies evaluating the phytotoxic effects of *P. australis* and other plants (Batish et al. 2002, Uddin, Caridi and Robinson 2012). The findings regarding speed of germination showed that they are the most sensitive index compared to other germination indices for assessing the phytotoxic effect and such type of findings are well documented in other studies (Chiapusio et al. 1997, Rashid, Asaeda and Uddin 2010a). In addition, radicle length and biomass were inhibited more than hypocotyl length and biomass parameters, a common finding in phytotoxicity studies (Burgos and Talbert 2000). In some cases, the 'hormesis' phenomenon, characterized by low-dose stimulation and high-dose inhibition, has been observed significantly by inflorescence extract in radicle length development and phytotoxins are known to induce hormesis as well (Duke et al. 2006). Although it is often assumed that the response of seeds or seedlings to plant extracts is due to allelopathy entirely but our results regarding the inhibition of germination indices and phenotypic characteristics with OP values higher than - 0.45 bar indicate that the effects caused by extracts might be partially a negative osmotic effect. Comparing the results of the extracts and the mannitol solution with same OPs showed some effects of mannitol was lower than those of the extracts indicate that the activity of the some extracts is not only the effects of OP alone, it includes the effects by phytotoxins. Some extracts concentration such as inflorescence, stem and root were not appreciably more effective in retarding germination, growth and physiological activity than were mannitol solutions of comparable OPs. The germination, growth and physiological activity were not depressed significantly at lower concentrations of mannitol solution but the significant inhibition occurred as the concentrations increased. The inhibition by extracts with high OPs are substantially overestimated regardless of

osmotic effects as is shown clearly in our study and the findings of Wardle et al.(1992). According to Anderson and Loucks (1966), the effects by the extracts with OP no greater than - 0.506 bar might be considered as a phytotoxic effect due to presence of toxic compounds in extracts and our results go beyond other findings in the literature indicating a significant effects on physiological activity of the tested species. So, the findings in our study demonstrate that the inhibition attributable to the osmotic influence of the extracts must be considered during the assessment of phytotoxicity and allelopathic potential of plant extracts in water.

ROS are normally produced within plants at very low levels in cells, but their production may be enhanced under biotic and abiotic stresses. In particular H_2O_2 , one major species of ROS, leads to oxidative damage causing lipid peroxidation and cellular damage such as electrolyte leakage and pigment degradation (Batish et al. 2006). ROS generation in the exposed plants to some phytotoxins and related oxidative stress has been recently proposed as one of the modes of action for plant growth inhibition (Weir, Park and Vivanco 2004). Our results in H_2O_2 production are compatible with other phytotoxicity studies where it has been reported that phytotoxins and aqueous extracts of allelopathic plants may cause oxidative damage through production of ROS and subsequent damage of cellular systems in target plants (Singh, Batish, Kaur, Arora and Kohli 2006, Sinha and Saxena 2006). The increase of ROS might be directly produced through acute damage of phytotoxins on cells of treated plants and they showed more sensitivity with increasing concentrations indicating that concentration of phytotoxins in ecosystem might be an important factor in demonstrating the allelopathic effects.

In addition, the decrease of dehydrogenase enzyme activity might be a reflection of cell damage due to extract exposure (Hong et al. 2008); it may be an index of cell vitality. The decrease of cellular activity upon exposure to different extracts implies an

interference with energy metabolism involved in the synthesis of macromolecules which results in reduced plant growth. Singh *et al.* (2002) demonstrated that respiratory ability of seedlings might be hampered with exposure to phytotoxins . Increased conductivity indicates that cellular membrane disruption results in excessive solute leakage. These results correspond with earlier findings that some phytotoxins cause ion leakage from plant tissue (Singh, Batish, Kaur, Arora and Kohli 2006) leading to induction of oxidative stress (Dayan *et al.* 2000). Bogatek *et al.* (2006) found that sunflower extracts increase LP with increasing MDA in mustard plantlets. Increased MDA content with increasing extract concentration supports the idea that seed germination might be affected through membrane LP (Bogatek, Gniazdowska, Zakrzewska, Oracz and Gawronski 2006, Uddin, Caridi and Robinson 2012). To avoid the adverse effects by ROS, the cells might utilise energy to retain the expression of some protective genes and synthesis of antioxidant enzymes and compounds (Hong, Hu and Li 2008) such as catalase and ascorbic peroxidases, which were not studied in this study. A wide range of OPs was used in our experiment linked with plant extract concentrations, to determine whether physiological inhibition resulted from limited water availability. We found that OPs had a potential effect on physiology even at a value of - 0.27 bar and afterwards but the variability of the inhibitory effects regarding electrolyte leakage, dehydrogenase enzyme activity, and MDA content between solution of mannitol and extracts of similar OP explained that they are not only for osmotic stress but rather phytotoxic effects of the extracts. Additionally, we can point out one of the best characterized phytotoxic mechanisms induced by extracts of *P. australis* is the direct inhibition of photosystem II (PSII) components that was found in our previous studies (Uddin, Caridi and Robinson 2012).

The greenhouse study more closely approximated natural conditions, demonstrating that *P. australis* greatly inhibited germination and suppressed the growth of lettuce seedlings. This experiment showed a substantial reduction of root length, biomass and several eco-physiological parameters. These results are consistent with several studies which found that *P. australis* lowered the root biomass of endemic *Eriocaulon carsonii* due to belowground competition (Davies et al. 2010) and showed negative effects on the germination and establishment of *Arabidopsis thaliana* *in vivo* conditions while conversely showing a positive effect under activated carbon treatment (Rudrappa, Bonsall, Gallagher, Seliskar and Bais 2007). Our previous study found that *P. australis* rhizosphere and infested soil contained total phenolics with a concentration of 1.17 and 1.26 mg g⁻¹ respectively that might be enough for an inhibitory effect on the associated plant species (Uddin, Caridi and Robinson 2012). This greenhouse study coupled with our previous results confirms the phytotoxic potential of *P. australis* for competitive effects on other plant species.

The presence of gallic acid in root exudates of *P. australis* was previously reported by Rudrappa et al. (2007) but no relative abundance among organs was studied in details. The present study confirmed the rank of abundance among different organs. The effects of the extracts might be a combined role of several other unidentified phytotoxins with the known one. Our results also show that leaf extracts had higher inhibitory effect than other organs. Higher amount of total phenolic compounds (Uddin, Caridi and Robinson 2012) and specific compound, gallic acid, in the leaves explain the stronger inhibition of leaves when compare to other extracts. More recently, Weidenhamer et al. (2013) contradicted the findings of Rudrappa et al. (2007) and Bains et al. (2009) stating that gallic acid was not present in high concentration in rhizosphere soil as well as in leaf and rhizome with a concluding remarks that gallic

acid might be not a primary explanation for invasion of *P. australis*. Weidenhamer et al. (2013) reported that leaves and rhizomes of *P. australis* contained 20 - 28 $\mu\text{g/g}$ FW and trace amount of gallic acid respectively whereas Bains et al. (2009) found 2,075-8,302 $\mu\text{g/g}$ FW in rhizome sample of invasive population. By contrast, the concentration of gallic acid in our leaf and rhizome samples that were approximately 12 and 13-fold higher respectively than those reported by Weidenhamer et al. (2013) whereas rhizome had a concentration of 12.57 $\mu\text{g/g}$ FW gallic acid that were 0.151% of the maximum concentration found by Bains et al. (2009). In light of our results combined with others, it can be said that the concentration and production of allelochemicals in soil and plant organ depends on the density, growth and phenology of alleopathic plant, habitat, soil type, climate, pattern of cultivation and agricultural practices (Inderjit and Dakshini 1995, Yaber Grass and Leicach 2011). In an advanced concept in alleopathy, a biogeographical approach has been considered as crucial for understanding the invasion process (Hierro et al. 2005).

Considering the overall results, it seems evident that leaf extract has higher phytotoxicity than other organs. This could explain how leaves may contribute the maximum level of phytotoxin and the associated effects. First, we may consider partially the 'enemy release hypothesis' that explains a way of success of invasive species (Colautti et al. 2004, Occhipinti et al. 2011). Generally, leaves are the main target organ of any plant for herbivores and as leaves of *P. australis* contain high amount phytotoxins, it is thought to gain a substantial advantage. Because *P. australis* populations are no longer directly suppressed by herbivores and consequently, they obtain a competitive advantage over natives that enables the plant to be more invasive attaining higher biomass production. This concept is supported by Hendricks et al. (2011) who suggested that phenolic compounds in leaf might be involved in chemical

defence of *P. australis* against herbivores and neighbouring plants. In addition, Chludil et al. (2012) reported that leaves of *Lupinus angustifolias* as with most species of Fabaceae are capable of producing chemical defences against herbivores and pathogenic microorganisms that facilitate the biological invasion of those species. Secondly, *P. australis* dominated ecosystems produce large volumes of above and belowground biomass (Park and Blossey 2008). Total biomass production of more than 200 t ha⁻¹ have been recorded (Engloner 2009) making *P. australis* one of the largest biomass producers in aquatic ecosystems. The worldwide distribution and the extremely large areas covered by *P. australis* stands may have a considerable effect on the accumulation of phytotoxins through decomposition in wetlands. Deposition of phytotoxins on this scale may actually create a 'leading edge' of plant death and damage (Cruz-Ortega et al. 2002, Rashid et al. 2010b). In addition, our field observations indicate that most of the wetlands occupied by *P. australis* have thick bed of residue especially leaves and we suspect that most phytotoxins are released into the soil through decomposition of leaf as it decomposes readily. Some long-term invaded wetlands in Australia have no other plant species recorded within the stands of *P. australis* compared to newly invaded sites that are floristically more diverse. The absence of other species in long-established stands may be due to the formation of more toxic and persistent breakdown products over time, mainly from accumulation of leaf materials and breakdown compounds.

In conclusion, our studies conferred that *P. australis* has phytotoxic effects on germination processes and seedling development of plant species, lettuce combined with osmotic effects of aqueous plant extracts by a range of - 0.45 to - 1.30 bar. Initial inhibition of imbibition by seeds reflects the phytotoxic effects that alter the ability of the seeds reaching the critical water level to increase metabolism and for initiation of germination. Consequently, alterations in reserve carbohydrate (TNC and WSC)

mobilization could negatively affect germination and root cell expansion. Increasing ROS as H₂O₂ in plantlets enhanced LP in the cells, further strengthened the extent of membrane injury as electrolyte leakage and decreased dehydrogenase enzyme activity. Considering all results, leaf extracts had the greatest inhibitory effects among all organs followed by rhizome, root, stem and inflorescence. These correlated and combined effects of aqueous extracts of *P. australis* cause oxidative stress in cells of impacted seedlings that can result in cell death and ultimately inhibit the growth of plants and may lead to plant death. *P. australis* may appear to exude phytotoxins that might have substantial negative effects on the germination and growth of lettuce. Again, the study confirms the presence of gallic acid in different organs of *P. australis* but further studies are required to identify more in details about the known and unknown compounds and their phytotoxicity. In addition, whether the induction of oxidative stress associated with the increased defence mechanisms by antioxidant enzyme activity in the cells of the plant persists is a question to be studied in further research. This study provides support for investigating the hypothesis that *P. australis* may exert allelopathic effects that facilitate invasion, and to identify specific further experiments such as a common phenomenon - allelopathy through residue decomposition in wetland that would be required to further examine the hypothesis of allelopathic effects.

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List of Table Captions

Table 1. Results of two way ANOVA (*F*-ratios) showing the effects of different organ materials (M) of *P. australis*, extract concentration (C) and their interaction (M × C) on germination indices, biometric and biochemical parameters in lettuce. Level of significance: ***, $P < .0001$

Table 2. Effect of osmotic potential at various concentrations on germination, growth and physiological parameters of lettuce. Values are mean ± standard error ($n = 3$). ***, **, * and ^{ns} indicate significant difference from control at $P \leq .001$, $P \leq .01$, $P \leq .05$ and non-significant respectively after applying the Dunnett test.

Table 3. Results of paired t-tests showing the effects of different organ of *P. australis* and extract concentration considering osmotically adjusted control on germination indices, biometric and physiological parameters in lettuce. Level of significance: *, **, ***, and NS at $P < .05$, $P < .01$, $P < .001$ and not significant; NC = not calculated due to high effects by osmotic potential rather than plant extracts and ST = stimulation. Units are as same as Table 2.

Table 1.

| Parameter | M | C | M × C |
|---------------------------------------|----------------------------|----------------------------|----------------------------|
| <i>Germination indices</i> | | | |
| % germination | $F_{4, 82} = 458.14^{***}$ | $F_{6, 82} = 707.20^{***}$ | $F_{24, 82} = 71.15^{***}$ |
| Speed of germination | $F_{4, 82} = 497.33^{***}$ | $F_{6, 82} = 745.98^{***}$ | $F_{24, 82} = 55.28^{***}$ |
| <i>Biometric parameters</i> | | | |
| Radicle length | $F_{4, 76} = 315.69^{***}$ | $F_{6, 76} = 276.26^{***}$ | $F_{22, 76} = 23.85^{***}$ |
| Hypocotyl length | $F_{4, 76} = 24.19^{***}$ | $F_{6, 76} = 9.54^{***}$ | $F_{22, 76} = 26.02^{***}$ |
| Radicle weight | $F_{4, 73} = 28.89^{***}$ | $F_{6, 73} = 36.92^{***}$ | $F_{21, 73} = 3.45^{***}$ |
| Hypocotyl weight | $F_{4, 76} = 43.86^{***}$ | $F_{6, 76} = 3.27^{***}$ | $F_{22, 76} = 14.78^{***}$ |
| <i>Biochemical parameters</i> | | | |
| H ₂ O ₂ content | $F_{4, 62} = 77.43^{***}$ | $F_{6, 62} = 116.23^{***}$ | $F_{20, 62} = 8.05^{***}$ |
| Enzyme activity | $F_{4, 71} = 46.41^{***}$ | $F_{6, 71} = 510.85^{***}$ | $F_{20, 71} = 14.68^{***}$ |
| Membrane injury | $F_{4, 71} = 131.99^{***}$ | $F_{6, 71} = 215.25^{***}$ | $F_{20, 71} = 12.83^{***}$ |
| Lipid peroxidation | $F_{4, 62} = 144.70^{***}$ | $F_{6, 62} = 196.76^{***}$ | $F_{20, 62} = 21.39^{***}$ |

Table 2.

| Osmotic potential (bar) | Germination (%) | Speed of germination | Radicle length (% of control) | Membrane injury (% of total leakage) | Dehydrogenase enzyme activity (% of control) | Lipid peroxidation (nmol MDA g ⁻¹ FW) |
|-------------------------|-----------------------------|----------------------------|-------------------------------|--------------------------------------|--|--|
| 0 | 100 ± 0 | 12.50 ± 0 | 100 ± 0 | 5.12 ± 0.03 | 100 ± 0 | 5.07 ± 0.25 |
| 0.11 | 97.33 ± 1.33 ^{ns} | 10.82 ± 0.70 ^{ns} | 100 ± 7.38 ^{ns} | 4.55 ± 0.30 ^{ns} | 96.33 ± 1.93 ^{ns} | 5.51 ± 0.37 ^{ns} |
| 0.17 | 97.33 ± 1.3 ^{ns} | 10.89 ± 0.36 ^{ns} | 97.16 ± 3.60 ^{ns} | 4.56 ± 0.21 ^{ns} | 94.50 ± 2.76 ^{ns} | 7.42 ± 0.69 ^{ns} |
| 0.22 | 94.67 ± 1.3 ^{ns} | 10.99 ± 0.15 ^{ns} | 100.44 ± 3.74 ^{ns} | 5.01 ± 0.10 ^{ns} | 93.04 ± 2.22 ^{ns} | 6.32 ± 0.23 ^{ns} |
| 0.27 | 94.67 ± 2.6 ^{ns} | 10.22 ± 0.43 [*] | 97.60 ± 5.69 ^{ns} | 4.26 ± 0.12 ^{ns} | 88.64 ± 2.86 [*] | 7.33 ± 0.28 ^{ns} |
| 0.37 | 94.67 ± 1.33 ^{ns} | 10.42 ± 0.32 [*] | 93.22 ± 1.52 ^{ns} | 5.90 ± 0.29 ^{ns} | 57.14 ± 2.28 ^{***} | 7.03 ± 0.26 ^{ns} |
| 0.45 | 97.33 ± 1.33 ^{ns} | 10.69 ± 0.44 [*] | 79.45 ± 2.65 [*] | 6.47 ± 0.33 [*] | 42.12 ± 1.93 ^{***} | 11.20 ± 1.06 ^{***} |
| 0.57 | 74.67 ± 2.67 ^{***} | 6.56 ± 0.52 ^{***} | 57.16 ± 2.47 ^{***} | 6.89 ± 0.37 ^{**} | 34.79 ± 1.93 ^{***} | 12.19 ± 0.65 ^{***} |
| 0.67 | 62.67 ± 3.53 ^{***} | 5.48 ± 0.48 ^{***} | 52.46 ± 1.89 ^{***} | 7.23 ± 0.28 ^{***} | 27.47 ± 1.26 ^{***} | 14.52 ± 0.66 ^{***} |
| 0.77 | 18.67 ± 2.67 ^{***} | 0.43 ± 0.04 ^{***} | 34.43 ± 1.68 ^{***} | - | - | - |
| 0.87 | 12.00 ± 2.31 ^{***} | 0.31 ± 0.03 ^{***} | 24.92 ± 1.62 ^{***} | - | - | - |
| 0.97 | 5.33 ± 1.33 ^{***} | 0.19 ± 0.03 ^{***} | 13.55 ± 1.08 ^{***} | - | - | - |

Table 3.

| Organ/ Concentration (%) | Germination | Speed of germination | Radicle length | Membrane injury | Dehydrogenase enzyme activity | Lipid peroxidation |
|-----------------------------|-------------|-------------------------|-------------------|--------------------|----------------------------------|-----------------------|
| Inflorescence | | | | | | |
| 1.25 | NS | NS | ST | NS | NS | NS |
| 2.5 | NS | NS | ST | NS | ** | * |
| 5.0 | NS | NS | ** | * | ** | NS |
| 10.0 | NS | NS | ** | * | *** | * |
| 15.0 | NS | NS | * | ** | * | NS |
| 20.0 | NC | NC | NC | ** | NC | NC |
| Leaf | | | | | | |
| 1.25 | NS | NS | * | * | ** | * |
| 2.5 | NS | * | ** | * | * | * |
| 5.0 | *** | *** | ** | * | * | ** |
| 10.0 | *** | *** | *** | ** | * | ** |
| 15.0 | - | - | - | - | - | - |
| 20.0 | - | - | - | - | - | - |
| Stem | | | | | | |
| 1.25 | NS | NS | NS | NS | ** | NC |
| 2.5 | NS | NS | NS | * | * | NC |
| 5.0 | NS | NS | NS | * | ** | NC |
| 10.0 | * | * | ** | NS | ** | NC |
| 15.0 | * | * | ** | * | ** | NC |
| 20.0 | NC | NC | NC | NC | - | - |
| Rhizome | | | | | | |
| 1.25 | NS | NS | * | ** | NS | NS |
| 2.5 | NS | NS | ** | ** | NS | NS |
| 5.0 | NS | NS | * | ** | ** | * |
| 10.0 | ** | ** | * | ** | ** | * |
| 15.0 | ** | ** | ** | * | ** | ** |
| 20.0 | NS | NS | NC | - | - | - |
| Root | | | | | | |
| 1.25 | NS | NS | NS | ** | ** | NC |
| 2.5 | NS | NS | NS | * | ** | NC |
| 5.0 | NS | NS | NS | * | *** | * |
| 10.0 | ** | * | NC | ** | NC | NC |
| 15.0 | NS | NS | NC | - | - | - |
| 20.0 | NC | NC | NC | - | - | - |

List of Figure Captions

Fig. 1 Effect of aqueous extracts (5 %) of different organs of *P. australis* on liquid imbibitions of lettuce seeds expressed in fresh weight [mg. (100 mg seed)⁻¹] throughout 32 h. Values are mean ± standard error ($n = 3$). ***, ** and * indicate significant differences from control at $P \leq .001$, $P \leq .01$ and $P \leq .05$ respectively after Dunnett test.

Fig. 2 Effect of aqueous extracts (5 %) of different organs of *P. australis* on (a) total carbohydrates (mg g⁻¹) and (b) water soluble carbohydrates (mg g⁻¹) of lettuce seeds at 0, 8 and 32 h imbibition. Values are mean ± standard error ($n = 3$). ***, ** and * indicate significant differences from control at $P \leq .001$, $P \leq .01$ and $P \leq .05$ respectively after Dunnett test.

Fig. 3 Dose-response relationships for effects of aqueous extracts of different organs of *P. australis* on (a) germination percentage, (b) speed of germination, and c radicle length of lettuce seeds. Values are mean ± standard error ($n = 3$). Horizontal lines indicate LC₅₀ concentration and values along arrows indicate LC₅₀ of different organs extracts. $r =$ correlation coefficient.

Fig. 4 Effects of aqueous extracts of different organs of *P. australis* at different concentrations on (a) H₂O₂ content (nmol g⁻¹ FW) and (b) dehydrogenase enzyme activity (% of control) in treated plantlets of lettuce. Values are mean ± standard error ($n = 3$). $r =$ correlation coefficient.

Fig. 5 Effects of aqueous extracts of different organs of *P. australis* at different concentrations on (a) membrane injury (% of total leakage) and (b) lipid peroxidation (nmol MDA g⁻¹ FW) in treated plantlets of lettuce. Values are mean ± standard error ($n = 3$). $r =$ correlation coefficient.

Fig. 6 Effects of root exudates of *P. australis* on (a) germination (%), (b) root and shoot length (cm), and (c) root and shoot weight (mg/plant) in lettuce grown alone, or together with *P. australis* either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 3$). Means with different letters were significant different in pairwise comparison ($P > 0.05$).

Fig. 7 Effects of root exudates of *P. australis* on (a) membrane injury of root (% of total leakage) and (b) total chlorophyll (mg L^{-1}) in lettuce grown alone, or together with *P. australis* either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 3$). Means with different letters were significant different in pairwise comparison ($P > 0.05$).

Fig.1

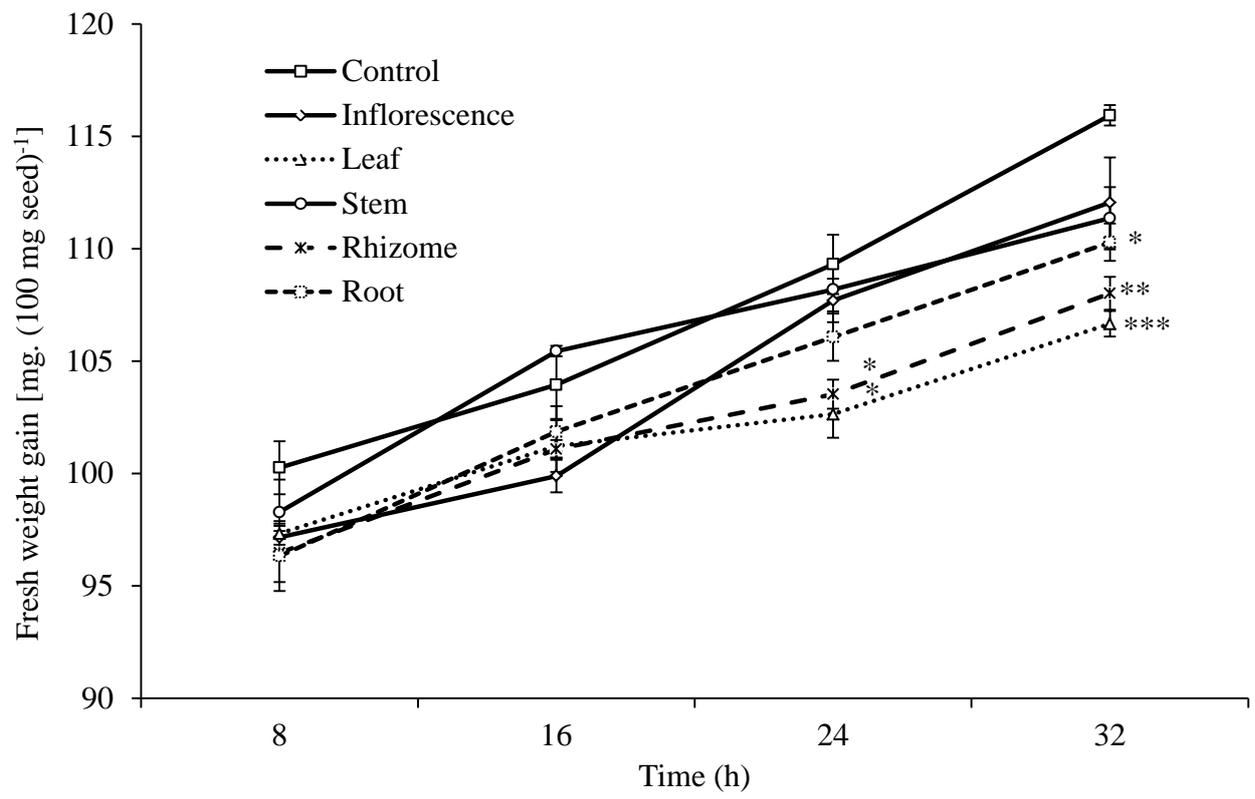


Fig. 2

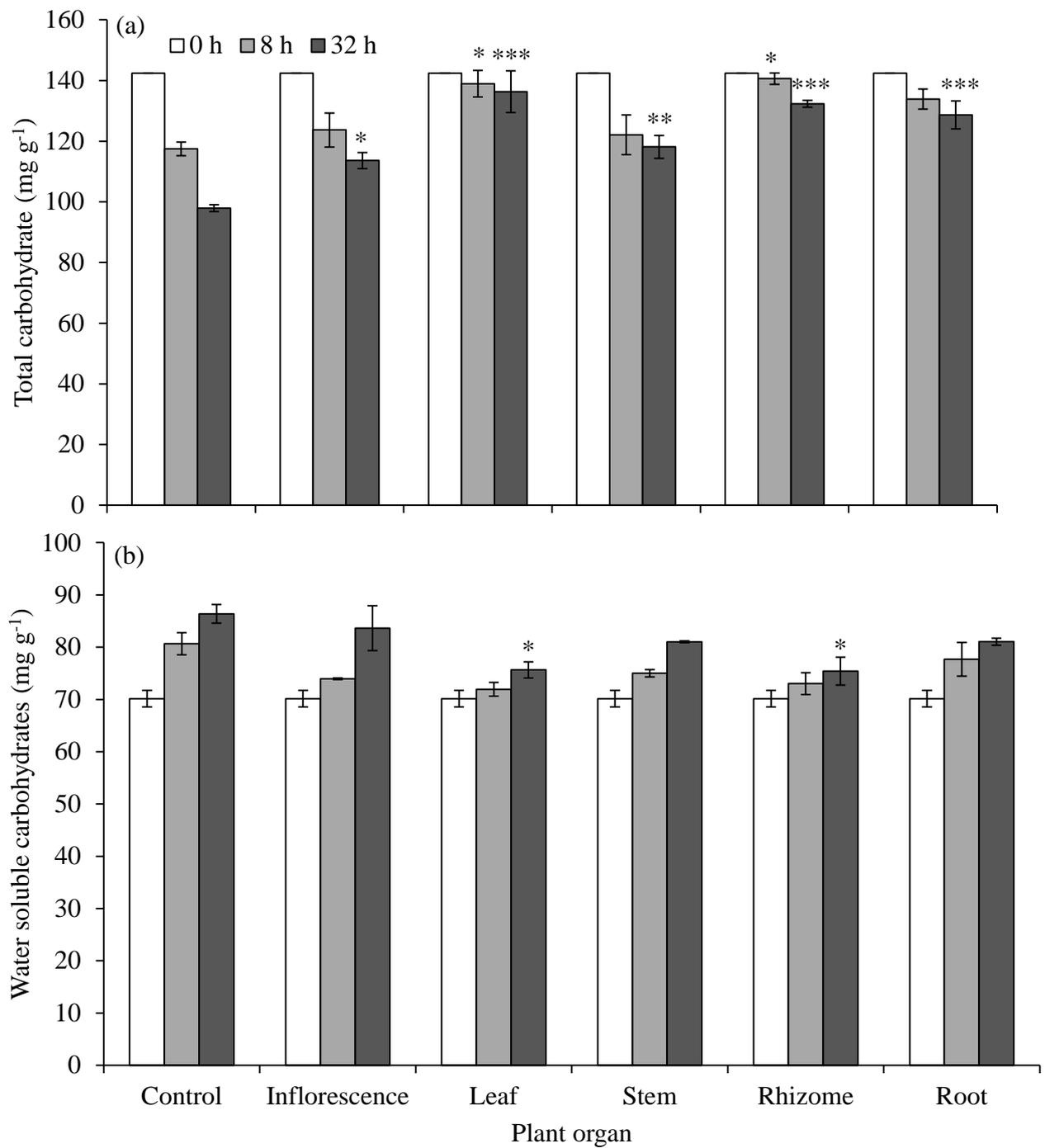


Fig. 3

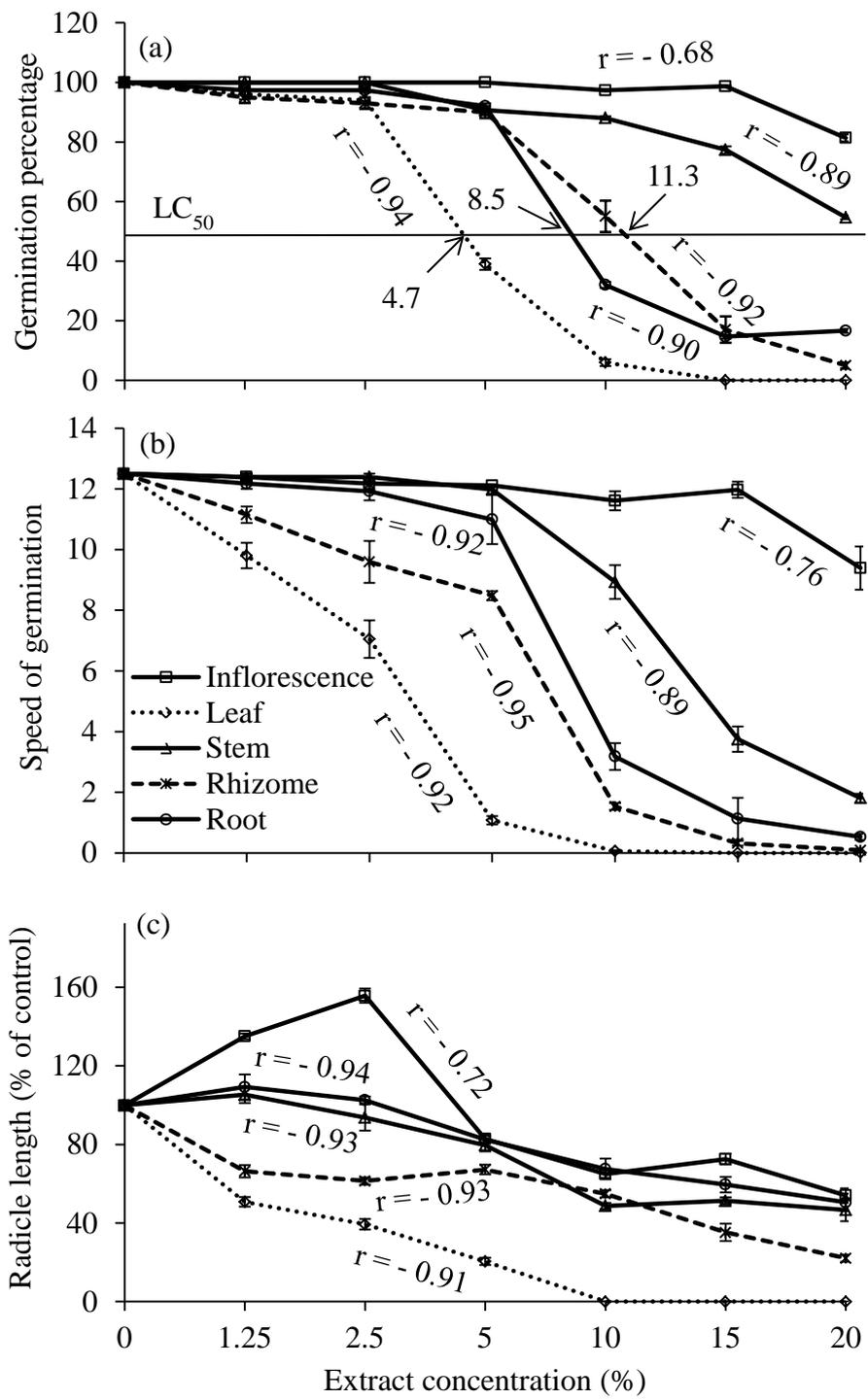


Fig. 4

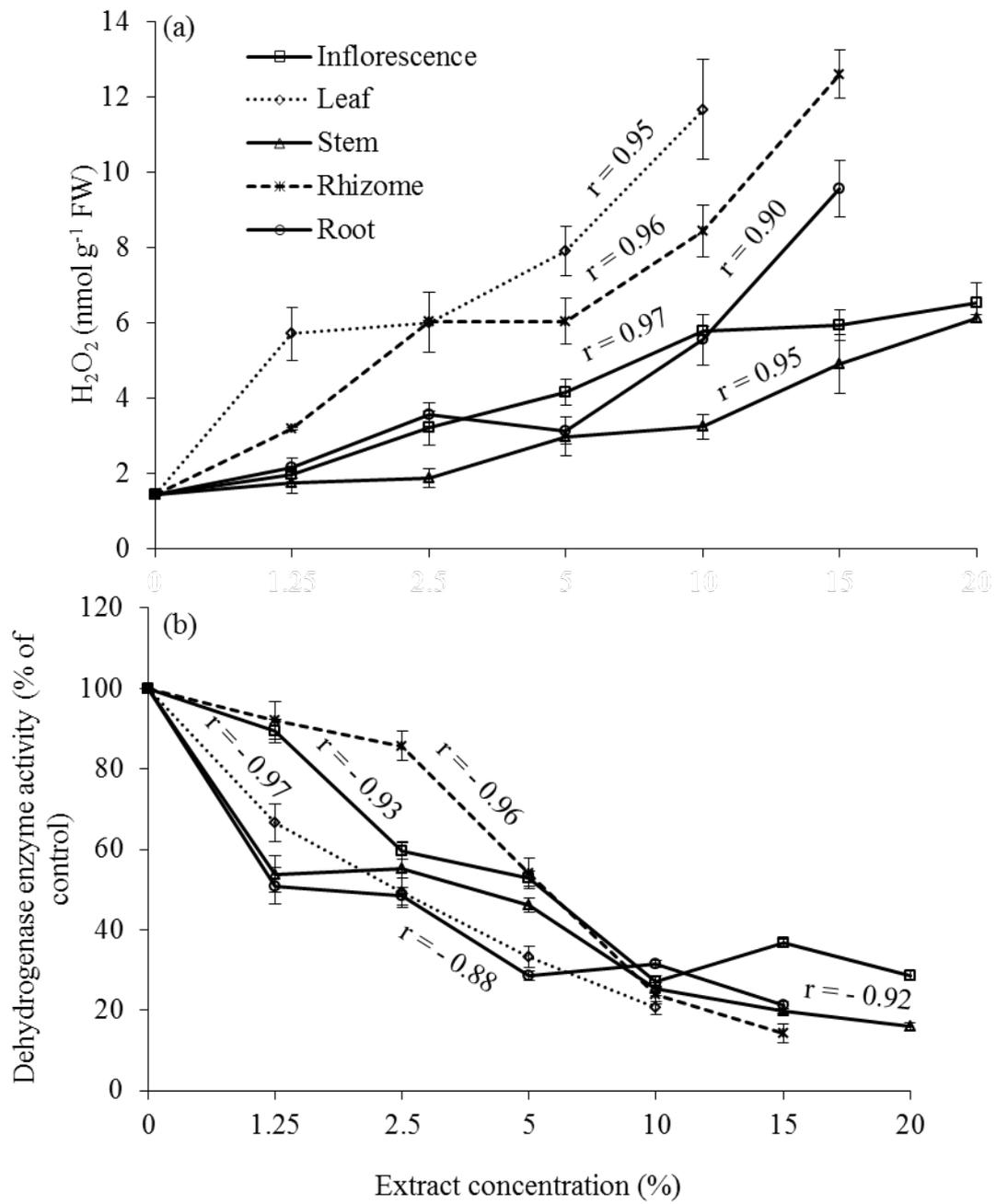


Fig. 5

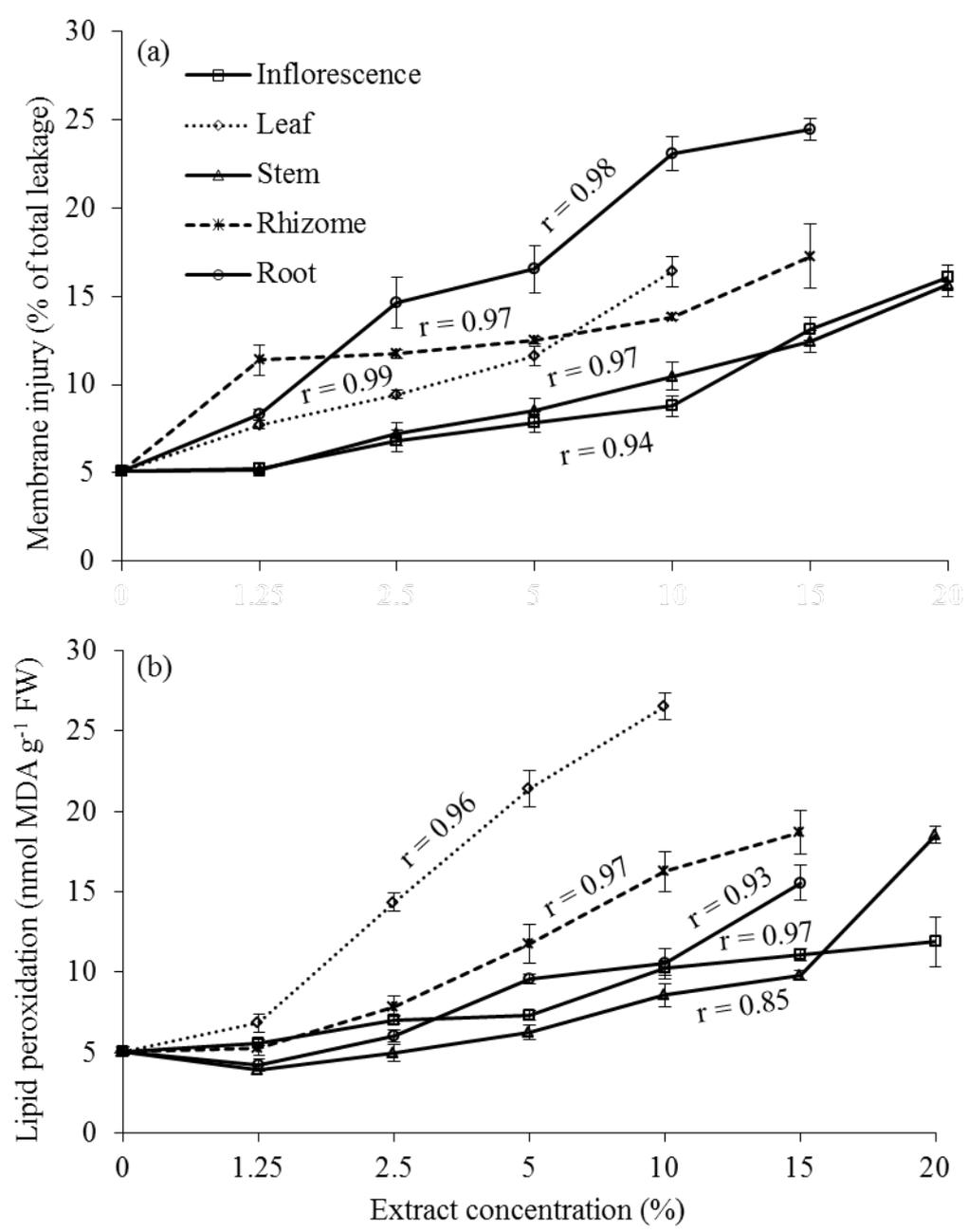


Fig. 6

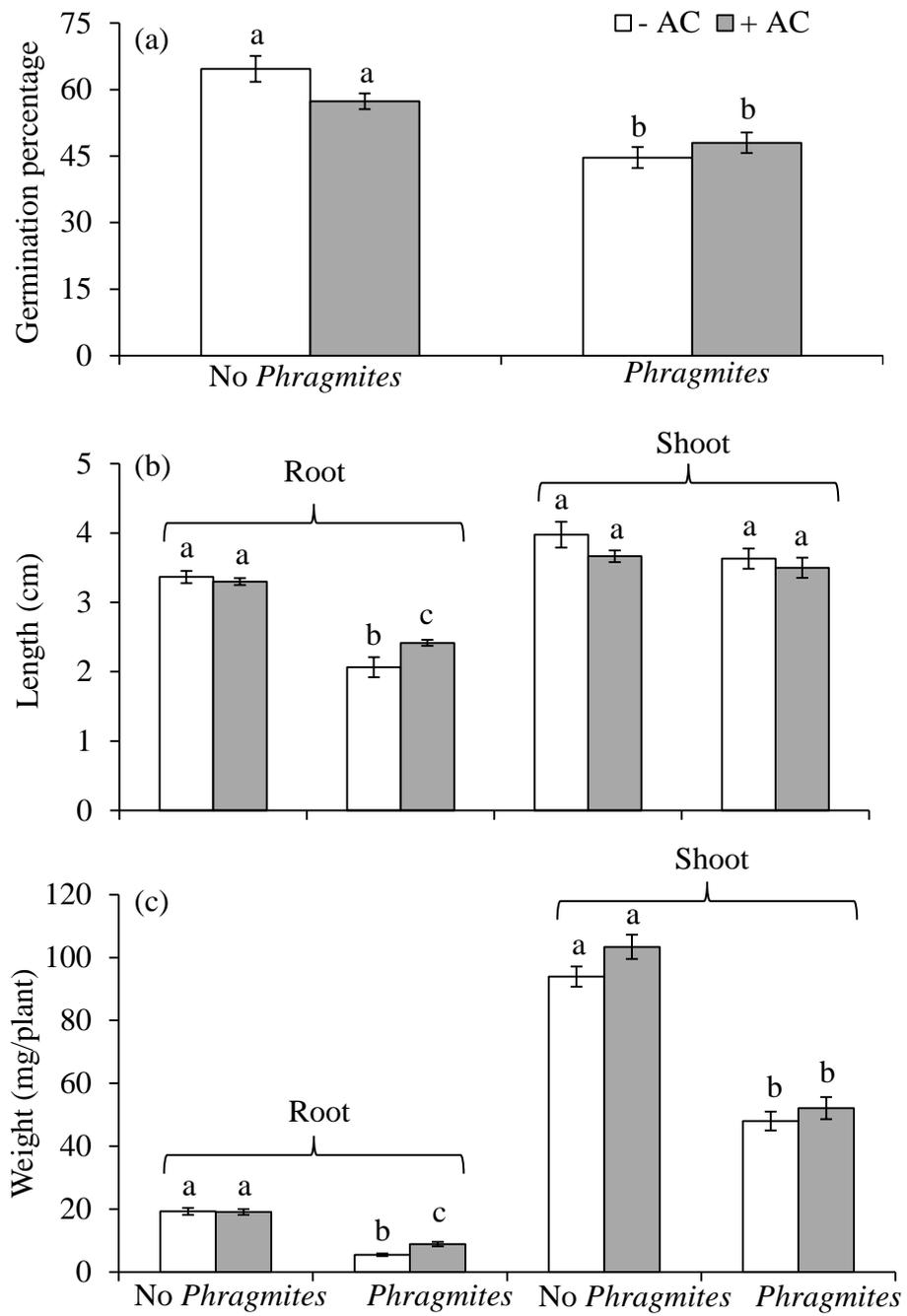
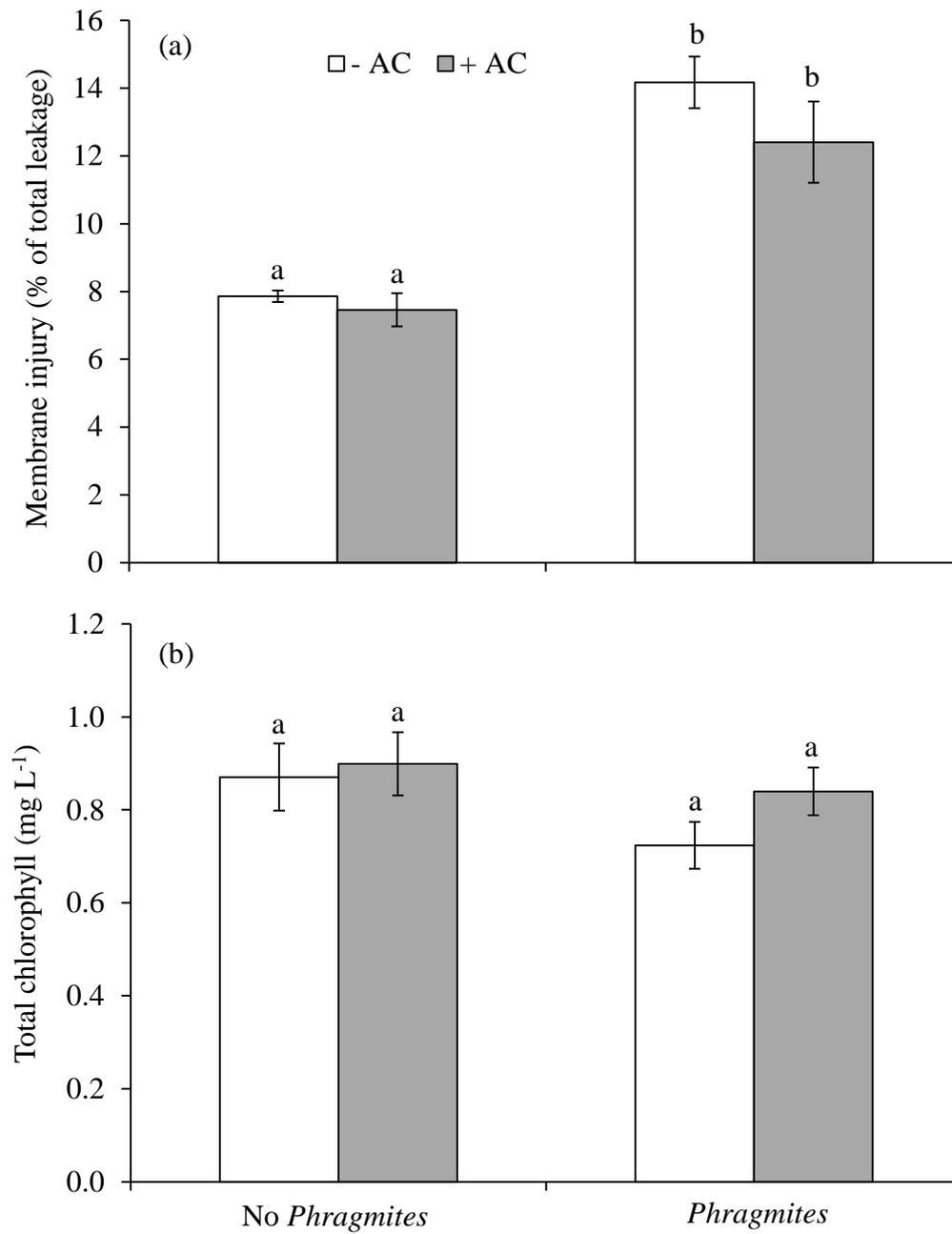


Fig. 7

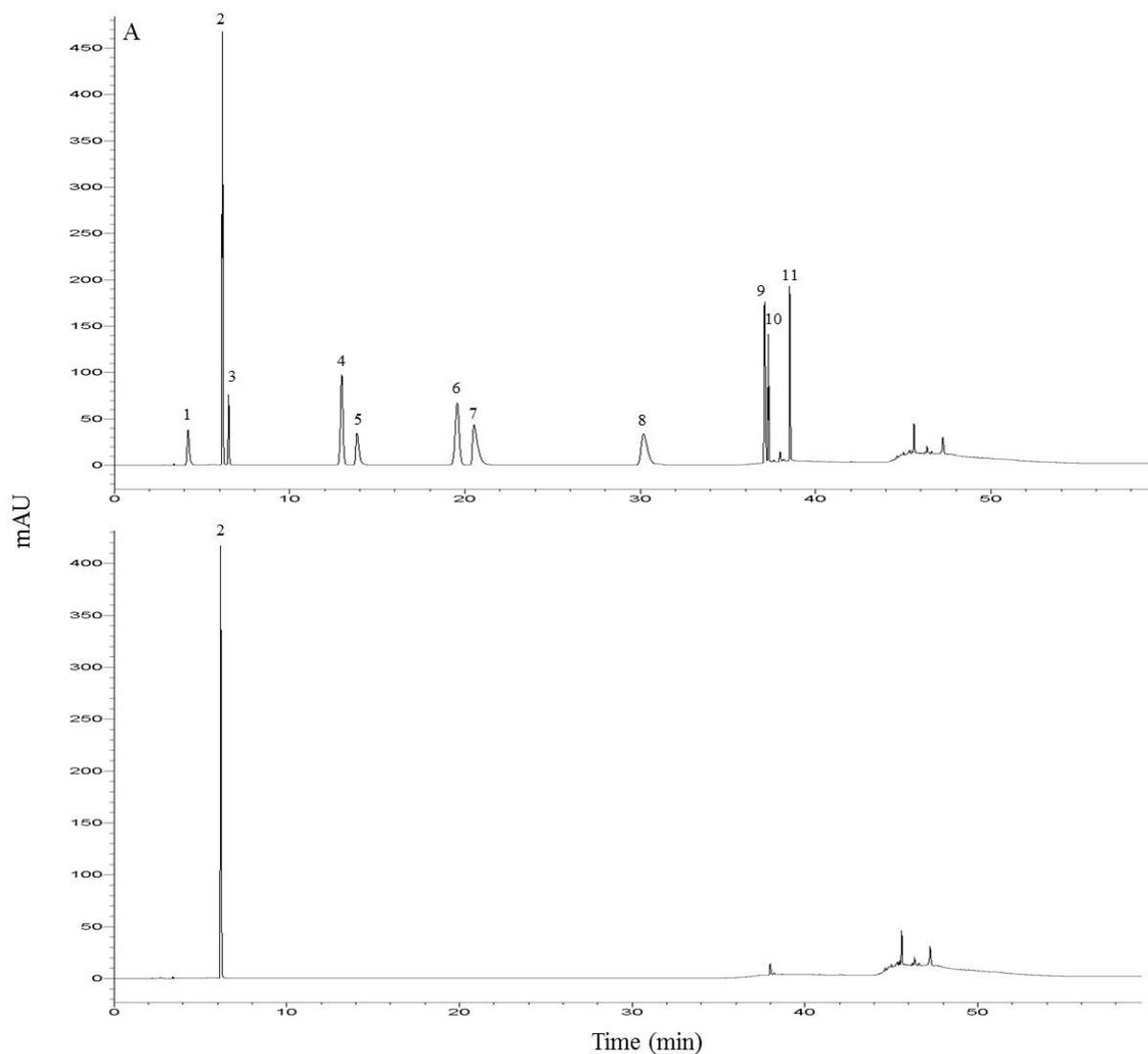


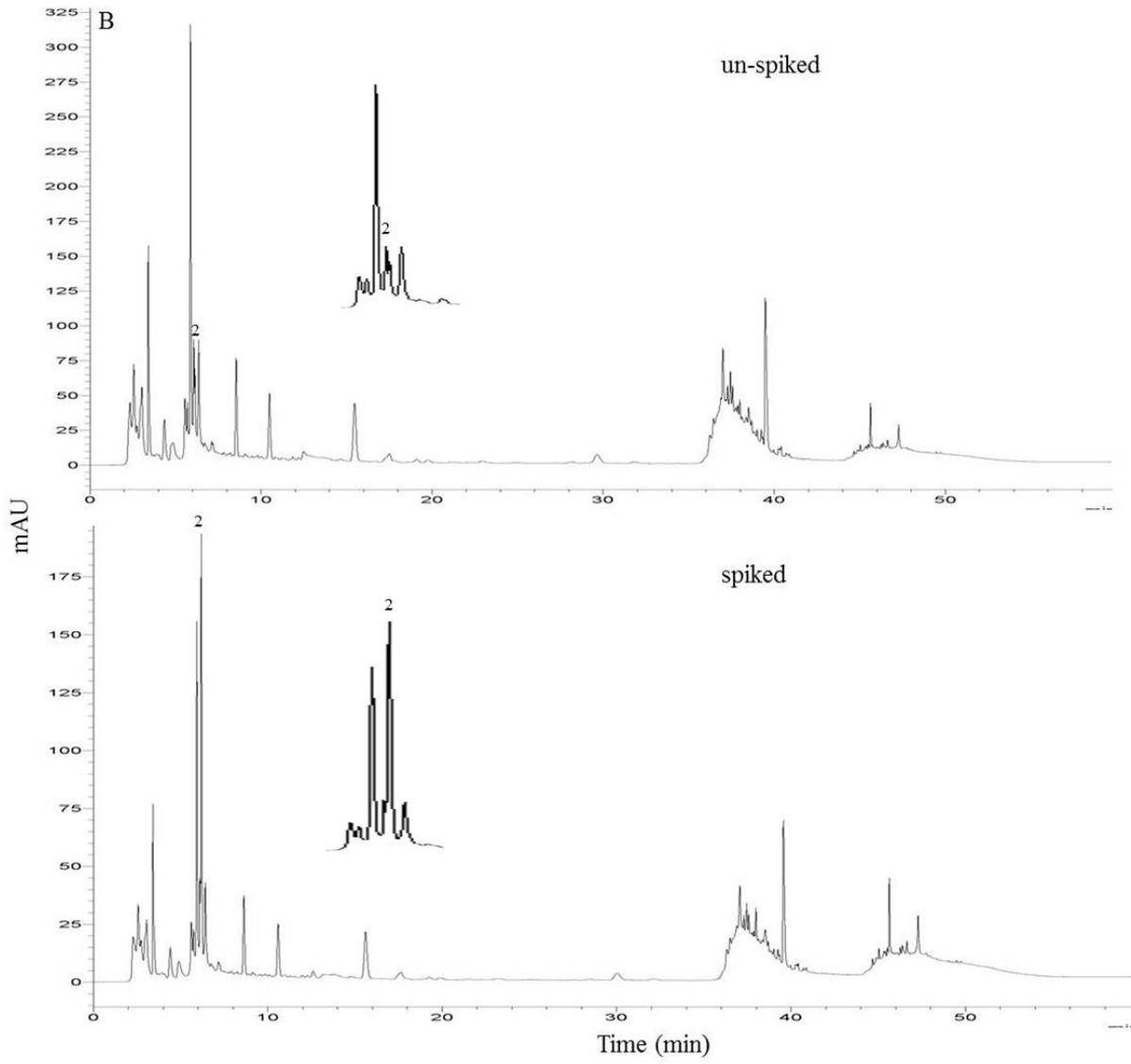
Supplementary Materials_TJPI_2013_0159.R1

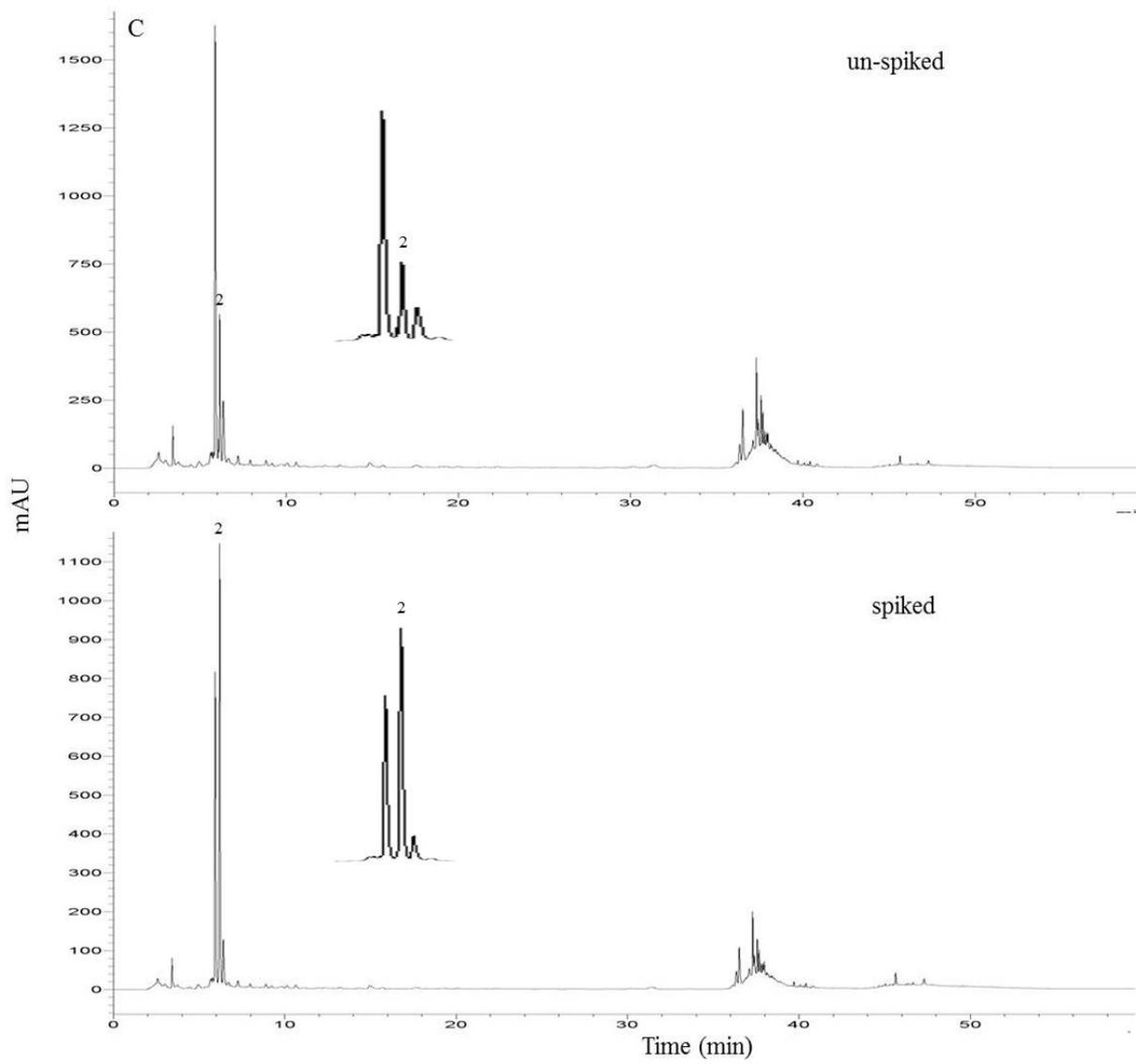
Table S1¹. Osmotic potential (- bar) at various concentrations of different organs of *Phragmites australis*

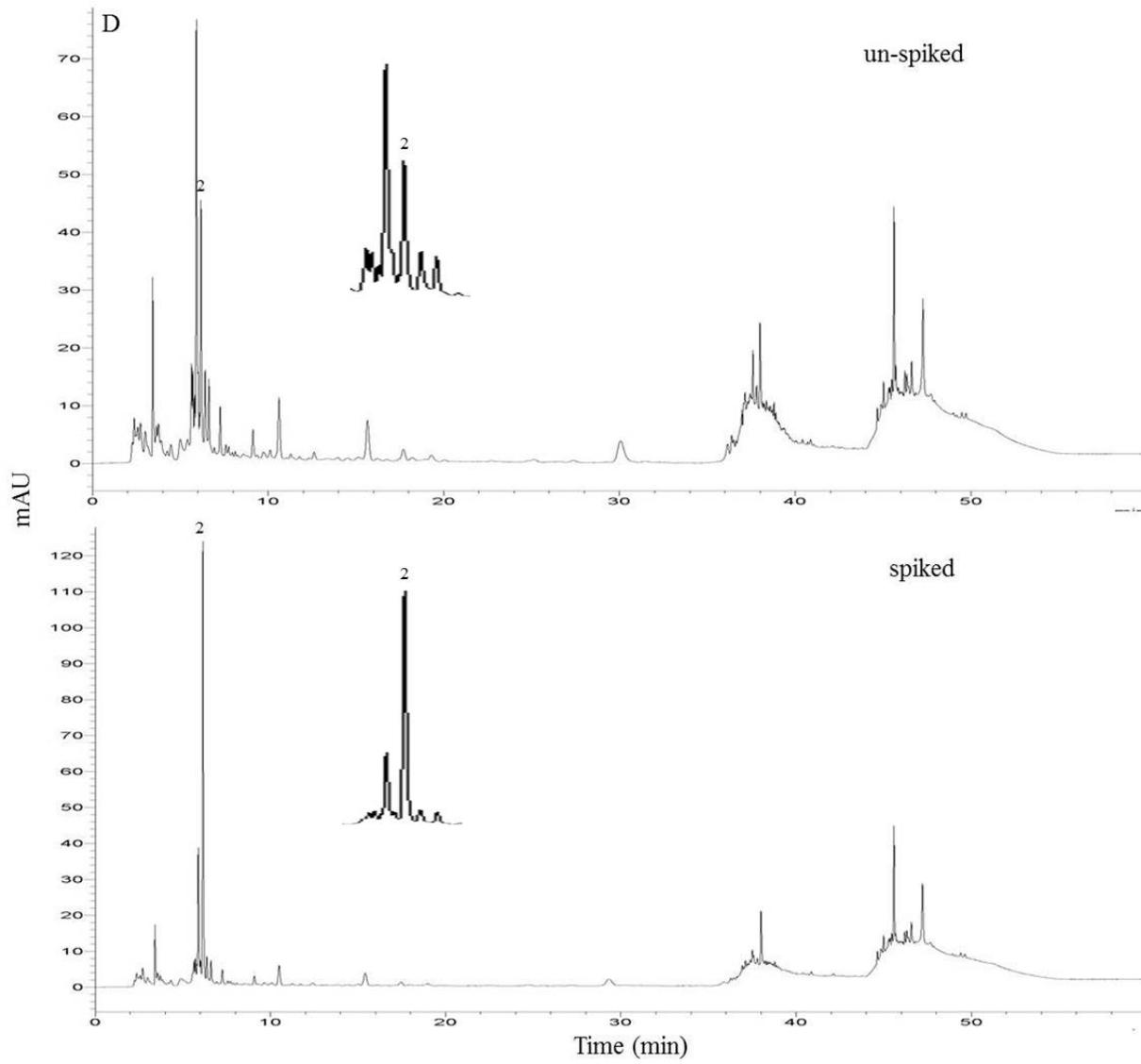
| Organ | Concentration (%) | | | | | | | | |
|---------------|-------------------|------|------|------|------|------|------|------|------|
| | 0 | 1.25 | 2.5 | 5 | 10 | 15 | 20 | Min. | Max. |
| Inflorescence | 0 | 0.05 | 0.09 | 0.18 | 0.31 | 0.46 | 0.68 | 0.05 | 0.68 |
| Leaf | 0 | 0.09 | 0.17 | 0.32 | 0.62 | 0.81 | 1.18 | 0.09 | 1.18 |
| Stem | 0 | 0.06 | 0.12 | 0.21 | 0.41 | 0.53 | 0.82 | 0.06 | 0.82 |
| Rhizome | 0 | 0.08 | 0.14 | 0.26 | 0.49 | 0.64 | 0.99 | 0.08 | 0.99 |
| Root | 0 | 0.10 | 0.18 | 0.35 | 0.63 | 0.82 | 1.27 | 0.10 | 1.27 |

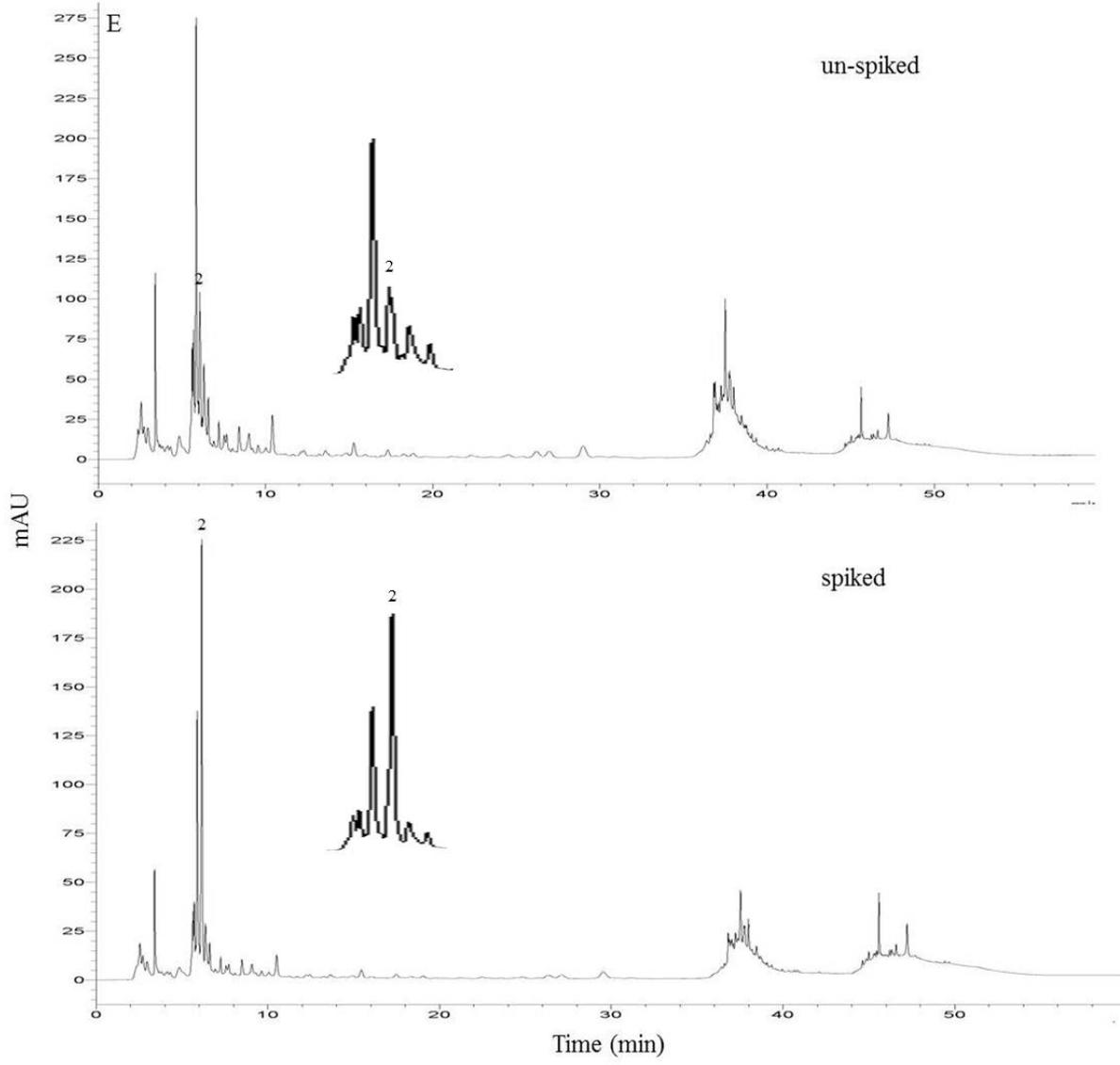
Figure S1¹. HPLC analysis. Chromatogram of the standards: **A.** 1. Arbutin; 2. Gallic acid; 3. HMF; 4. Catechin; 5. Gentistic acid; 6. Chlorogenic acid; 7. Epicatechin; 8. Coumaric acid; 9. Ferulic acid; 10. Rutin; and 11. Phloridzin. Chromatogram of the extracts of different organs of *Phragmites australis*: **B.** Inflorescence (un-spiked and spiked); **C.** Leaf (un-spiked and spiked); **D.** Stem (un-spiked and spiked); **E.** Rhizome (un-spiked and spiked); and **F.** Root (un-spiked and spiked) with insets only in plant samples showing enlargement of the range of the peaks in the vicinity of gallic acid. The elution pattern was monitored at 220 nm and extracts were spiked with gallic acid.











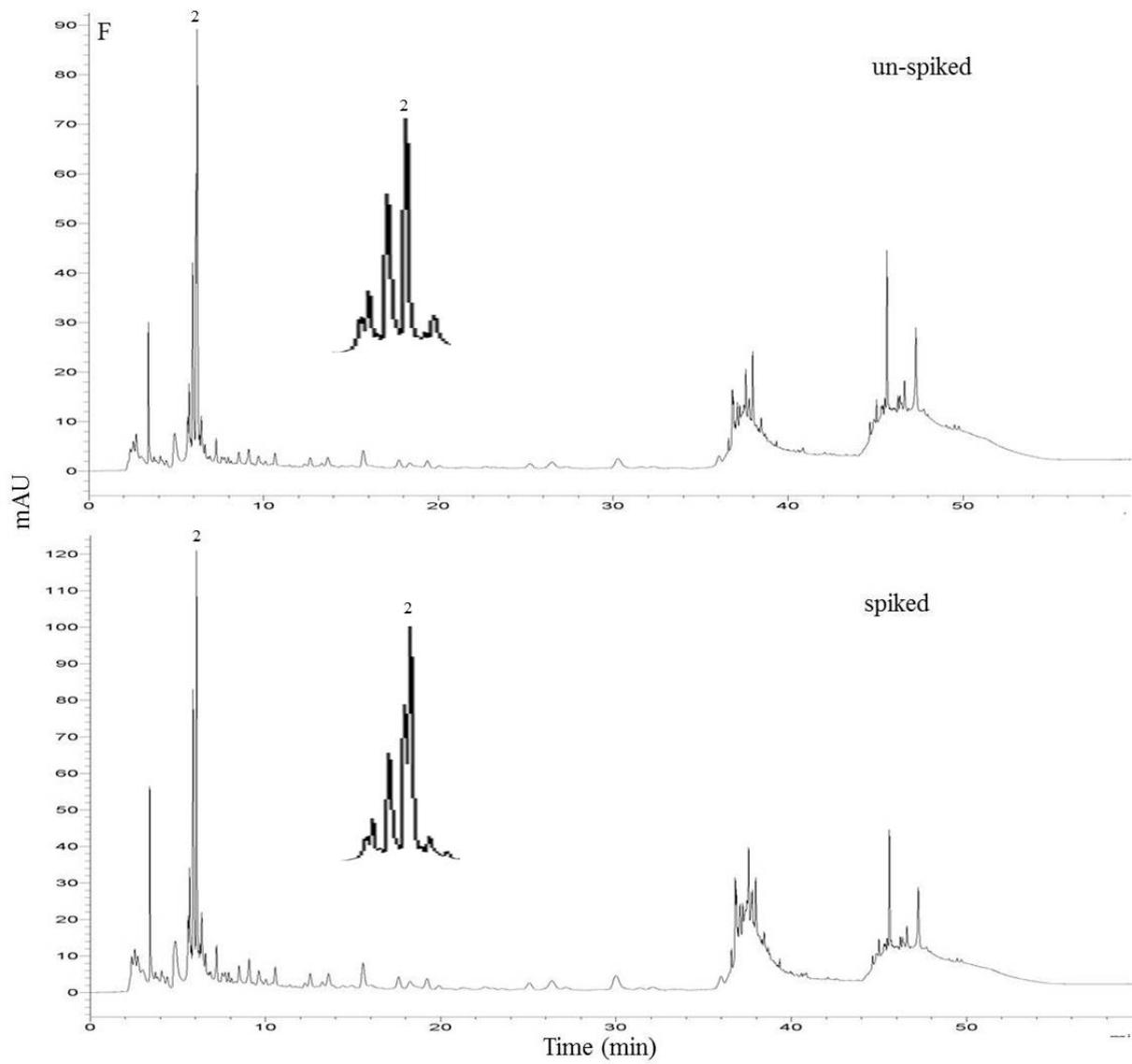
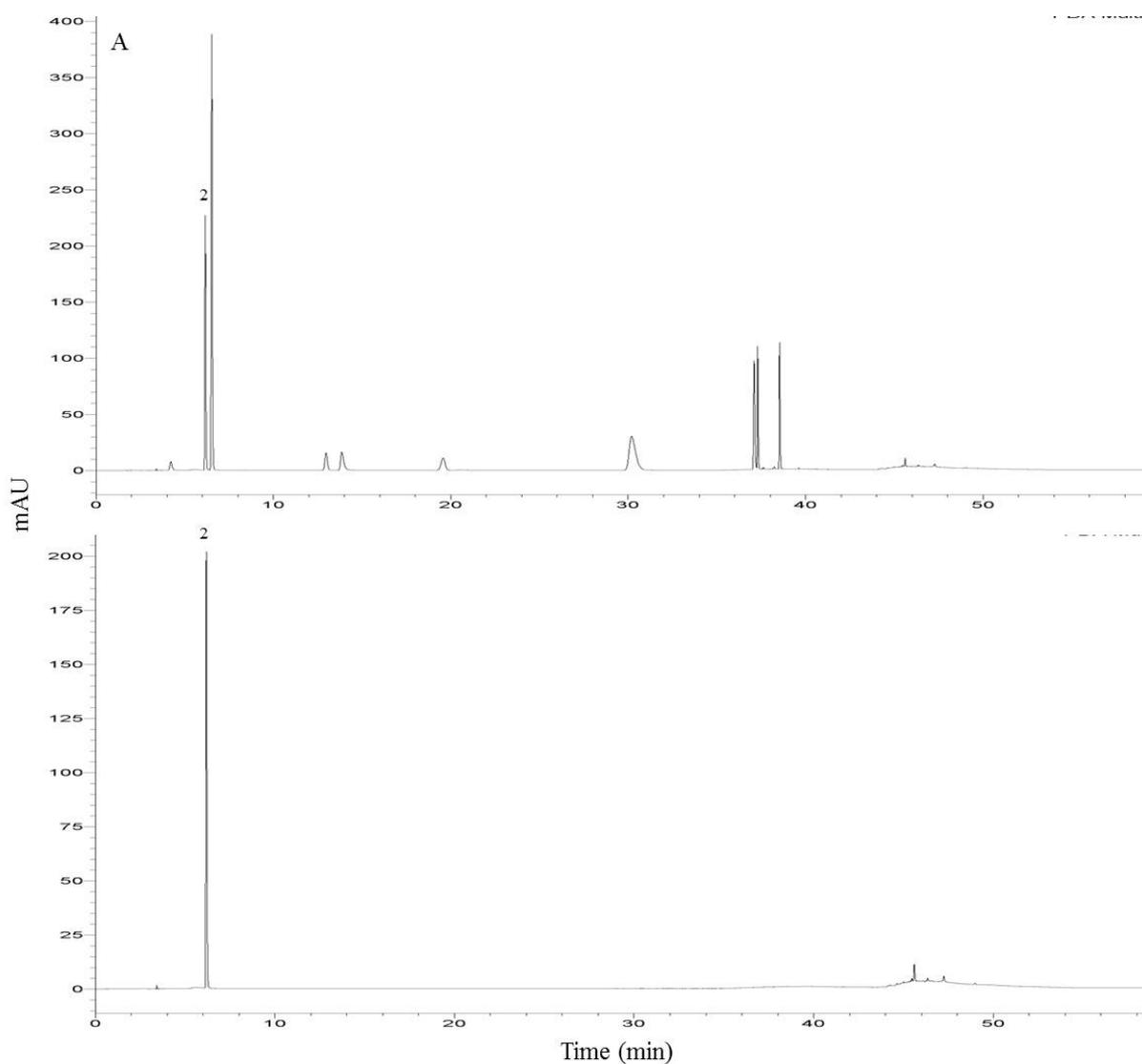
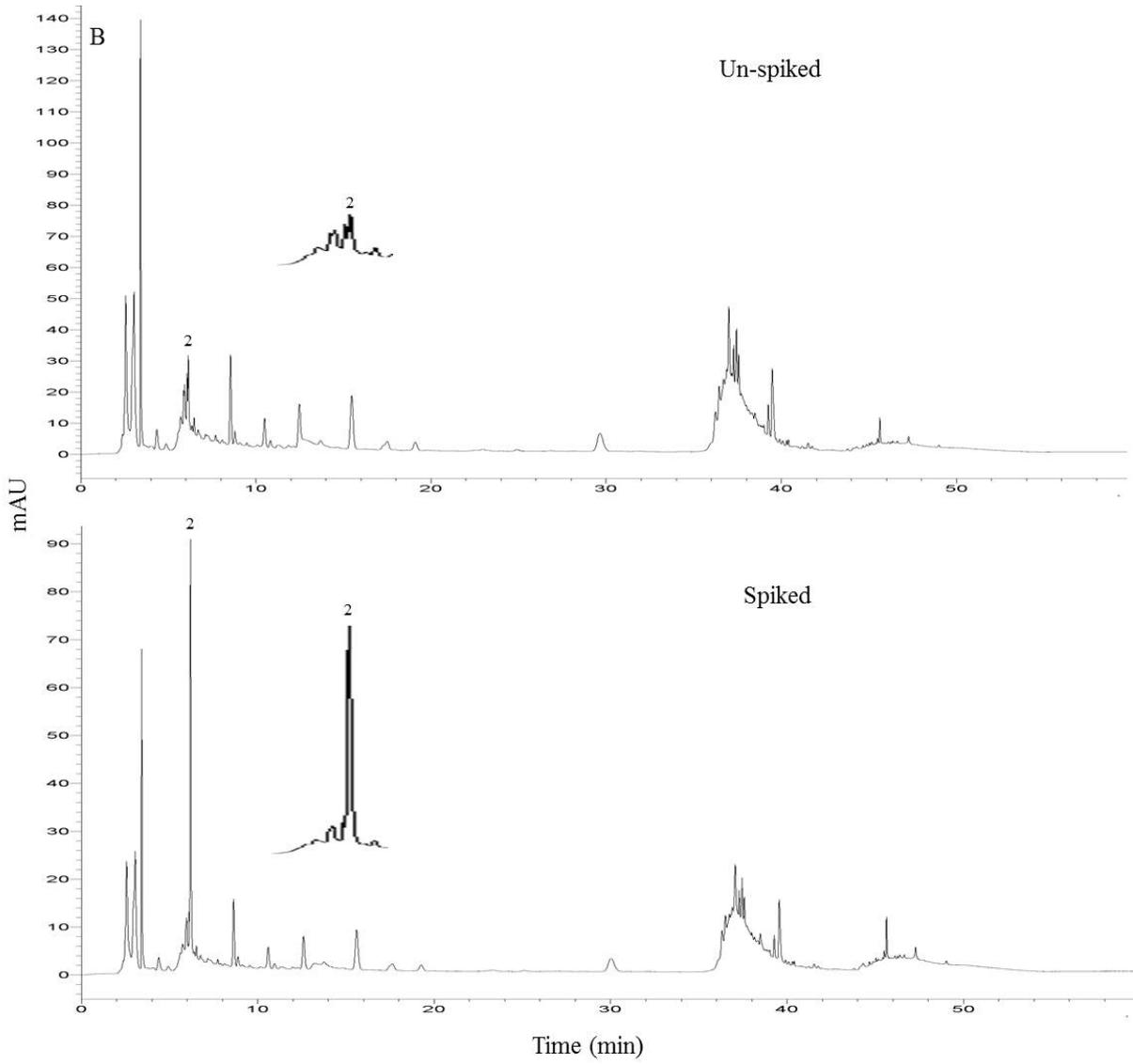
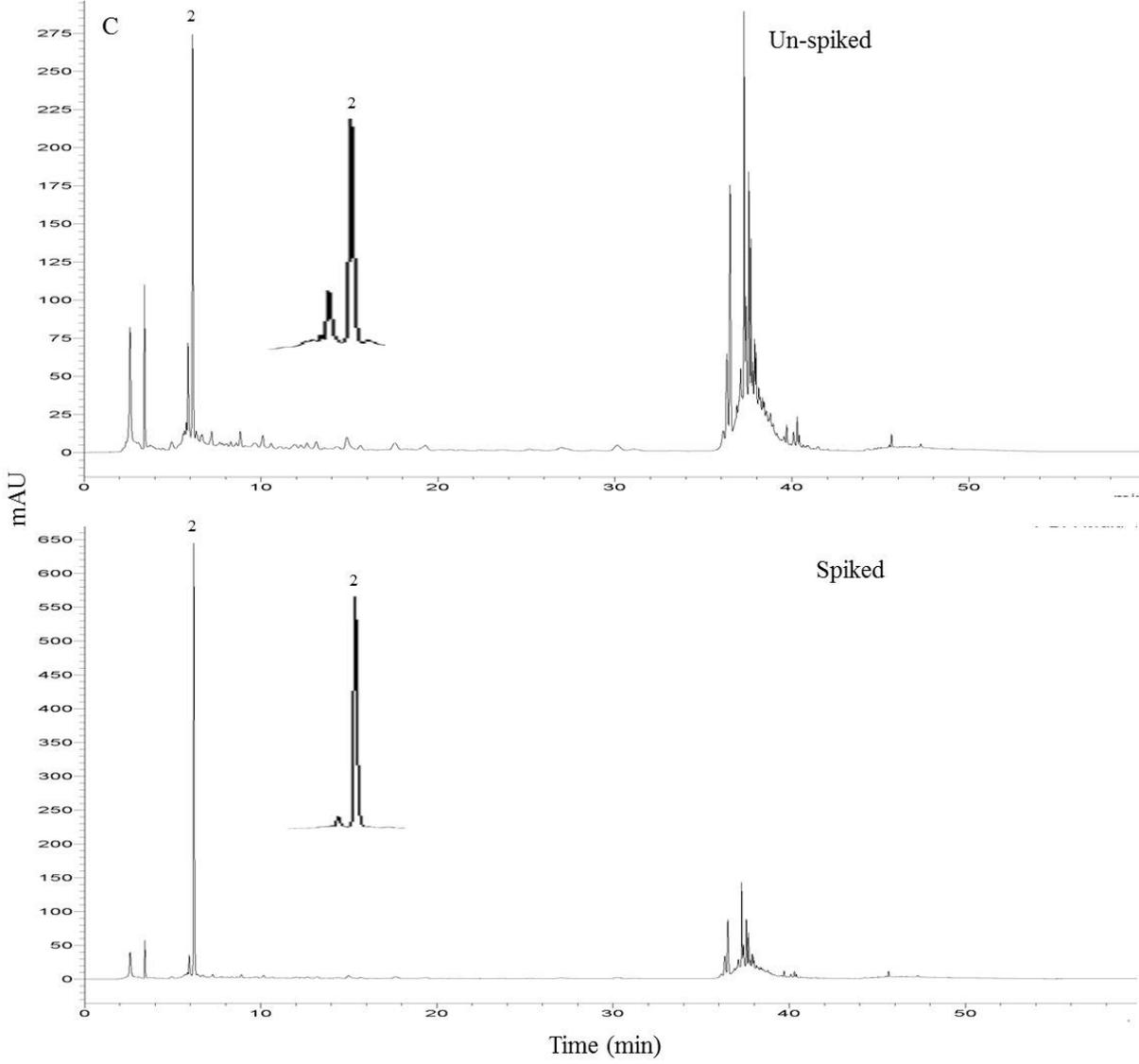
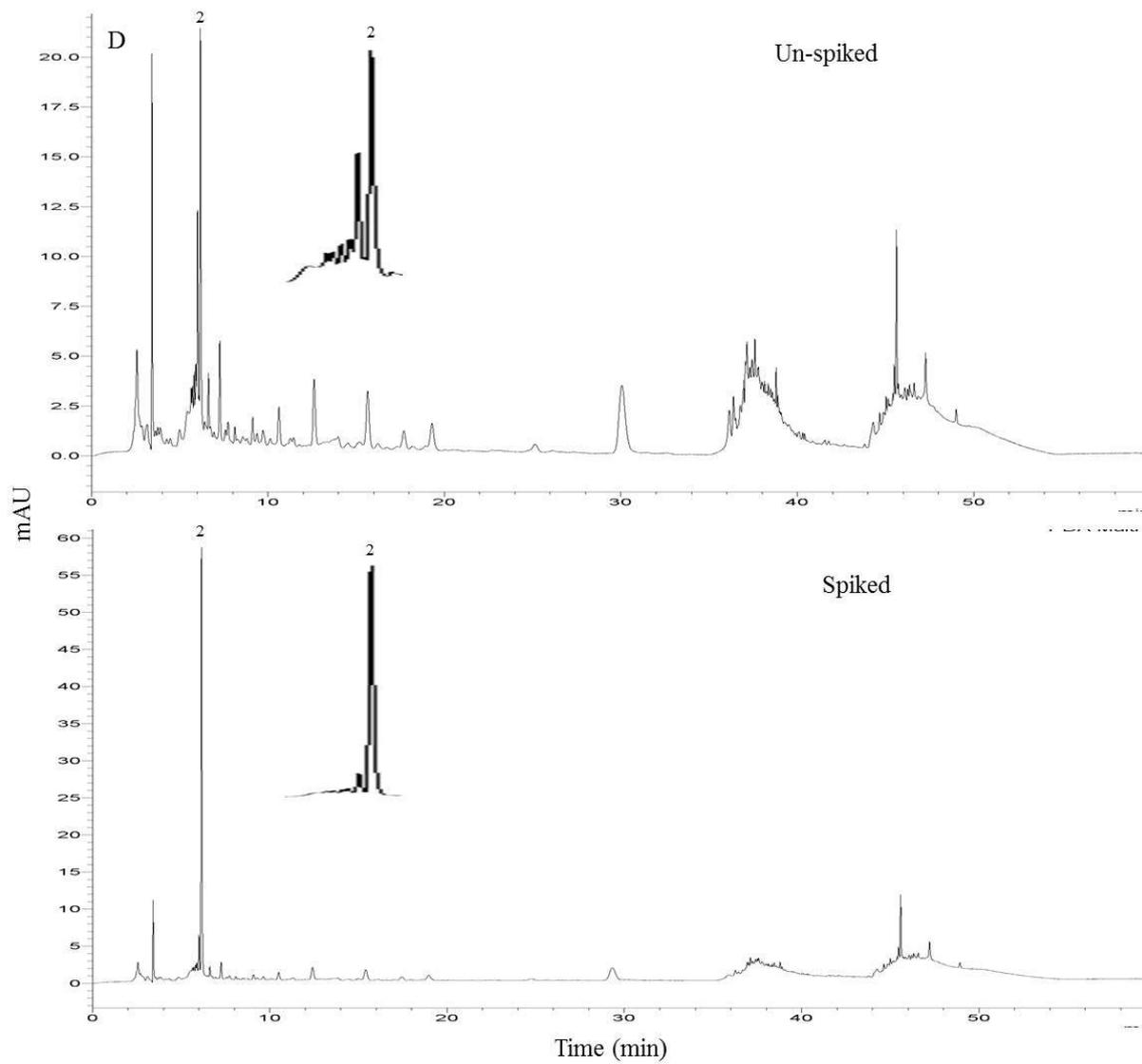


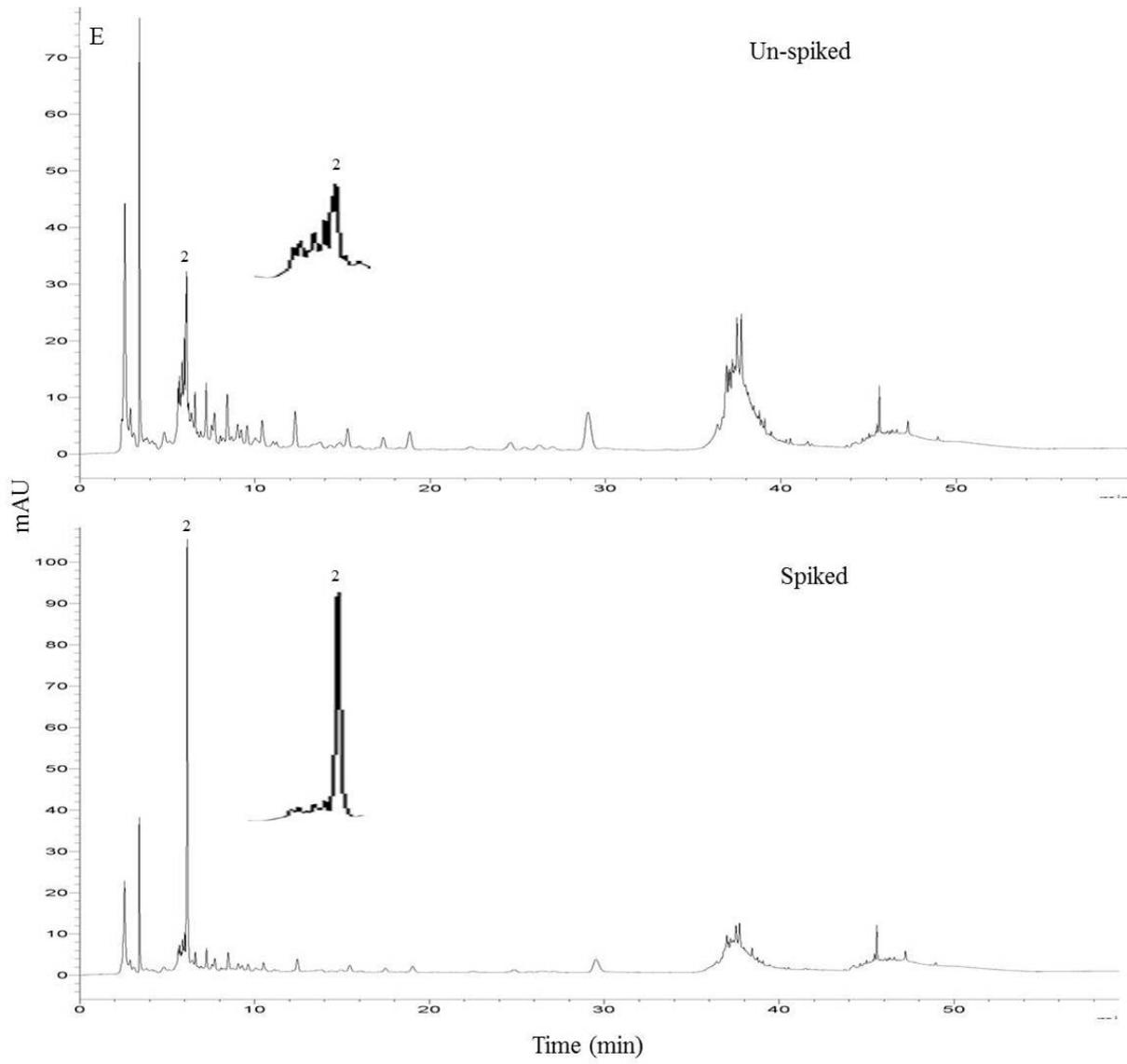
Figure S2¹. HPLC analysis. Chromatograms of the standards and the extracts of different organs of *Phragmites australis*: **A.** Gallic acid; **B.** Inflorescence (un-spiked and spiked); **C.** Leaf (un-spiked and spiked); **D.** Stem (un-spiked and spiked); **E.** Rhizome (un-spiked and spiked); and **F.** Root (un-spiked and spiked) with insets only in plant samples showing enlargement of the range of the peaks in the vicinity of gallic acid. The elution pattern was monitored at 271 nm and extracts were spiked with gallic acid.











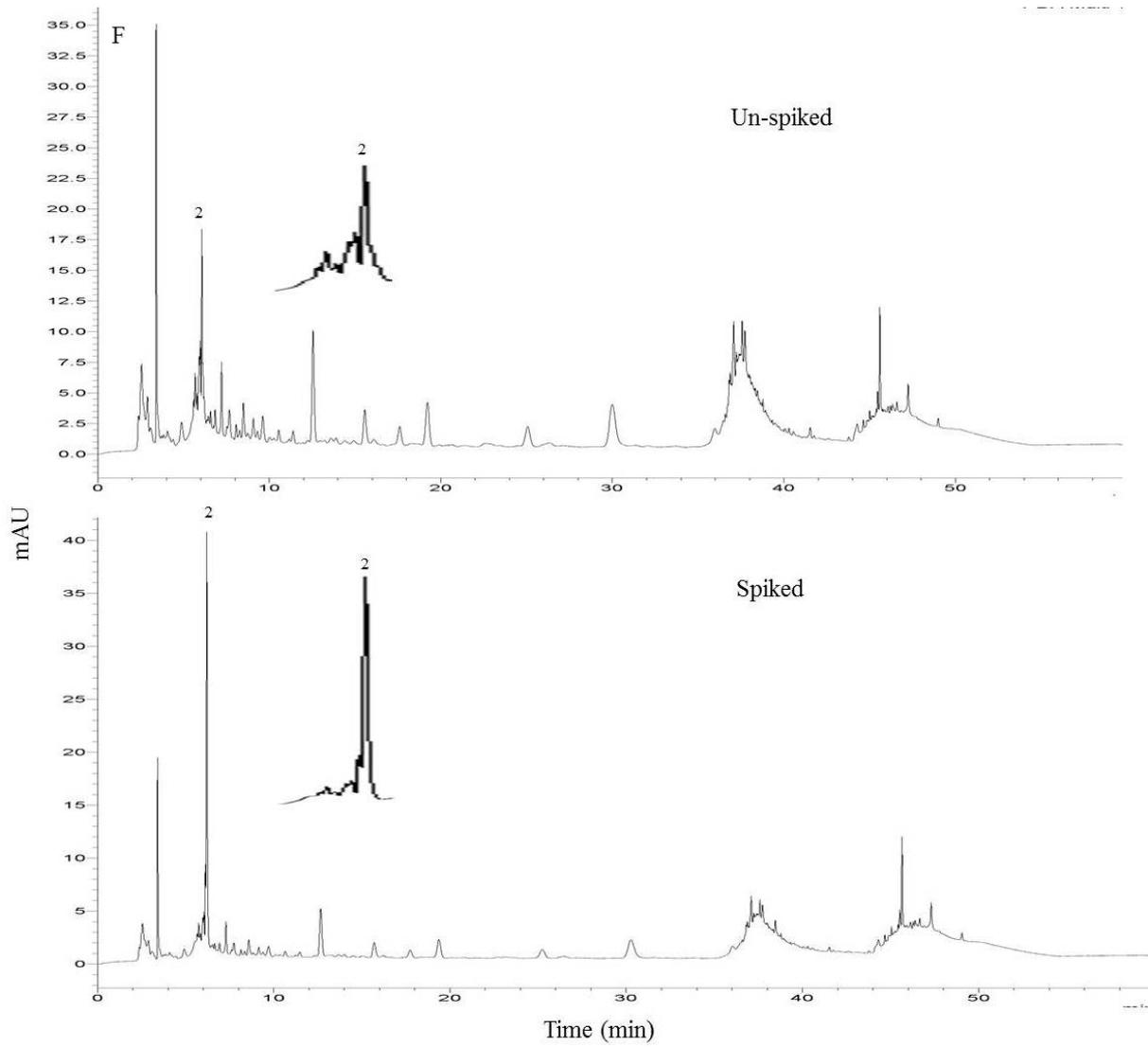
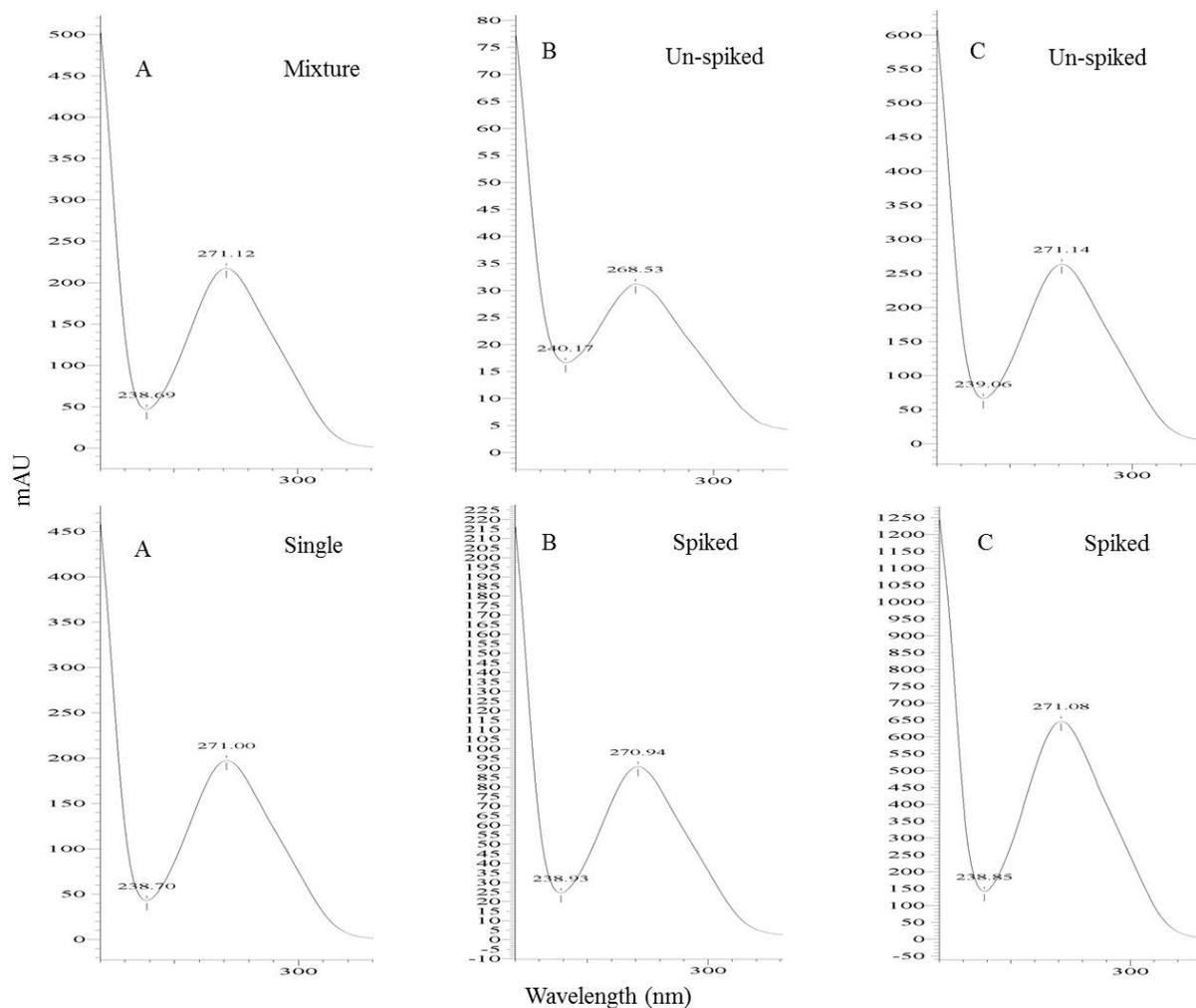
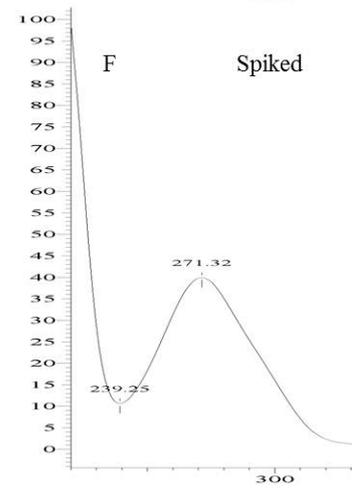
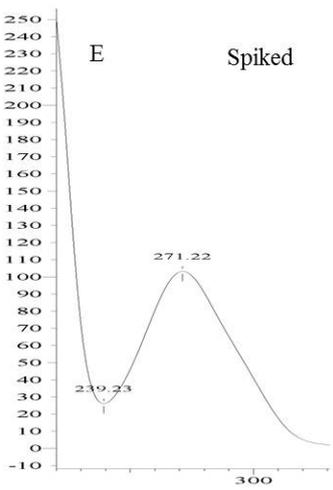
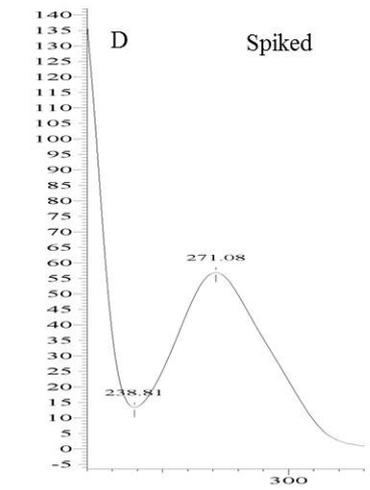
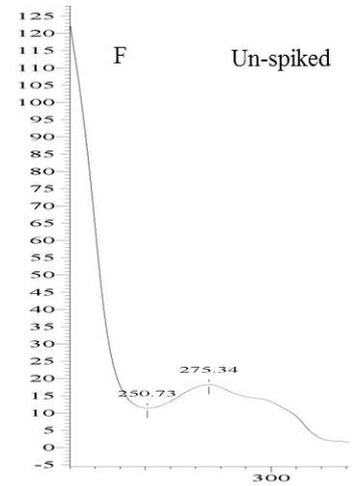
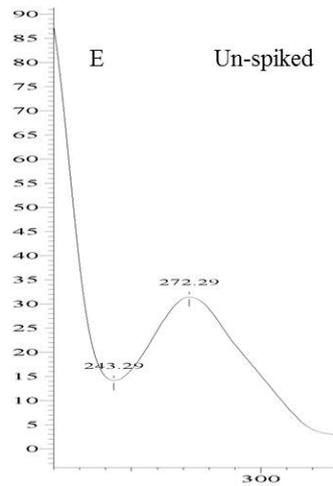
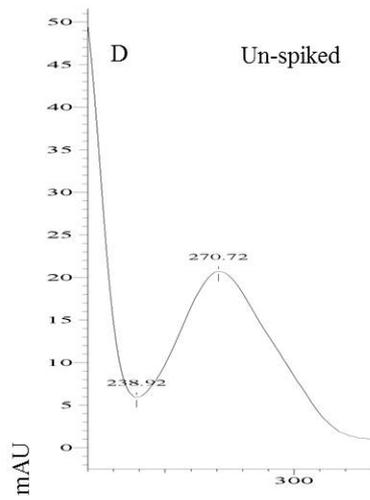


Figure S3¹. HPLC analysis. UV spectrums of gallic acid as standard and in extracts of different organs of *Phragmites australis*: **A.** Mixture of standards and single; **B.** Inflorescence (un-spiked and spiked); **C.** Leaf (un-spiked and spiked); **D.** Stem (un-spiked and spiked); **E.** Rhizome (un-spiked and spiked); and **F.** Root (un-spiked and spiked). The extracts were spiked with gallic acid.





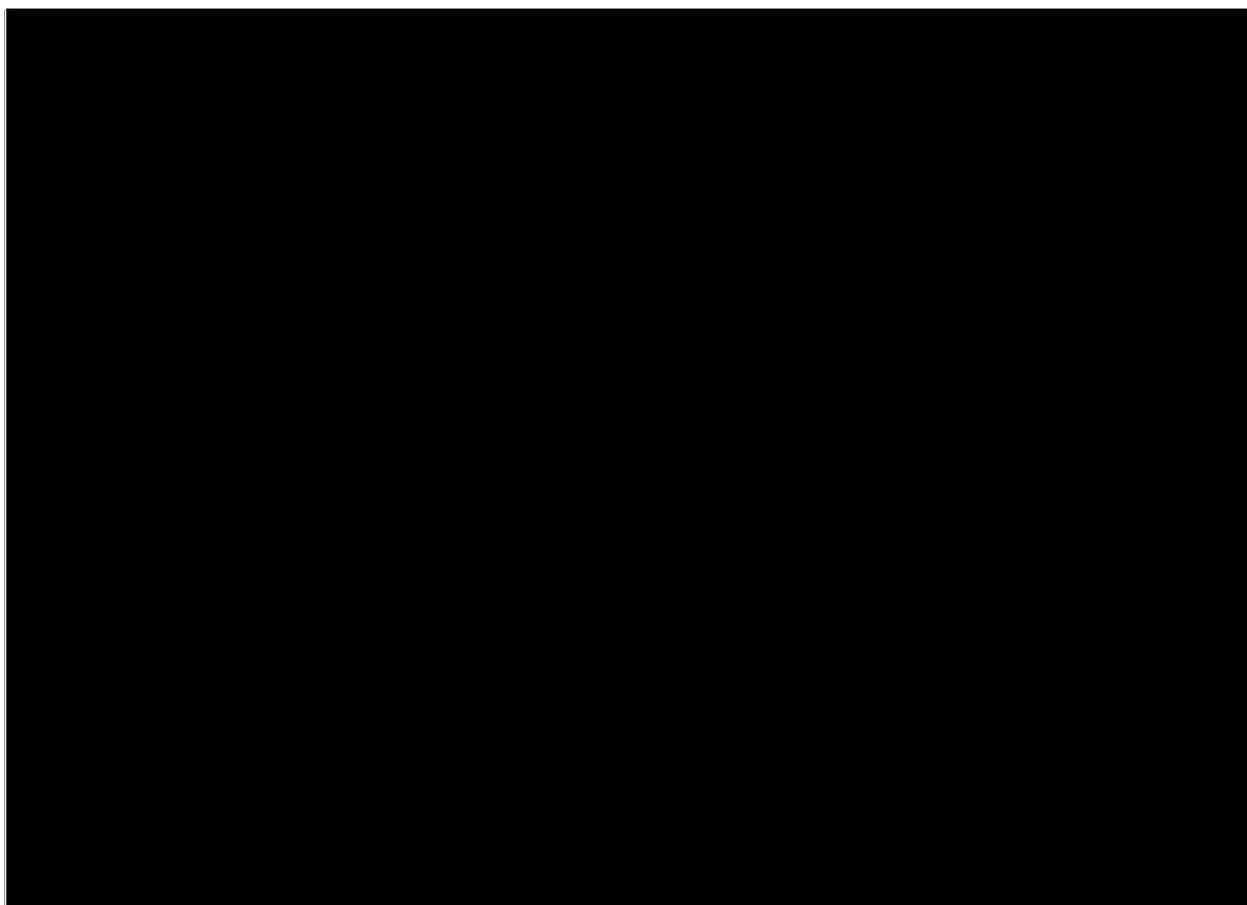
Wavelength (nm)

Chapter Four

Is Phytotoxicity of Phragmites australis Residue Influenced by Decomposition Condition, Time, and Density?

Introduction

We investigated the concentration and dynamics of allelochemicals, including other physicochemical variables of extracts during decomposition of *Phragmites* residue in soil systems using aerobic and anaerobic conditions in the laboratory. We also investigated the effects of the extracts on root elongation of *Lactuca sativa*, and seed germination and growth of *Poa labillardierei*. Additionally, we studied the effects of long-term (6-months) decomposed residues with consideration of residue density in soil, on the germination and growth of *Melaleuca ericifolia*.



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PART B:

**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS
 INCORPORATED IN THESIS BY PUBLICATION**

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Md. Nazim Uddin

Signature: 

Date: 24/07/2014

Paper Title: Is phytotoxicity of *Phragmites australis* residue influenced by decomposition condition, time, and density?

In the case of the above publication, the following authors contributed to the work as follows:

| Name | Contribution % | Nature of contribution |
|---------------------|----------------|---|
| Md. Nazim Uddin | 77 | Concept development; plant residue and soil collection; conducting experiments, chemical and ion analysis; data collection, statistical analysis and interpretation; and manuscript writing, editing and submitting for publication |
| Randall W. Robinson | 15 | Concept development and manuscript editing |
| Domenico Caridi | 3 | Manuscript editing |
| Md. A Y Harun | 5 | Data collection and manuscript editing |

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): College of Science & Engineering, Victoria University, Melbourne, Victoria, Australia

and will be held for at least five years from the date indicated below:

| | | Date |
|-------------|---|------------|
| Signature 1 |  | 18/07/2014 |
| Signature 2 |  | 18/07/2014 |
| Signature 3 |  | 21/7/2014 |
| Signature 4 |  | 18/07/2014 |

Is phytotoxicity of *Phragmites australis* residue influenced by decomposition condition, time, and density?

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Abstract. *Phragmites australis* is an invasive wetland plant and allelopathy appears to contribute to its invasiveness. We studied dynamics of physico-chemical characteristics and phytotoxicity through residue decomposition of *Phragmites* with and without soil under different conditions and density over time. Physico-chemical variables (water soluble phenolics, dissolved organic carbon, specific ultraviolet absorbance, pH, electrical conductivity, osmotic potential and some anions, namely PO_4^{3-} , Cl^- , NO_2^- , NO_3^- and SO_4^{2-}) of extracts were more consistent and showed normal range in aerobic rather than anaerobic conditions. ‘Residue alone’ and ‘residue with soil’ extracts exhibited significant inhibition on germination and growth of *Poa labillardierei* and *Lactuca sativa* initially but reduced over time in aerobic conditions whereas the inhibition increased sharply and remained almost stable in anaerobic conditions ($P \leq 0.001$). Regression analyses showed that water-soluble phenolic was significant predictor of the inhibitory effects on germination and growth of the tested species compared with other variables in the extracts. Long-term decomposed residues exhibited significant effects on germination and growth of *Melaleuca ericifolia* ($P \leq 0.01$) depending on residue density in soil. The results demonstrated that decomposition condition and soil incorporation coupled with residue density may play a crucial role

over time in dynamics of physico-chemical variables and associated phytotoxicity. The study contributes to understanding of the ecological consequences of phytotoxins in residue decomposition, partially explaining the invasion process of *Phragmites* in wetlands and thereby improving in wetland management.

Additional keywords: aerobic-anaerobic condition, ecosystems, residue density, soil, wetlands.

Introduction

Phragmites australis (hereafter called *Phragmites*), an invasive plant, dominates a wide variety of wetland ecosystems in temperate regions throughout the world (Adams and Bate 1999). It grows in aquatic, semi-aquatic and even moist terrestrial environments. *Phragmites* grows across a broad climatic and altitudinal range in Australia (Hocking 1989); being especially common in south-eastern Australia (Morris *et al.* 2008; Uddin *et al.* 2013). Although *Phragmites* is generally considered native in Australia, some authors still consider it a weed and there are unanswered questions concerning the nativity of all gene pools (Hocking *et al.* 1983). It is a noxious weed in North America (Ailstock *et al.* 2001), European countries (Haslam 1972) and most part of Canada (Mal and Narine 2004). The rapid spread of *Phragmites* in most of the wetlands in Australia in recent times is a great conservation concern. *Phragmites* generally forms mono-specific stands in ecosystems and consequently suppresses neighbouring plant species (Meyerson *et al.* 2000). The ecological impacts of *Phragmites* invasions are many, the most notable being habitat and subsequent biodiversity loss (Mazzoleni *et al.* 2007) and native species extinction (Silliman and Bertness 2004). A range of mechanisms may be given to explain the invasiveness of plant species in general, including resource competition, allelochemical phytotoxicity and alteration of ecosystem processes (Levine *et al.* 2003). Allelopathy is increasingly seen as a major pathway for the

invasive process, especially in plants classified as 'weeds' (Bais *et al.* 2003; Callaway and Ridenour 2004; Rama Devi and Prasad 1996). Allelochemical phytotoxicity may arise by leaching of living plant parts, root exudates, volatilisation, residue decomposition, microbial activity and even certain agricultural practices (Inderjit 1996).

Formation of dense plant residue and subsequent decomposition is a key ecosystem process for carbon fractionation and nutrient cycling influencing seed germination (Zancola *et al.* 2000), seedling growth, establishment and regeneration of plant species (Cuneo and Leishman 2012; Facelli and Pickett 1991). The conditions for decomposition vary from habitat to habitat, especially in wetland soils where the supply of oxygen can be significantly reduced, i.e. anaerobic conditions (McLatchey and Reddy 1998). Notably, anaerobic conditions have been associated with high soil toxicity (Patrick 1971). More specifically, volatile fatty acids and other organic acids may be produced during anaerobic condition due to limited microbial synthesis (Patrick and Koch 1958). Residue decomposition, the introduction of carbon, has been considered to be the most effective method of releasing allelochemicals (Reigosa *et al.* 1999).

Phragmites-dominated ecosystems produce large volumes of above and belowground biomass (Park and Blossey 2008). Total biomass production of more than 200 t ha⁻¹ have been recorded (Engloner 2009) making *Phragmites* one of the largest biomass producers in aquatic ecosystems. The worldwide distribution and the extremely large areas covered by *Phragmites* stands may have a considerable effect on the accumulation of phytotoxins through decomposition in wetlands. Deposition of allelochemicals on this scale may actually create a 'leading edge' of plant death and damage (Cruz-Ortega *et al.* 2002). Our field observations indicate that most of the wetlands occupied by *Phragmites* have a thick bed of residue and we suspect that allelochemicals are released into the soil and water through decomposition. Some long-

term invaded wetlands in Australia have no other plant species recorded within the stands of *Phragmites* compared with newly invaded sites that are floristically more diverse. The absence of other species in long-established stands may be due to the formation of more toxic and persistent breakdown products over time. Our previous studies showed that aqueous extracts of different organs of *Phragmites* inhibit germination, growth and some biochemical parameters of different test and associated plant species (Kettenring *et al.* 2011; Uddin *et al.* 2012) and gallic acid in different organs of *Phragmites* has been identified as a potent phytotoxin (Kettenring, McCormick *et al.* 2011; Rudrappa *et al.* 2007). Some earlier publications revealed that *Phragmites*-invaded soil contains large amounts of allelochemicals that are potentially phytotoxic to plants (Armstrong and Armstrong 1999; Bains *et al.* 2009; Rudrappa, Bonsall *et al.* 2007). Once evidence of allelopathy is obtained from plant growth studies, more supportive analytical data are needed from analyses of allelochemical concentration and dynamics in the soil in validating a hypothesis of allelopathic interferences.

Environmental processes for decomposition of plant residues seem to play a significant role in enhancing the toxicity of allelochemicals from *Phragmites*. Anaerobic decomposition in wetlands plays a critical role in determining plant community structure through amelioration of phytotoxicity of allelochemicals but no studies have been conducted so far considering this issue in *Phragmites*-dominated wetland. Most phytotoxicity studies examine the effect of plant allelochemicals only on seed germination and growth using bioassays in isolated Petri dishes and consequently ignore the effects of soil microorganisms (Kaur *et al.* 2009) but the role of soil and soil microorganisms may significantly degrade and transform allelochemicals in soil systems (Ehlers 2011). Toxicity of allelochemicals is a function of both concentration

and flux rates in the soil and rhizosphere. Therefore, it is important to monitor the dynamics of allelochemicals during decomposition in soil to get a clear idea about the phenomenon of allelopathy. Detailed knowledge about the dynamics of allelochemicals from residue decomposition as an invasive mechanism of *Phragmites* is essential to restore and/ or maintain the wetland.

We investigated the concentration and dynamics of allelochemicals, including other physico-chemical variables of extracts during decomposition of *Phragmites* residue, in soil systems using aerobic and anaerobic conditions in the laboratory. We also investigated the effects of the extracts on root elongation of the model plant, *Lactuca sativa* (hereafter called lettuce) and seed germination and growth of associated plant species, *Poa labillardierei* (hereafter called *Poa*). Additionally, we studied the effects of long-term (about 6-months) decomposed residues with consideration of residue density in soil, on the germination and growth of another associated plant species, *Melaleuca ericifolia* (hereafter called *Melaleuca*) to confirm the presence and potential of allelochemicals even after long-term decomposition in soil. In this study, we conducted the above-mentioned ecologically realistic experiments to address the following questions: (1) What are the differential effect of aerobic vs. anaerobic condition in decomposition processes?; (2) What are the effects of soil incorporation in residue decomposition?; (3) What is the impact of extracts from decomposed materials on seed germination and seedling growth? and (4) Do allelochemicals retain differing levels of concentration depending on residue density after long-term decomposition and does this differential affect the germination and growth of associated plant species disproportionately? These results could be important for effective conservation management in wetland invaded by *Phragmites*.

Materials and methods

Study site, plant and soil sample collection

We collected fallen plant residues (above ground tissue i.e. leaves and stems), mostly leaves, in June 2011 from natural stands adjacent to Cherry lake (37° 51' 30"S, 144° 50' 5"E), a freshwater wetland in Altona, Melbourne, Australia. The salinity level in surface water varied from 0.200 ‰ to 0.236 ‰ and the wetland is saturated with water seasonally. All samples were placed into sealable plastic bags for transportation to the laboratory. Plant samples were sorted from another plant residue and debris then kept at room temperature to air dry until constant dry weight. After desiccation the sorted samples were cut into small pieces (< 2 cm) and preserved in plastic ziplock bag until experiment. Soil samples were collected from the top layer of *Phragmites*-infested and *Phragmites*-free areas of the same study site, and dried in room temperature.

Seed selection and collection

Commercially available *Lactuca sativa* var. all the year round (lettuce), (DT Brown Seeds, South Windsor, NSW, Australia), was used because of its sensitivity and common use in phyto-toxicity bioassay. The use of known susceptible species gives at least some insight and being a 'known' entity allows for easy comparison to other studies. In addition, lettuce is easily grown, minimising the risk of observed growth differences being due to factors other than the treatments applied in the experiments.

Poa labillardierei is a species of tussock grass that is endemic to Australia (Groves *et al.* 1973). It is widely distributed in the south-eastern corner of Australia, mainly in coastal and wetland zones (May and Campbell 1991). *Melaleuca ericifolia* is a native shrub that is distributed extensively in coastal and near-coastal freshwater and brackish-water wetlands across south-eastern Australia. Most species of the genus *Melaleuca* are

reliant on seed alone for reproduction; however, *Melaleuca* can form extensive clonal stands through the production of ramets that can become physically independent of the parent genet once sexual recruitment has taken place (Robinson *et al.* 2008). These two associated species naturally coexist with *Phragmites* in most of the wetlands of south-eastern Australia. Recent studies postulated that invasions of *Phragmites* in *Melaleuca*-dominated wetland might directly disadvantage *Melaleuca* through allelopathy from root exudates or indirectly by altering interactions with soil microbes (Morris, Boon *et al.* 2008). It is critical that the relationship between *Phragmites* and *Melaleuca* be established if already depleted *Melaleuca* wetlands are to be conserved. Seeds of *Poa* were collected from Iramoo native plant nursery, Victoria University, Melbourne, Australia. Seed capsules of *Melaleuca* were collected in May 2011 from Cherry Lake and stored in paper bags at room temperature for 1-week. Seeds were shaken from the capsules, sieved to remove empty capsules and other detritus and stored in room temperature for experiment.

Laboratory decomposition experimental design

Three treatment series (residue alone, residue with soil and soil alone) and two types of decomposition (aerobic and anaerobic) were maintained in the laboratory. In the case of the first treatment, 4 g of dried and ground *Phragmites* residue (above-ground tissue i.e. leaves and stems) was placed in a 500 mL glass jar with 100 mL distilled water. For the second treatment, 4 g of residue was placed in 100 g soil and 50 mL distilled water was added to initiate field conditions. For the soil alone treatment, 100 g soil was moistened with 50 mL distilled water. Microbial inoculum (10%) was obtained from a mixture of water and *Phragmites*-infested soil. The inoculum solution in equal amount was added to each treatment and incubated in room temperature. Distilled water was added to the pot to compensate for the evaporative loss. Three replicates were set for each treatment

and sampling was carried out each week for the 5-week incubation period. Aqueous extracts were obtained by adding 100 ml distilled water to the second and third treatment and shaking in an orbital shaker for 1 h at room temperature. The samples were then centrifuged for 15 min at 3951 g (3000 rpm) at room temperature, sterilised (microfiltration with 0.22 µm pore filter) and stored at - 20°C until chemical analyses and bioassay.

Physicochemical analyses of the extracts

pH and electrical conductivity (EC) was determined with a pH meter (Pocket digital pH meter, 99559, Dick-smith electronics, Australia) and conductivity meter (TPS Digital conductivity meter, 2100, TPS Pty Ltd., Australia). Osmotic potential (OP) was calculated using the equation ($OP = EC * - 0.36$) according to McIntyre (1980). Measurements of dissolved organic carbon (DOC) concentration were conducted using a TOC analyser (TOC-V with TN detector, Shimadzu, Kyoto, Japan). Specific ultra violet absorbance (SUVA) was determined by dividing its UV absorbance at 254 nm (measured using UV/Visible spectrophotometer, Biochrom Libra S12, England) with its DOC concentration (Strauss and Lamberti 2002). Major ions (PO_4^{3-} , Cl^- , NO_2^- , NO_3^- and SO_4^{2-}) were analysed using an ion chromatograph (Shimadzu Ion Chromotograph, Kyoto, Japan). Total phenolics (TP) and water soluble phenolics (WSP) of plant residues (live and dead), extracted with 70% acetone and distilled water respectively were measured according to Singleton and Rossi (1965) with gallic acid used as the standard.

Bioassays with the aqueous extracts

Pre-germinated lettuce seedlings (10) with visible radicles not exceeding 1 mm in length were placed on Whatman No. 1 filter paper moistened with 5 mL sample extracts

at equal distance into a sterile 9 cm Petri dish. All extracts were buffered with 0.01M sodium phosphate buffer and pH values of the extracts including distilled water (control) were adjusted to 6.5 with 1N NaOH and 1N HCl. Three replicates were maintained for all treatments in complete randomized design (CRD). The prepared dishes were wrapped with paraffin film and kept in a germination growth chamber set to 25/15°C day/night temperature and a 12h photoperiod with illumination of $84\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. After 7 days, root-shoot length and root-shoot biomass were measured. Chlorophyll in the leaves of emerged seedlings was determined using the method of Inskeep and Bloom (1985). In the case of the *Poa* germination and growth experiment, 20 surface sterilised seeds were placed in the above-mentioned conditions with three replicates for all treatments. Germinated seeds were counted each alternative day for 16 days until the point where cumulative germination levelled off in all treatments. Germination indices (germination percentage and rate) and biometric characteristics (length and biomass of root and tiller) were measured at the end of the experiment.

Greenhouse decomposition experimental design and bioassay in laboratory

The chopped *Phragmites* residues (< 2 cm long) were mixed with 1-kg potting mix soil in a 1.5 L pot containing five different densities (0%, 1.25%, 2.5%, 5% and 10%) by weight with three replicates. Pots were kept in a naturally lit greenhouse at $23 \pm 3^\circ\text{C}$ and $12 \pm 2^\circ\text{C}$ day/night temperature as well as watered regularly with an auto-irrigation system to keep soil moist at a level of $55 \pm 5\%$. They were randomized once a week and unwanted germinants were removed. After 6 months, the decomposed residues were dried at room temperature for use in the experiments. Glass dishes (12 cm by 6 cm) containing 100 g of soil with prepared decomposed residues in same concentrations were moistened uniformly with distilled water and incubated for 48 h at 25°C with two sets of independent experiments, one for germination and another for growth and

establishment. Subsequently, *Melaleuca* seeds were sown on the soil surface and the dishes were wrapped with transparent plastic film and incubated to 25/15°C day/night temperature and a 12 h photoperiod with illumination of 84 $\mu\text{mol s}^{-1}\text{m}^{-2}$. After 2 weeks, the germination experiment was terminated and the number of germinant was counted. In another experiment, the seedlings were thinned in equal number (10 per dish) for growth and establishment observation. Biometric parameters (root-shoot length and biomass) and chlorophyll were measured after 3 months.

Data analyses

All the experiments were conducted in a CRD with at least three replicates. Mean \pm standard error (s. e.) was computed by using raw data. Data were transformed according to the requirements of ANOVA and other statistical tests. Three-way ANOVA was done to test the main effects and interactions among decomposition conditions denoted by C (aerobic versus anaerobic), materials denoted by M (residue alone, residue with soil, and soil alone) and decomposition time denoted by T (5-week period) on the germination and growth inhibition of *Poa* seeds and lettuce seedlings. Three-way ANOVA was also used to find out the effects on the physico-chemical composition of the extracts. One-way ANOVA was performed to test the phytotoxic effect of long term decomposed materials on the germination and growth parameters of *Melaleuca*. Linear regression was used to measure the phytotoxic effect of the extracts on different plant parameters as well as the effect of decomposition on different physico-chemical characteristics of the extracts. Pearson correlation was performed to examine relationships among the measured variables. All data were analysed using IBM SPSS statistics 20.0 software.

Results

Dynamics of physico-chemical characteristics of the extracts

TP and WSP concentrations in live and dead plant materials of *Phragmites* were significantly different and leaves had higher concentrations than the stems (Fig. 1). TP and WSP in live leaves were 32.1 and 11.41 mg g⁻¹ whereas dead leaves had 18.77 and 4.59 mg g⁻¹ respectively (Fig. 1). In the case of aerobic decomposition, rapid reduction of WSP concentration was noticed in the 'residue alone' treatment, but the rate of reduction slowed after the second week and continued until the third week, leading to an almost constant level for the rest of the experiment time. The same phenomenon was observed for 'residue with soil' treatment although constant level was reached slightly earlier. Phenolics in the 'soil alone' treatment remained almost constant over the period (Fig. 2a). The final stable level of phenolics in 'residue alone' and 'residue with soil' decomposition were 150 µg mL⁻¹ and 40 µg mL⁻¹ respectively. In the case of anaerobic conditions, the initial (200.80 and 74.89 µg mL⁻¹) and final (181.18 and 60.53 µg mL⁻¹) concentrations of WSP were almost the same after following an inconsistency pattern in 'residue alone' and 'residue with soil' treatments respectively while the 'soil alone' treatment followed that for aerobic decomposition (Fig. 2a). The quantity of WSP in anaerobic decomposition was always higher than in aerobic. WSP showed strong significant correlations with EC ($r = 0.90^*$), OP ($r = 0.90^*$), DOC ($r = 0.862^*$) and SUVA₂₅₄ ($r = 0.72^*$) at 1% level of probability. WSP was significantly influenced individually and interactively ($F_{10, 72} = 165.63, P < 0.001$) in all factors.

DOC in both aerobic and anaerobic condition showed a similar pattern of change to WSP for all treatments (Fig. 2b). However, an exception was found for the 'residue alone' treatment in the first week when DOC reduced more sharply than that of WSP (Fig. 2b). Consequently, the final DOC concentration in the 'residue alone'

treatment in anaerobic condition was much lower than the initial level. It was observed that the concentrations fluctuated for both treatments but they were always higher in extracts in anaerobic conditions. The ranges of DOC were 1249.83-331.75 mg L⁻¹ and 1652.00-538.00 mg L⁻¹ in residue alone as well as 407.97-117.24 mg L⁻¹ and 609.83-112.62 mg L⁻¹ in residue with soil for aerobic and anaerobic conditions respectively. There was significant interaction ($F_{10, 72} = 27.19, P < 0.001$) and main effect on DOC. SUVA₂₅₄ is an average absorptivity for all the molecules that comprise the DOC in the extracts and it has been used as a surrogate measurement of DOC aromaticity. A significant correlation was found between DOC with pH ($r = 0.32^{**}$), EC ($r = 0.78^{**}$), OP ($r = 0.78^{**}$), WSP ($r = 0.86^{**}$), and SUVA₂₅₄ ($r = 0.78^{**}$) of extracts. The results clearly showed a relative increase in the values of SUVA₂₅₄ with decomposition over time in all treatments of aerobic and anaerobic conditions but in the case of anaerobic conditions it fluctuated (Fig. 3). Increasing SUVA₂₅₄ indicates increasing aromaticity of DOC.

pH, EC and OP of all extracts showed dynamic changes over the decomposition period. Boxplots have been presented to compare the distribution of data on these parameters acquired from all treatments (Fig. 4). In the case of aerobic conditions, the changes followed a regular trend but large variations were observed in anaerobic conditions over the whole decomposition period. pH levels ($F_{10, 72} = 46.78, P < 0.001$), EC ($F_{10, 72} = 10.34, P < 0.001$) and OP ($F_{10, 72} = 10.34, P < 0.001$) were significantly affected by the interaction of decomposing materials, time and condition. In most cases, pH increased in aerobic conditions and decreased under anaerobic conditions, whereas EC and OP showed the opposite. Temporal variations of nutrient concentrations (PO₄³⁻, Cl⁻, N (NO₂⁻, NO₃⁻) and SO₄²⁻) were found in both conditions and are illustrated in boxplots (Fig. 4). Nitrogen disappeared sharply in all treatments of aerobic

decomposition with an appearance again in the late stages of decomposition whereas in anaerobic conditions it was absent from the start with very negligible amounts periodically. PO_4^{3-} concentrations in aerobic conditions decreased rapidly and was completely eliminated after 3-weeks incubation, whereas concentrations were high and fluctuated over the whole decomposition period in anaerobic conditions. Similarly, SO_4^{2-} reduced quickly in aerobic conditions, whereas anaerobic condition showed very slow reduction. In contrast, Cl^- was almost constant in all conditions.

Phytotoxicity of aqueous extracts

Decomposing materials, conditions and time all exerted highly significant ($P < 0.001$) effects individually and interactively on root and shoot length of lettuce (Table 1). Root dry biomass was inhibited significantly (Table 1) whereas shoot dry biomass was stimulated, but root-shoot biomass ratio showed a significant interactive effect (Table 1). Phytotoxicity was relatively higher in all treatments at the initial stage and gradually decreased in aerobic condition but exhibited marked variability in anaerobic conditions. Root and shoot length were significantly inhibited by the 'residue alone' and 'soil with residue' treatment in both conditions with much stronger negative effects in anaerobic conditions (Fig. 5). In the case of aerobic conditions, the average root length inhibitions were 31.84% and 17.24% in 'residue' and 'residue with soil' treatment respectively whereas in anaerobic condition they were 63.95% and 41.98% accordingly. Significant correlation was found between WSP and root length of lettuce in both conditions but anaerobic conditions showed higher phytotoxicity than aerobic (Fig.6). The phytotoxicity of all treatments was closely correlated to their corresponding WSP. In general, high WSP exerted severe inhibition, while less inhibition or no inhibition or even stimulation was observed as WSP content decreased. Both root and shoot length had significant correlation with EC, OP, DOC and SUVA_{254} ; however, shoot length was

significantly correlated with WSP as well. Root biomass was significantly correlated only with pH. Total chlorophyll content was inhibited by decomposing materials ($F_{2,72} = 17.50, P < 0.001$) and time ($F_{5,72} = 6.84, P < 0.001$) but not condition ($F_{1,72} = 0.65, P < 0.422$), while interactions between terms were significant, $M \times C$ ($F_{2,72} = 3.49, P < 0.036$); $M \times T$ ($F_{10,72} = 2.45, P < 0.014$) and $C \times T$ ($F_{5,72} = 2.33, P < 0.05$). Total chlorophyll was significantly correlated with all measured physiochemical parameters.

Aqueous extracts of all treatments, except soil alone, significantly affected the germination rate compared with germination percentage in *Poa* (Fig. 7). The interactive effect on germination rate was more noticeable whereas individual effects existed in the case of materials and time (Table 2). Length (root and tiller) and biomass were significantly influenced more so than germination indices through both interactive and individual effect of all factors (Table 2). 'Residue alone' in both conditions reduced significantly all germination indices and biometric parameters than 'residue with soil' treatments but the inhibition was lowered as time proceeded (Figs.7, 8). Anaerobic conditions had more suppressive effects on root and tiller length than germination indices. The germination rate, root and tiller length and biomass were always higher and almost steady in aerobic conditions compared with anaerobic conditions in all treatments. Root length showed the most sensitivity to phytotoxins in extracts, especially in anaerobic conditions. In the case of aerobic decomposition, the root length inhibitions were 85.90% and 54.66% in residue alone and residue with soil treatments respectively at the beginning of the experiment whereas they were 90.85% and 76.13% for anaerobic conditions. However, all types of treatments in aerobic decomposition showed a significant and rapid decrease in their inhibitory effects during decomposition with low or even no inhibition whereas anaerobic decomposition caused inhibition unsteadily even at the end of the experiment. The regression analyses data showed that

WSP and DOC characteristics of all extracts were significant predictors of the inhibitory effect of germination and growth parameters of *Poa* (Table 3). Root and tiller length were more sensitive than germination indices to chemical changes.

Phytotoxic effect of long-term decomposed residues

Long-term decomposition of materials showed a significant effect on germination of *Melaleuca* seed (Fig. 9a). Germination varied significantly across the density treatments ($F_{4, 10} = 4.58, P < 0.023$), being reduced in all density treatments compared with the control, except at 1.25% concentration. A significant effect (18% mortality) was observed on the survival rate of *Melaleuca* seedlings in 5.0% and 10.0% residue compared with the control. The root and shoot length, and shoot biomass were not significantly affected across the treatments but the effect was significant on root biomass by 5.0% and 10.0% residue ($F_{4, 10} = 12.38, P < 0.001$) (Fig. 9b). In addition, chlorophyll content (chlorophyll *a*, chlorophyll *b* and total chlorophyll) was inhibited only in 10.0% residue (Fig. 9c).

Discussion

Dynamics of physico-chemical compositions

Phenolic content decreased in dead plant materials compared with live materials since phenolics apparently drain to wetland soils and water due to precipitation and other factors. The use of dead plant materials in our experiments, as collected from the field just after the natural fall, reflected natural decomposition and associated effects on the germination of plant species. Conditions during decomposition can strongly influence the phytotoxic potential of plant residues (Patrick 1971). In aerobic conditions, organic compounds disappear rapidly and microbial material is synthesized, whereas in anaerobic conditions, due to oxygen deficiency, volatile fatty acids and other organic

acids accumulate and suppress microbial synthesis and consequently enhance formation of different organic acids and phenolic compounds that have been found to have phytotoxic potential (Patrick 1971). Our findings aligned with other studies (Welbank 1963) that found that anaerobic decomposition of plant residue in soil is critical for phytotoxin formation and persistence; however, we also found that formation of these phytotoxins is highly dependent on time and density of residue.

Phytotoxins, (e.g. acetic, propanoic, *n*- and *iso*-butyric and *n*-caproic acids and sulphides) derived from decaying materials of *Phragmites* and organic matter deposited in sediment from eutrophic conditions in lakes might amplify the growth, morphological and anatomical symptoms of die-back of *Phragmites* itself as well as associated plant species at a concentration of ~ 1mM (Armstrong and Armstrong 2001). Certainly, allelochemicals from crop plants such as rye and corn may inhibit growth of lettuce at concentrations between 25 and 50 ppm (Chou and Patrick 1976). Soluble phenolics in our study exhibited phytotoxicity after decomposition similar to Bains et al. (2009) but also retained phytotoxicity even after 6 months of decomposition. The constant accumulation of phenolics in the field and their effects might strongly influence plant to plant interactions, especially in wetlands (Jarchow and Cook 2009). What we have observed is that decomposition of *Phragmites* residue did not diminish phytotoxicity completely in the short-term that also aligned with other studies that demonstrated phenolics through decomposition may persist for months or long time in soil environment (Rashid *et al.* 2010b).

Several studies have identified that death and decay of underground organs of *Phragmites* may produce a higher level of organic acid than normal and that these may be toxic to the associated plants and *Phragmites* itself (Armstrong *et al.* 1996; Čížková *et al.* 1999). *Phragmites* death can arise through eutrophic conditions that can lead to a

reduced organic layer depth through accelerated decomposition by phytoplanktonic blooms (Armstrong and Armstrong 1999). It has been observed that die-back of *Phragmites* can be attributed to phytotoxins from organic matter decomposition especially from *Phragmites* and algal blooms (Ostendorp 1989). Our study predicts that the same phenomenon might happen in *Phragmites*-dominated wetlands even in the absence of algal blooms.

Larger amounts of phenolics are released from decomposing plant residue than from throughfall in any natural plant community. Soluble phenolics in soil systems experience four different fates, namely biodegradation, transformation, adsorption and dissolution; finally, they remain in the ecosystem as a part of DOC (Hättenschwiler and Vitousek 2000). The decomposition kinetics pattern indicates the presence of labile and recalcitrant carbon fractions in the plant residue. The initial rapid loss of DOC found in this study corresponds to the decomposition of water-soluble amino acids, amino sugars and carbohydrates whereas the slow decline indicates the decomposition of structural components (Watkins and Barraclough 1996). The DOC pattern in aerobic versus anaerobic decomposition suggests that conditions are an important factor in determining the rate of decomposition (Otsuki and Hanya 1972). Our results found that anaerobic decomposition produced higher amounts of DOC than that of aerobic decomposition. The DOC produced from this plant residue had phytotoxic effects on plant growth. Molar absorptivity of the extracts had a linear relationship with aromaticity, providing an important clue about the chemical nature of DOC. The results of SUVA₂₅₄ demonstrate that increasing aromaticity is mainly due to the presence of highly conjugated aromatic structures that exhibit allelochemical effects on plant processes (Brunner *et al.* 1996).

pH results showed similarity with other studies (Bonanomi *et al.* 2006; Godshalk and Wetzel 1978) that found that pH increased due to the presence of organic compounds and decreased as a result of the production of acetic, formic and propanoic acids. The higher values of EC and OP in anaerobic condition and lower values in aerobic condition found in our study, whilst not unusual, have been rarely reported (Bonanomi *et al.* 2011). These parameters probably reflect the concentration of mineralised nutrients and certain water-soluble compounds in the extracts, a possibility suggested by other studies (Ebid *et al.* 2007; Rashid *et al.* 2010a).

Phytotoxicity assessment

Allelochemical phytotoxicity dynamics were wide-ranging within the experimental treatments. In aerobic conditions, phytotoxicity with 'residue alone' treatment steadily decreased as the decomposition proceeded. 'Soil alone' had no significant inhibition or stimulation and 'residue with soil' treatment showed a lower inhibition than residue alone in all sampling times. In the case of anaerobic conditions, the phytotoxicity was more severe in the 'residue alone' treatment than in aerobic conditions and an inverse trend was observed. 'Soil alone' had less inhibition in the first week whereas 'residue with soil' treatment exhibited a strong negative effect. We found that all treatments except 'soil alone' caused a strong inhibition of root length of lettuce and *Poa* compared with other biological parameters such as germination percentage and rate. These findings suggest that seed germination might happen in *Phragmites*-infested wetland but the seedlings may lose their fitness and survivability when in competition with other species that facilitate the invasion process. Some studies have shown that phytotoxicity increased with decomposition and then decreased (An *et al.* 2001) but our findings clearly show decreased phytotoxicity over time in all treatments in aerobic conditions and increased phytotoxicity over time in anaerobic conditions. The findings in this study

are more in line with several studies that found that phytotoxicity rapidly lowered in aerobic condition but increased and became stable in anaerobic conditions (Bonanomi, Sicurezza *et al.* 2006; González *et al.* 1995; Souto *et al.* 1994).

The results presented here clearly indicate that phytotoxicity is significantly influenced by materials, conditions and time of decomposition. Addition of soil lowered the phytotoxicity in both aerobic and anaerobic conditions. This lowering of phytotoxicity could be attributable to an alternation in phytotoxin production, driven by abiotic and biotic factors in soil (Blum *et al.* 1999), especially soil microorganisms through their role in decomposition and transformation of organic matter (Kaur, Kaur *et al.* 2009). The contrasting results between ‘residue alone’ and ‘residue with soil’ in both decomposing conditions in our studies support that microbial activity is the driving force of phytotoxicity dynamics. The correlation matrix among physico-chemical and biological parameters showed direct casual relationships that are deemed critical to understanding the very nature of phytotoxic nature of phytotoxins (An, Pratley *et al.* 2001; Bonanomi, Sicurezza *et al.* 2006; Brunner, Luster *et al.* 1996; Ohno and Doolan 2001). In addition, we might note that WSP and DOC of the extracts do not fully account for the phytotoxicity of ‘residue alone’ and ‘residue with soil’ treatments, suggesting that some other factors might be involved. Again, residue decomposition in wetlands is strongly controlled by environmental conditions, such as temperature, pH, salinity, and moisture content (Windham and Lathrop 1999). However, as all our experiments were carried out under standard conditions such as optimum temperature, light, water content and residue, further studies are required to monitor the dynamics of the chemical changes in decomposition and their associated inhibitory effects on seed germination and growth under limited environmental conditions because they have different implications in the rate of decomposition and their related impacts.

Low pH in anaerobic conditions may be effective in allelochemical solubility and may increase phytotoxicity (Armstrong and Armstrong 1999), a suggestion strongly shown and supported by our findings. Higher aromaticity is associated with highly conjugated aromatic structures such as coumarins, tannins, and flavonoids, may exhibit allelochemical stress on cell division and elongation, resulting in an abnormal radical growth (Brunner, Luster *et al.* 1996). In addition, high OPs in anaerobic conditions correspond to higher inhibition in terms of germination, growth and even in physiology (Kettenring, McCormick *et al.* 2011; Wardle *et al.* 1992). However, our overall results clearly show a dynamic phytotoxic pattern during the decomposition where the effects in anaerobic conditions were more persistent than in aerobic conditions.

The results of long-term decomposition experiments demonstrate that *Phragmites* residues could be phytotoxic on germination, growth and establishment, even after 6-months of decomposition, but it depends on residue density in soil. Limited inhibition or stimulation of germination and growth is often documented due to addition of low rates of residue, because this contributes to addition of available nutrient and improving soil structure whereas the greater the quantity of residue, the more phytotoxicity (Mason-Sedun and Jessop 1988). Other studies (Zuo *et al.* 2008) observed that allelopathic potential of wheat stubble remained for 5-9 years but that decomposition and accumulation rates were considerably slower despite being in an aerobic condition. The long-lasting phytotoxic effect might interact with other factors, such as availability of inorganic toxic compounds (Marschner 1995). Persistence and accumulation of phytotoxins may be frequent in wetlands due to water-logging. Additionally, the phytotoxic effects in natural systems might be higher than the observations in our study due to continuous accumulation of phytotoxins in the environment. Soil-mediated allelopathic effects were documented in an earlier study

that demonstrated decreasing seed germinability and growth of *Acacia sophorae* by *Chrysanthemoides monilifera ssp. rotundata* litter (Vranjic *et al.* 2000). It has been argued that phytotoxic compounds from plants could rapidly diminish in soil systems with very little effect on the plant community (Bonanomi, Sicurezza *et al.* 2006), even though allelopathic potential from decomposed materials may persist for more than 1 year (Zuo, Wang *et al.* 2008). The constant accumulation of allelochemicals, as implied by this study, could suppress some critical phases of species regeneration such as seed germination, seedling growth and establishment and root development.

The overall findings of our study demonstrate that physicochemical variables of the aqueous extracts in the decomposition processes are very dynamic, especially in the anaerobic conditions. Aerobic condition of all treatments showed a consistent pattern whereas there was a high level of inconsistency in anaerobic condition. Pattern consistency among physicochemical variables was largely dependent on the condition and materials, revealing that the factors influencing variables across the decomposition over time may play a crucial role in natural wetland ecosystems. Phytotoxicity assessments with three species, lettuce, *Poa* and *Melaleuca*, demonstrate that phytotoxin accumulation through residue decomposition may pose a threat to vegetation community structure, over a long time period by affecting seed germination and seedling growth in nature, especially if anaerobic conditions exist. Furthermore, phytotoxic activity of *Phragmites* residue exists even in long-term decomposition depending on density in the soil. In light of our results, we can point out that plant litter releases not only nutrients but also phytotoxic substances that might be considered in assessing the inhibitory effects on plant growth. Future studies of allelochemicals, particularly identification and associated phytotoxicity in different phases of decomposition as well as field-based observations (water-soil chemistry and associated

phytotoxicity) are needed to more fully understand the role of these allelochemicals in the environment. A multidirectional approach is necessary for a better understanding of phytotoxicity dynamics through decomposition processes in natural ecosystems, especially the specific role of soil microorganisms and limiting environmental conditions. Our results may imply that *Phragmites* residue decomposition contributes phytotoxins and associated physicochemical change in soil and water systems and thereby, causing suppression of germination and growth of associated plant species. The results may go some way to explaining the long-term effects of *Phragmites*-dominated wetlands on community ecology and more specifically allelochemical suppression of competing species. It may also be applicable to the agriculture practices to assess the suppressive effects of organic matter used as crop cover and crop residue.

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Table Caption

Table 1. ANOVA results of percentage inhibition of root-shoot length and biomass of lettuce by the extracts during decomposition

Table 2. ANOVA results of germination percentage and rate, root and tiller length, and biomass of *Poa labillardierei* affected by the extracts during decomposition

Table 3. Results (F-value with significant level; coefficient of determination) of regression analysis among germination indices, biometric parameters of *Poa labillardierei* and chemical changes of treatments during decomposition. Level of significance: *, $P < .0001$ and degree of freedom (df) = 1, 106

Table 1.

| Source | <i>df</i> | Root length | | Shoot length | | Root mass | | Root-shoot mass ratio | |
|---------------|-----------|-------------|----------|--------------|----------|-----------|----------|-----------------------|----------|
| | | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> |
| Materials (M) | 2 | 354.72 | < 0.001 | 201.41 | < 0.001 | 14.74 | < 0.001 | 6.11 | 0.004 |
| Condition (C) | 1 | 1049.82 | < 0.001 | 43.33 | < 0.001 | 7.76 | 0.007 | 4.81 | 0.031 |
| Time (T) | 5 | 96.07 | < 0.001 | 79.07 | < 0.001 | 12.80 | < 0.001 | 10.27 | < 0.001 |
| M × C | 2 | 1.65 | 0.198 | 25.06 | < 0.001 | 60.75 | < 0.001 | 80.66 | < 0.001 |
| M × T | 10 | 33.54 | < 0.001 | 43.96 | < 0.001 | 16.13 | < 0.001 | 20.14 | < 0.001 |
| C × T | 5 | 40.02 | < 0.001 | 15.24 | < 0.001 | 21.09 | < 0.001 | 19.70 | < 0.001 |
| M × C × T | 10 | 18.23 | < 0.001 | 12.73 | < 0.001 | 15.24 | < 0.001 | 17.74 | < 0.001 |

Table 2.

| Source | <i>df</i> | Germination % | | Germination rate | | Root length | | Tiller length | | Biomass/plant | |
|---------------|-----------|---------------|----------|------------------|----------|-------------|----------|---------------|----------|---------------|----------|
| | | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> | <i>F</i> | <i>p</i> |
| Materials (M) | 2 | 17.44 | < 0.001 | 123.36 | < 0.001 | 301.12 | < 0.001 | 706.36 | < 0.001 | 81.21 | < 0.001 |
| Condition (C) | 1 | 0.44 | 0.51 | 0.44 | 0.507 | 169.85 | < 0.001 | 94.36 | < 0.001 | 14.05 | < 0.001 |
| Time (T) | 5 | 16.06 | < 0.001 | 32.86 | < 0.001 | 71.44 | < 0.001 | 54.00 | < 0.001 | 8.69 | < 0.001 |
| M × C | 2 | 2.62 | 0.079 | 2.43 | 0.095 | 76.14 | < 0.001 | 11.77 | < 0.001 | 8.08 | < 0.001 |
| M × T | 10 | 3.94 | < 0.001 | 8.04 | < 0.001 | 38.81 | < 0.001 | 19.78 | < 0.001 | 1.35 | 0.21 |
| C × T | 5 | 2.27 | 0.056 | 1.30 | 0.274 | 8.34 | < 0.001 | 11.64 | < 0.001 | 3.21 | < 0.01 |
| M × C × T | 10 | 1.81 | 0.074 | 5.83 | < 0.001 | 7.08 | < 0.001 | 16.30 | < 0.001 | 1.30 | 0.24 |

Table 3.

| | Chemical variables | |
|-----------------------------------|-------------------------|--------------------------|
| | Water soluble phenolics | Dissolved organic carbon |
| Germination and growth parameters | | |
| <i>Germination indices</i> | | |
| Percentage | F = 19.56*; 0.15 | F = 29.98*; 0.22 |
| Rate | F = 87.47*; 0.45 | F = 120.79*; 0.53 |
| <i>Biometric parameters</i> | | |
| Root length | F = 123.01*; 0.53 | F = 252.26*; 0.70 |
| Tiller length | F = 166.07*; 0.61 | F = 183.56*; 0.63 |
| Total plant length | F = 189.32*; 0.64 | F = 336.18*; 0.76 |
| Biomass plant ⁻¹ | F = 63.80*; 0.37 | F = 68.85*; 0.39 |

Figure Captions

Fig. 1. Total phenolics (light-grey square) and water-soluble phenolics (dark-grey square) in live and dead plant materials of *Phragmites australis*. Values are mean \pm s. e. ($n = 3$).

Fig. 2. Dynamics of (a) water soluble phenolics ($\mu\text{g mL}^{-1}$) and (b) dissolved organic carbon (mg L^{-1}) in aerobic (open symbols) and anaerobic (closed symbols) decomposition of three experimental treatments: residue alone (square), residue with soil (triangle) and soil alone (circle). Values are mean \pm s. e. ($n = 3$).

Fig. 3. Dynamics of specific ultraviolet absorbance ($\text{L mg}^{-1} \text{cm}^{-1}$) in aerobic (open symbols) and anaerobic (closed symbols) decomposition of three experimental treatments: residue alone (square), residue with soil (triangle) and soil alone (circle). Values are mean \pm s. e. ($n = 3$).

Fig. 4. Boxplot comparison of pH, electrical conductivity, osmotic potential, and nutrient (PO_4^{3-} , Cl^- , N (NO_2^- , NO_3^-) and SO_4^{2-}) concentrations in (a) aerobic and (b) anaerobic conditions in decomposition process. They depict medians, 25th and 75th percentiles and minimum and maximum value. Data of each parameter refer to all stages of decomposition.

Fig. 5. Effects of aqueous extracts on root growth of lettuce seedlings during decomposition processes according to aerobic (open symbols) and anaerobic (closed symbols) conditions of three experimental treatments: residue alone (square), residue with soil (triangle) and soil alone (circle). Values are mean \pm s. e. ($n = 3$).

Fig. 6. Correlation between root growth of lettuce seedlings and water soluble phenolics in aerobic (open symbols) and anaerobic (closed symbols) decomposition of three

experimental treatments: residue alone, residue with soil and soil alone. Values are mean of each parameter that refers to all stages of decomposition.

Fig. 7. Effects of aqueous extracts on (a) germination percentage and (b) germination rate of *Poa labillardierei* during decomposition processes according to aerobic (open symbols) and anaerobic (closed symbols) conditions of three experimental treatments: residue alone (square), residue with soil (triangle) and soil alone (circle). Values are mean \pm s. e. ($n = 3$).

Fig. 8. Effects of aqueous extracts on (a) root length, (b) tiller length, and (c) dry biomass of *Poa labillardierei* during decomposition processes according to aerobic (open symbols) and anaerobic (closed symbols) conditions of three experimental treatments: residue alone (square), residue with soil (triangle) and soil alone (circle). Values are mean \pm s. e. ($n = 3$).

Fig. 9. Effect of decomposed residue on a) germination, b) root biomass and c) total chlorophyll of *M. ericifolia*. Values are mean \pm s.e. ($n = 3$). **, and ns indicate significant difference from control at $P \leq .001$ and non-significant respectively after applying the Dunnett test.

Fig. 1.

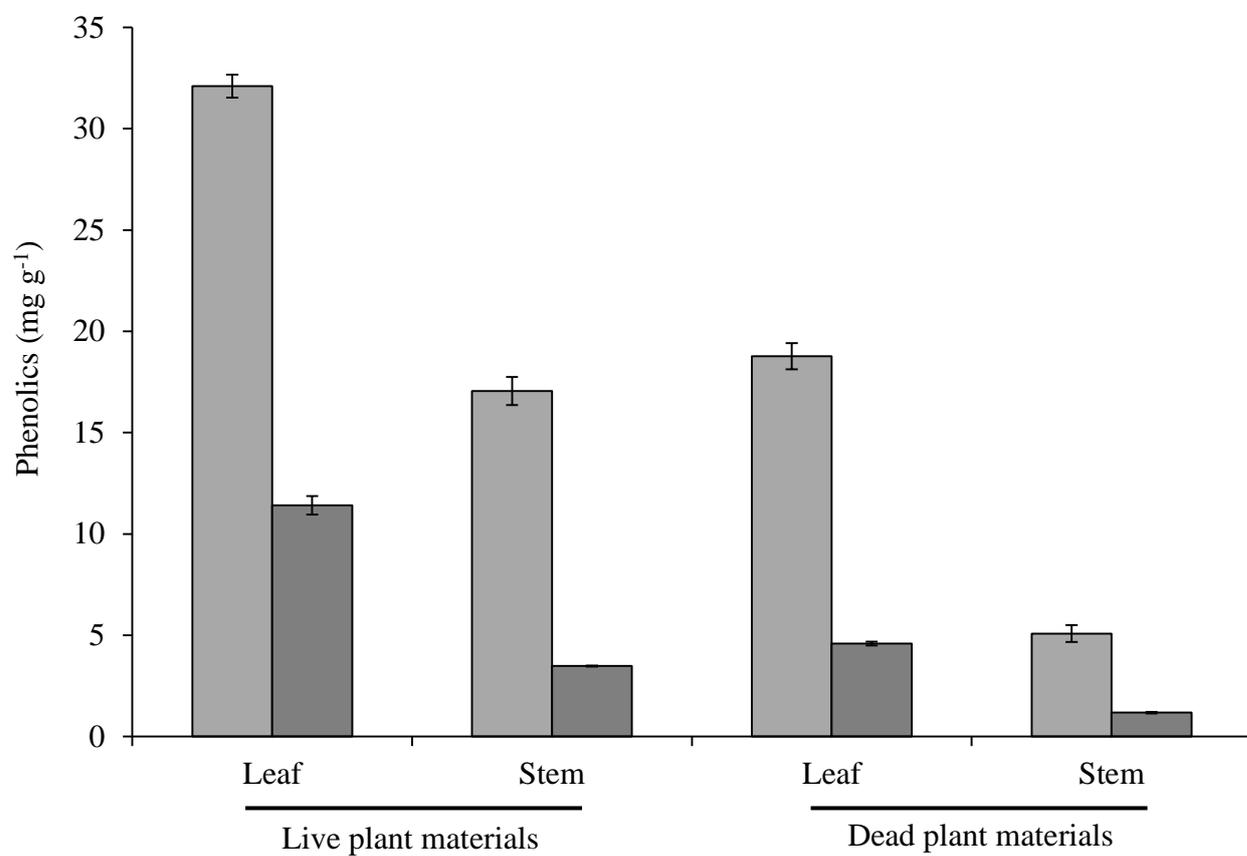


Fig. 2.

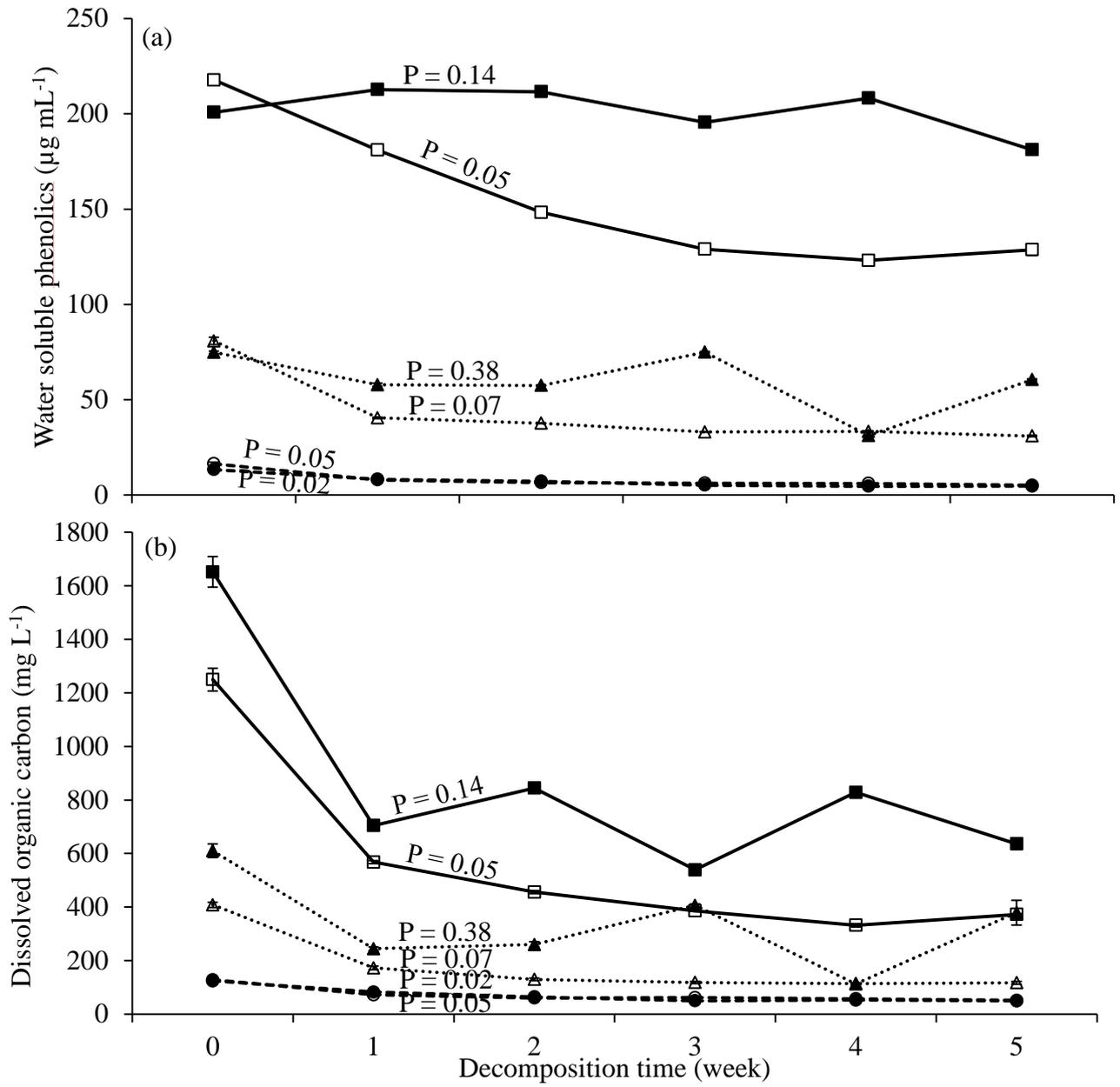


Fig. 3.

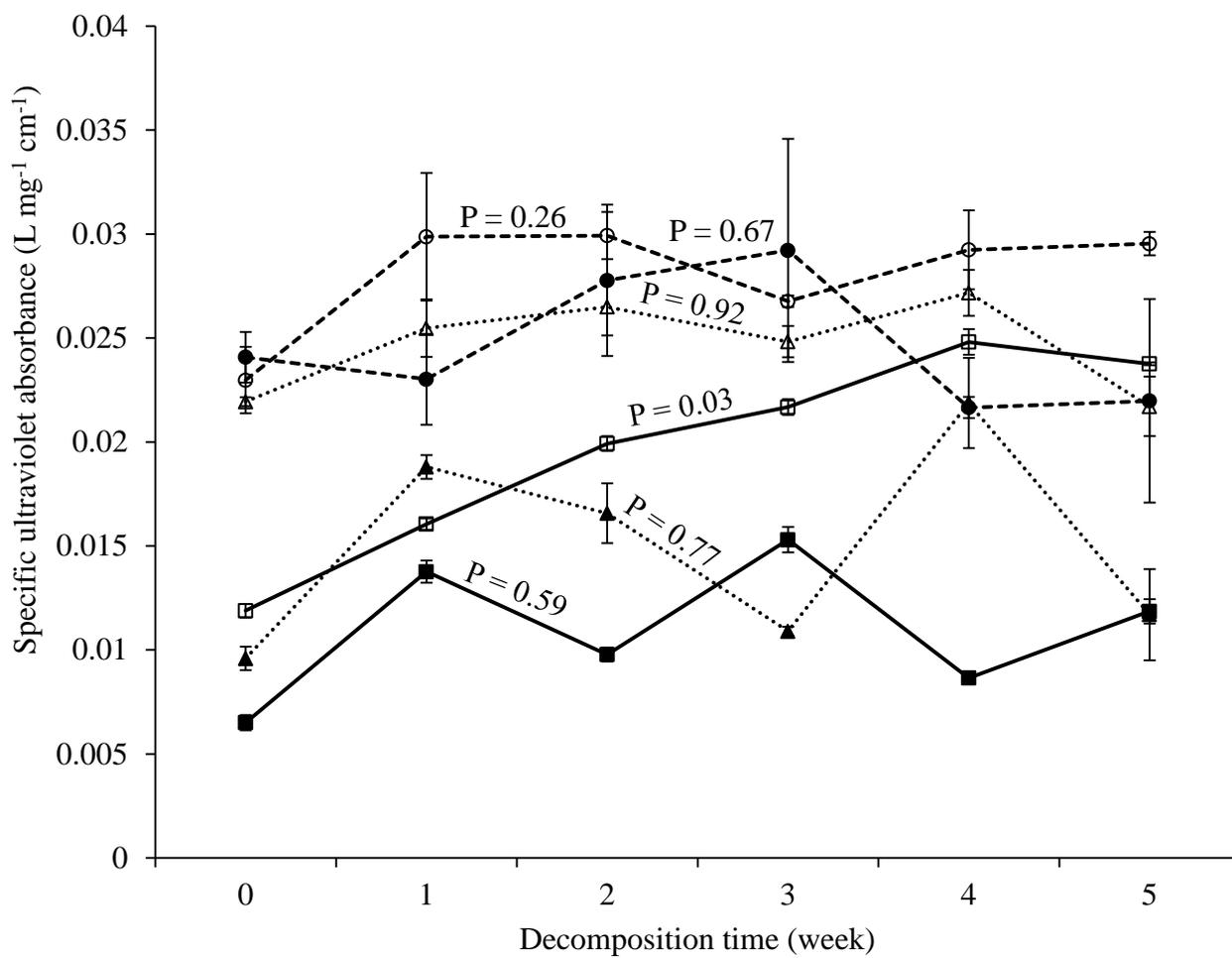


Fig. 4.

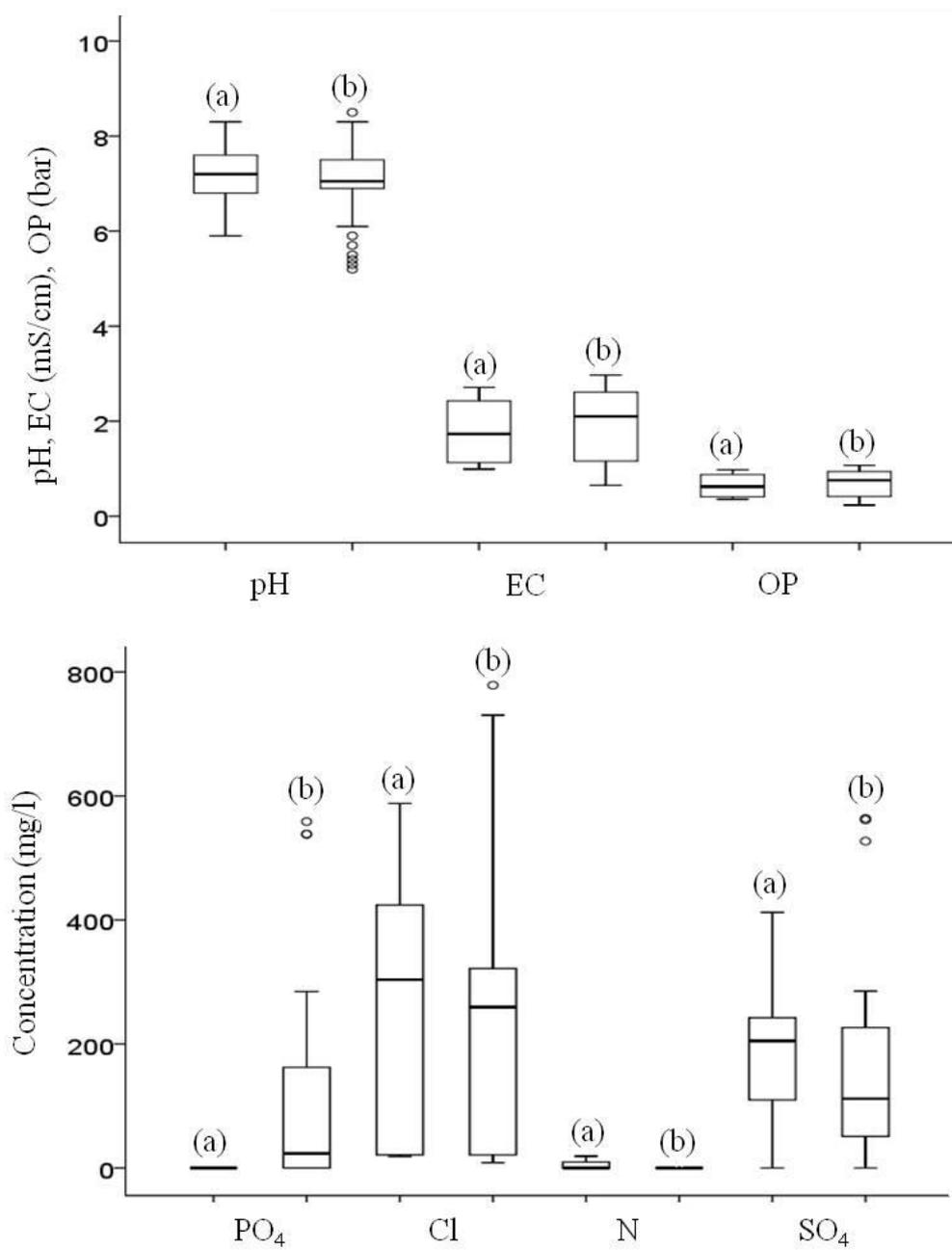


Fig. 5.

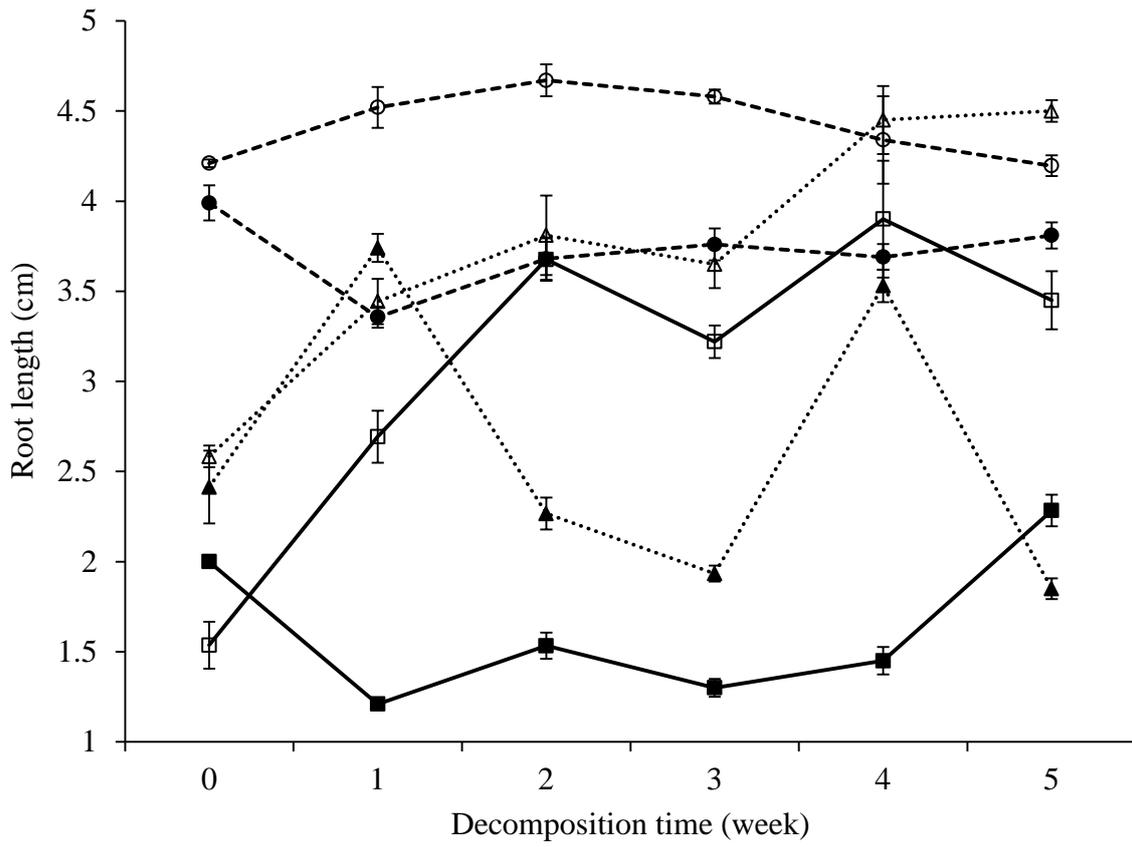


Fig. 6.

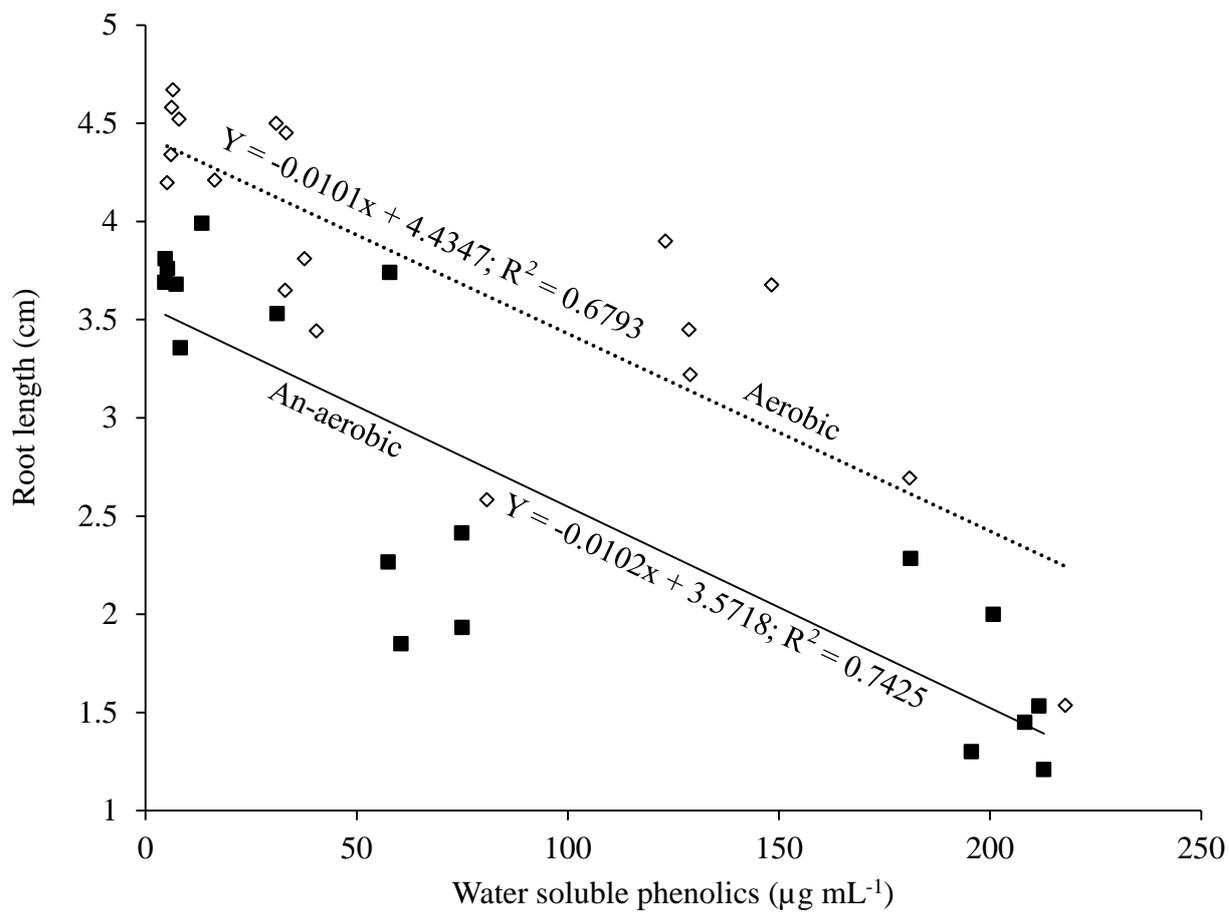


Fig. 7

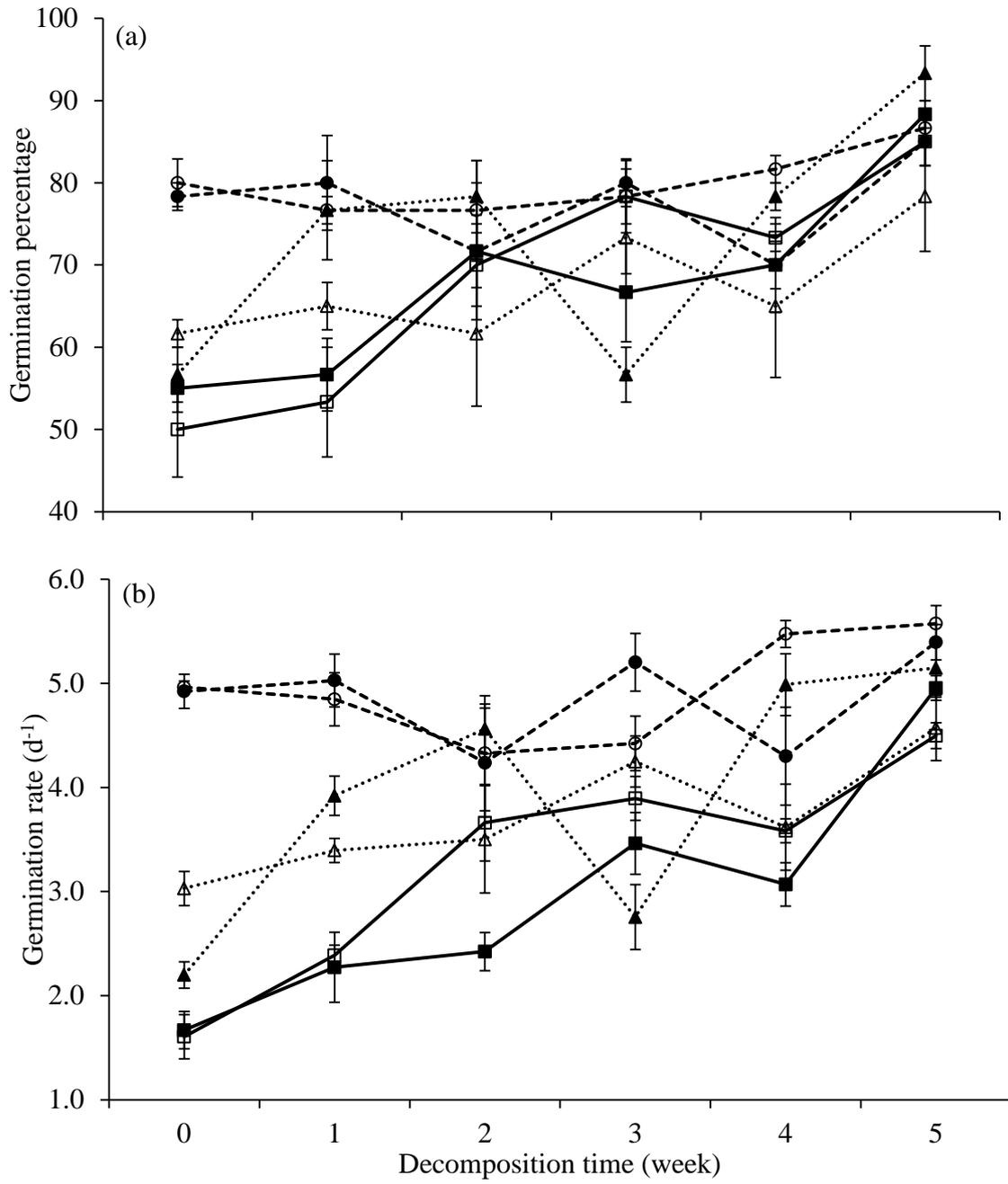


Fig. 8

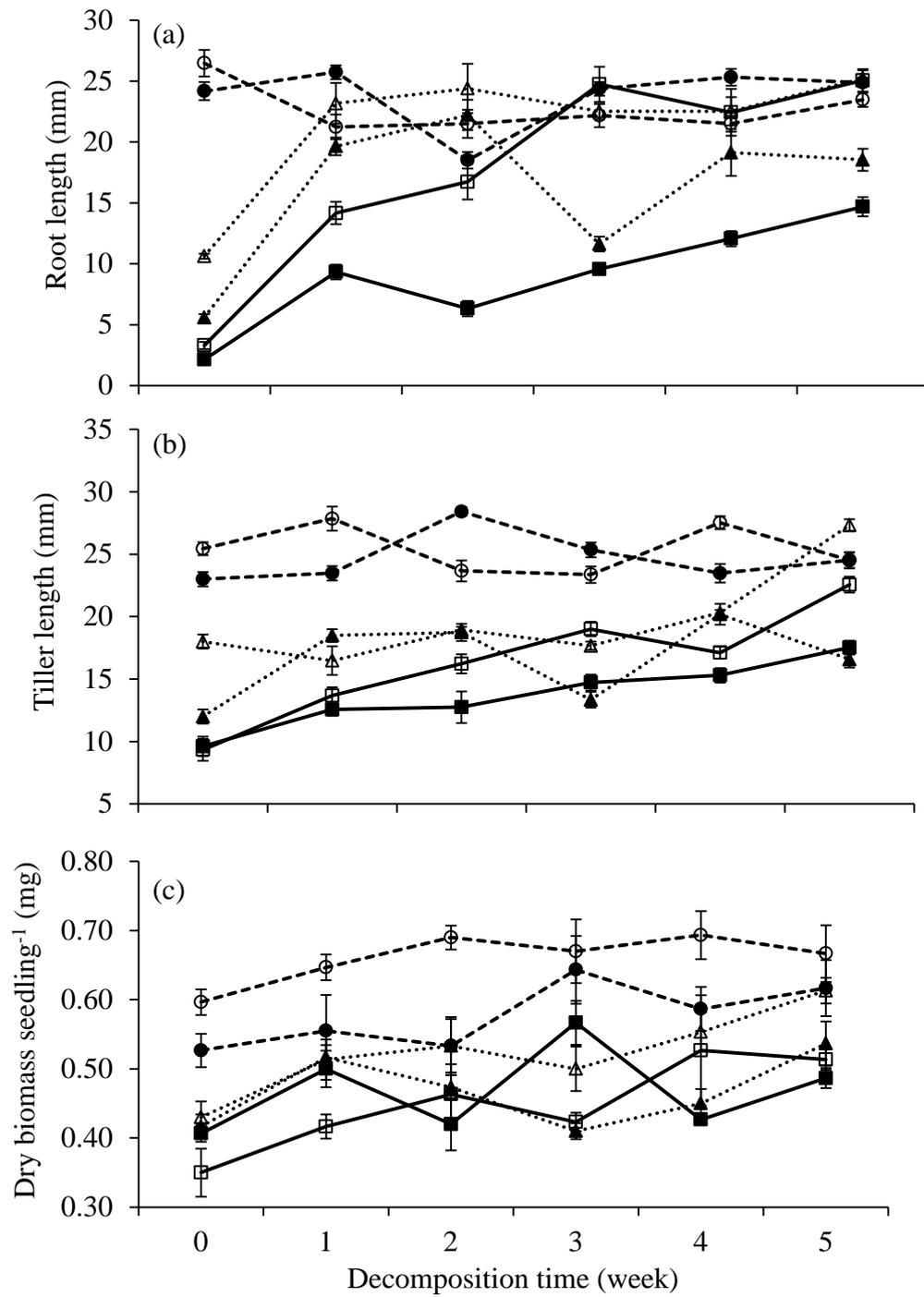
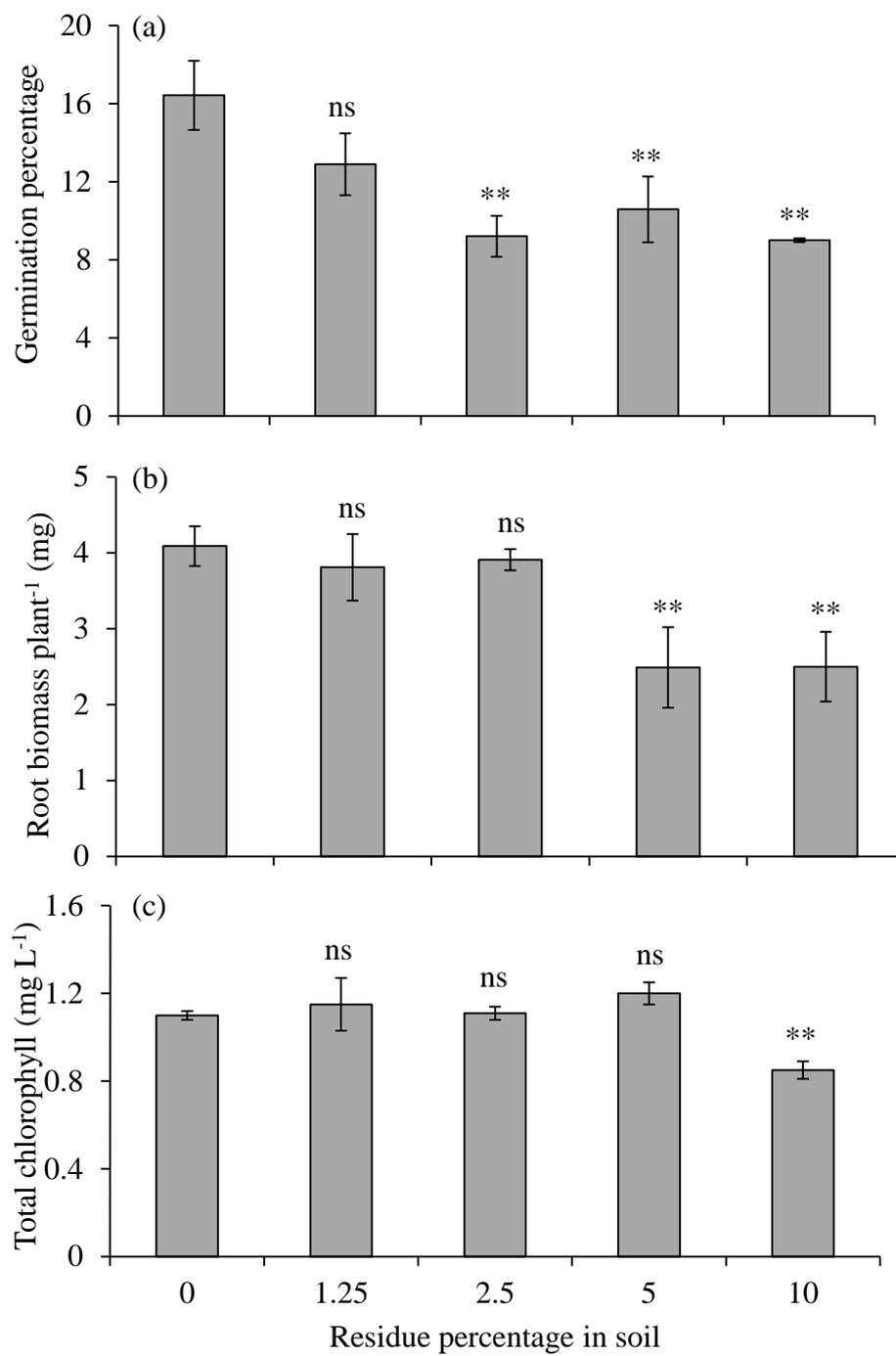


Fig. 9.



Chapter Five

Chemistry of a Phragmites australis Dominated Wetland and its Phytotoxicity may Suggest Field Evidence of Allelopathy

Introduction

This manuscript explored the allelopathic interference of *Phragmites australis* on plant communities by assessing the chemical characteristics of soil and water of invaded communities in the field, and its phytotoxicity tests in laboratory.

The following manuscript entitled '**Chemistry of a *Phragmites australis* dominated wetland and its phytotoxicity may suggest field evidence of allelopathy**' by Md. N. Uddin, Randall W. Robinson, and Md. A. Y. A. Harun has been submitted for publication in *Journal of Ecology* and it is now under review.

PART B:
**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS
 INCORPORATED IN THESIS BY PUBLICATION**

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Md. Nazim Uddin

Signature: 

Date: 24/07/2014

Paper Title: Chemistry of a *Phragmites australis* dominated wetland and its phytotoxicity may suggest field evidence of allelopathy

In the case of the above publication, the following authors contributed to the work as follows:

| Name | Contribution % | Nature of contribution |
|---------------------|----------------|--|
| Md. Nazim Uddin | 85 | Concept development; plant, soil and seed collection; conducting experiments and chemical analysis; data collection, statistical analysis and interpretation; and manuscript writing, editing and submitting for publication |
| Randall W. Robinson | 10 | Concept development; sample collection; and manuscript editing |
| Md. A. Y. Harun | 5 | Data collection & manuscript editing |

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

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|-------------|---|------------|
| Signature 1 |  | 18/07/2014 |
| Signature 2 |  | 18/07/2014 |
| Signature 3 |  | 18/07/2014 |

Chemistry of a *Phragmites australis* dominated wetland and its related phytotoxicity may suggest field evidence of allelopathy

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Summary

1. Allelopathy is one of the mechanisms that help to explain the invasion success of a plant species. The production and release of allelochemicals by invasive species can have negative effects on soil and water chemistry and can thereby inhibit resident plant communities. Allelopathic interference of *Phragmites australis* on plant communities has been addressed by assessing the chemical characteristics of soil and water of invaded communities in the field, and its phytotoxicity tests in laboratory.

2. This study analysed the chemical characteristics of soil and water and monitored both over four seasons taking into consideration the phenological cycle of *P. australis* in the field. We also conducted a series of bioassays in relation to phytotoxicity of soil and water held *P. australis* allelochemicals on different plant species in the laboratory.

3. Significant changes in soil and water chemistry were observed in invaded compared with uninvaded area. Soil-water and plant-leachate significantly inhibited germination of *Lactuca sativa* at higher concentration and thereby affected α -amylase activity. The adventitious root formation of *Phaseolus aureus* was suppressed by plant-leachate, soil-water and soil surface water collected from *P. australis* invaded environments. Seasonal impact on allelopathic interference of *P. australis* in terms of germination and growth of

L. sativa, *Melaleuca ericifolia*, and *Poa labillardierei* showed distinct but inconsistent variation. Soil sterilization experiments indicated that soil biota might play an important role in reducing the phytotoxicity in natural soil.

4. Synthesis. A synthesis from field studies and their associated laboratory experiments may provide a more logical understanding towards invasion mechanisms of *P. australis* through allelopathy, leading to more realistic management decisions.

Key-words: biodiversity, biological invasion, phenolic compounds, soil biota, soil characteristics

Introduction

Biological invasion by non-native species is a worldwide phenomenon that threatens to dramatically change communities and ecosystems (Mack et al., 2000; Alvarez and Cushman, 2002). It is widely acknowledged that a thorough understanding of the mechanism of invasion must include the specific negative effects of the invasive plant on native communities and functioning of the invaded ecosystems (Hejda et al., 2009). Generally, the suppression of native plants by the dominance of the invasive species is a common phenomenon on the invaded ecosystems. In some cases, species richness may be increased in an invaded area that leads some researchers to question whether most invasive species are really a prominent threat to the conservation of the native biodiversity (Davis et al., 2011; Powell et al., 2013). Recent studies have considered invasion from a variety of viewpoints that include the natural enemies theory (Colautti et al., 2004), characteristics of the invader (Kolar and Lodge, 2001), resource competition (Davis et al., 2000), and the characteristics of invaded communities (Lonsdale, 1999).

Allelopathy, the release of phytotoxins by plants, has been proposed as an alternative theory for the success of many invasive plants (Callaway and Aschehoug, 2000). There are some important aspects of allelopathy studies that are crucial to be addressed if methods could be established to determine their impact. One aspect of allelopathy presently not generally addressed is phytotoxicity assessment in relation to field concentration of phytotoxins released from the allelopathic plants. Most of the research works performed on phytotoxicity assessments are carried out in the laboratory and greenhouse but more ecological realistic experimental designs are needed to understand what is occurring in natural ecosystems. In addition, field chemistry associated with soil and water is the key in determining the qualitative and quantitative availability of allelochemicals in the vicinity of neighbouring species and is critically important to provide the better understanding in allelopathy (Inderjit, 2001; Callaway et al., 2004; Inderjit, 2005) and understanding suppression of germination, growth and establishment of the associated species in the field (Lambers et al., 1998). As the interactive effects of plants, water, and soil are not independent in natural ecosystems, it is essential to integrate them in experiments on the biological invasion of any wetland invasive species. With these complex interactions, we preceded to design a study to find out the underlying mechanism of the invasion of *Phragmites australis* that reflects the complexity of the 'natural' ecosystem.

Phragmites australis, a ubiquitous wetland plant, has been considered one of the most invasive species in the world (Uddin et al., 2012), however, the origin and original distribution of the species is still unclear (Plut et al., 2011). It is a perennial graminaceous plant, 3 to 4 m tall, which reproduces mainly through rhizomes and, at low frequency, through seeds. This large wetland grass grows in all temperate zones of the world, and is especially common in North America, Europe and Australia (Hocking

et al., 1983; Morris et al., 2008; Kulmatiski et al., 2011). The distribution and abundance of *P. australis* has expanded over the last 150 years and in most areas it forms dense monocultures (Saltonstall et al., 2005). Due to *P. australis* invasions, habitats have been diminished or altered significantly with direct and indirect impacts on flora and fauna causing loss of biodiversity and ecosystem functions (Warren et al., 2001; Silliman and Bertness, 2004). Interestingly, some chemicals produced by residue decomposition of *P. australis* may be responsible for die-back of *P. australis* itself (Armstrong and Armstrong, 1999) as well as associated plant species (Uddin et al., 2014c). Photo-degradation of secreted phytotoxins by *P. australis* can cause severe phytotoxicity to other plant species (Rudrappa et al., 2009).

Our earlier research established that *P. australis* achieves its inference success by inhibiting the germination, growth, physiology and establishment of certain plant species through water-soluble compound synthesis (Uddin et al., 2012; Uddin et al., 2014a), root exudation (Rudrappa et al., 2007; Uddin et al., 2014b), and residue decomposition (Uddin et al., 2014c). Gallic acid, an important inhibitor, has been identified as a major contributor in the invasion process of *P. australis* (Rudrappa et al., 2007; Uddin et al., 2014a). In addition to ecological impacts, *P. australis* may cause substantial economic damage or loss. An estimated cost about \$25 billion has been spending each year in the USA for management of invasive species (Pimentel et al., 2005). Many multi-million dollar projects have been undertaken in wetland restoration initiatives with particular reference to *P. australis* invasion in wetlands throughout the world, including the USA, Europe, Canada and Australia (Streever, 1997; Van der Putten, 1997; Silliman et al., 2009). It is generally acknowledged that those restoration projects that involve control of invasive plant such as *P. australis* might be limited due to lack of specific knowledge of the species regarding allelopathy (Walker et al., 2007).

Restoration of ecosystems after the removal of invasive species, particularly those that are allelopathic, relies heavily on a sound knowledge of biochemical processes (Siemens and Blossey, 2007).

The literature on the *P. australis* invasion processes that includes allelopathy is not robust enough to provide reliable information that could integrate the existing knowledge to sound on-ground reality. Within this context, we designed our studies through two distinct methods namely observational and experimental studies in the field and laboratory respectively. Measuring the chemical changes in the soil and water of invaded wetlands allows the comparison of the chemistry of invaded and uninvaded *P. australis* habitat and thereby, may assist to identify potential effects of an invading species. Integrating the field studies with the laboratory studies allow for a more logical explanation for invasion of *P. australis* than would be able to be achieved by field study alone.

Materials and methods

STUDY SITE AND SAMPLE COLLECTION

We conducted our studies on the natural stands of *P. australis* adjacent to Cherry lake (37° 51' 30"S, 144° 50' 5"E). Cherry lake is a part of the coastal wetlands at Altona, a suburb of Melbourne, Victoria, Australia. It covers an area of 60 ha within a large reserve of 176 ha. Prior to European settlement it was a low-lying and seasonally flooded swampy area supplied from runoff from Cherry Creek and flood flows from Kororoit Creek. The water regime was highly variable and the wetland could be dried for period greater than 12 months. As the surrounding area expanded and developed there was greater demand for protection of local area from flooding. As a result, in 1963, parts of the swamp area were drained by the Board of Works (now Melbourne

Water). In addition, levees were constructed and a concrete channel was built to carry flood flows to the nearby Port Philip Bay. Thus, it became an important flood retarding basin and one of the most popular passive recreation facilities in the western suburbs. The soil of Cherry Lake consists of sand, gravel, clay, and coal and is one of the volumetrically largest and deepest formations in the Port Philip region. Among the vegetation of that time, the *Phragmites australis* occupied a small portion of that area which has been expanding vigorously. As a result, it has been changing the floristic composition by invading the wetland and ultimately makes the other native population more vulnerable. All plants, soil materials and water were collected seasonally on the basis of phenological cycles of *P. australis* from areas considered visually homogeneous with respect to shoot density and age. All the sites (invaded and uninvaded) sampled had the same soil texture class and subjected to similar environmental conditions.

OBSERVATIONAL STUDIES IN THE FIELD

Seasonal impact on soil-water and soil surface water

This study was conducted to determine the impact of seasonal variations on chemistry of rhizosphere soil-water and soil-surface water of *P. australis* invaded zones. Sampling was done in four different seasons, namely spring (September to November), summer (December to February), autumn (March to May), and winter (June to August). Generally, rhizosphere soil-water was extracted by centrifugation (3000 rpm for 30 minutes) of wet soil collected from field followed by sterilization with microfiltration (0.22 μm) but exception of summer sample. The summer soil was extracted with addition of distilled water at saturation level as these samples were almost dry. In addition, we collected soil surface water during the spring and winter season from the same populations with these samples being processed in the laboratory following the

same procedures as listed above. All samples were preserved in freezer at - 80⁰ C for chemical analysis and bioassay experiments. Soil-water and soil-surface water were also collected from *P. australis* nearby un-invaded zone as a control. Only summer samples (full-strength) were diluted with distilled water during bioassays and they were referred as half-strength and quarter-strength. pH and electrical conductivity (EC) were measured with a pH meter (Pocket digital pH meter, 99559, Dick-smith electronics, Australia) and conductivity meter (TPS Digital conductivity meter, 2100, TPS Pty Ltd., Australia) respectively. Osmotic potential (OP) was calculated using the equation (OP = EC * - 0.36) according to McIntyre (1980). Dissolved organic carbon (DOC) was measured using a TOC analyser (TOC-V with TN detector, Shimadzu, Kyoto, Japan). Water soluble phenolics (WSP) were measured according to Singleton and Rossi (1965) with gallic acid used as the standard.

EXPERIMENTAL STUDIES IN LABORATORY

Germination with α -amylase activity bioassay by whole plant leachate and soil-water

Whole plants collected at mature stage (summer) were dried at room temperature, and chopped into small pieces (< 2 cm long). To formulate leachate, 5 g dried plant was submerged in 100 mL distilled water (5 %) and agitated for 24 h on an orbital shaker (Orbital Mixer, EOM5, Ratek Instruments Pty. Ltd, Vic-3155, Australia) at room temperature. The leachate was filtered through cheese-cloth, centrifuged at 10,000 rpm for 10 min (Beckman Avanti 30 High Speed Compact Centrifuge, 364105, Beckman Coulter Inc., USA) and sterilized (microfiltration with 0.22 μ m pore filter). pH and OP of the leachate were measured. During experiment set-up, the 5% base concentration (full-strength) was diluted with distilled water to obtain concentrations of 2.5% (half-strength), and 1.25% (quarter-strength). All extracts were buffered with 0.01M sodium

phosphate buffer and pH values of the extracts including distilled water (control) were adjusted to 6.5 with either 1N NaOH or 1N HCl. We used a wide range of doses to ensure that they would encompass the lowest dose for an observable effect, as well as the highest dose for maximal effect (Belz et al., 2007). The OPs of all concentrations were in the range of - 0.076 to - 0.264 bar that could not affect germination and growth of the test species (Uddin et al., 2014a).

Seeds of *Lactuca sativa* were sterilized with 1.5% (v/v) sodium hypochlorite for 1 min and subsequently washed. Five mL of each solution (plant and soil) with different concentrations (distilled water as control) was placed into a sterile 9-cm Petri dish containing two sterile sheets of filter paper (Whatman No. 1). Three replicates (each having 20 surface sterilized seeds) were used for each treatment. Petri dishes were sealed with parafilm (Pechiney, Plastic Packaging Company, Menasha, WI 54952) and placed in a growth chamber (Westinghouse, Electrolux home products, Australia) in a completely randomized design (CRD) set to 25°C in dark condition. The Petri dishes were randomized each day for reducing the spatial effect. The germinated seeds were counted at 18, 27, and 45 h and harvested for α -amylase activity. Extraction and measurement of α -amylase activity was performed by following the method of Poonpaiboonpipat et al. (2013).

Adventitious rooting bioassay with whole plant leachate and soil-water

Surface sterilised seeds of *Mucuna pruriens* (Linn.) were germinated in Petri dishes on Whatman filter paper soaked with distilled water and maintained in growth chamber at 25/15°C day/night temperature and a 12 h photoperiod with illumination of 84 μ mol $s^{-1}m^{-2}$. The primary roots were removed after 5 days, then 5 seedling explants were inserted into 50 mL Schott bottle containing 20 mL of different strengths test solutions (plant leachate, summer soil-water, and soil surface water) but distilled water as control with

three replicates. In the case of soil surface water, the spring sample was used as there was no surface water at summer. The bottles were incubated as of germination bioassay. *Mucuna pruriens* (Linn.) hypocotyls were selected as an experimental material because these are widely used as a non-woody cutting plant in studies aimed at understanding the biometric aspects of adventitious rooting formation (Batish et al., 2008b; Singh et al., 2009). The plant characteristics were evaluated in terms of number of roots per explant and average root length after five days.

Germination and growth bioassay with seasonal collected soil

Air-dried seasonal soil (40 g) (sieved at 4 mm and excluded from extraneous material) was placed in mini punnet (6.5 cm by 4.5 cm) and moistened with 25 mL distilled water. After 6 h incubation at 25⁰C, 20 seeds of *Poa labillardierei* and *L. sativa*, and 100 seeds for *Melaleuca ericifolia* were placed on and incubated as above mentioned conditions. The germinants were counted after 1, 2, and 4 weeks for *L. sativa*, *P. labillardierei* and *M. ericifolia* respectively and after which they were harvested for measuring biometric parameters.

Bioassays evaluating the effect of soil microbes in allelopathy

Growth responses of different assay species were studied in sterile and non-sterile soil amended with whole plant extract to observe the role of soil microbes in plant growth. Soil sterilization has recently been used as a tool to establish the role of microbial communities in determining allelopathic effects (Inderjit, 2006). Soil collected from the *P. australis* un-invaded zone was autoclaved three successive times (120⁰C, 103 kPa pressure for 30 min). Both non-sterile and sterile soil (40 g) was treated with 25 mL of whole plant leachate of different strength (distilled water as control) and incubated for 6 h. Subsequent to the above treatment, 10 seeds of *L. sativa* and *P. labillardierei* were

sown on soil and maintained in the growth chamber as above mentioned conditions with three replicates. Data on germination and biometric parameters were collected at 7 d for *L. sativa* and 14 d for *P. labillardierei*.

STATISTICAL ANALYSES

Two and three way analysis of variance (ANOVA) was used for analysis of the difference between group means and their associated procedures such as variation among and between groups. T-tests were used to evaluate the differences in growth response of *L. sativa* and *P. labillardierei* in the sterilization experiment between treatments.

Results

CHANGES OF FIELD CHEMISTRY

Soil-water pH was consistently lower in invaded areas throughout the season, with the annual mean for invaded, 7.02 ± 0.26 and uninvaded, 7.19 ± 0.23 (Fig. 1a). Higher EC (1.61 ± 0.09 vs. 1.52 ± 0.11 in mS cm^{-1}) and OP (0.54 ± 0.03 vs. 0.54 ± 0.04 in bar) were observed in invaded area compared with uninvaded ones. Annual mean for WSP (3.48 ± 0.21 vs. 1.14 ± 0.11 in $\mu\text{g mL}^{-1}$) and DOC (54.06 ± 3.95 vs. 21.58 ± 2.59 in mg L^{-1}) were also significantly higher in *P. australis* invaded areas compared with uninvaded (Fig. 1b, c). WSP and DOC were significantly higher in spring among the seasons whereas pH was significantly lower in autumn (Fig. 1). In addition, the levels of measured parameters in soil surface water were higher in invaded area compared with uninvaded area (data not shown). All parameters in soil-water and soil surface water significantly varied across the seasons in invaded area but differential interactive effects (season \times sample type \times invasion) were observed (Table 1).

GERMINATION AND α -AMYLASE ACTIVITY

Whole plant leachate and soil-water inhibited germination percentage of *L. sativa* even at low strength solution at 18 h observation but only full strength solution had potential to significantly affect germination at 45 h (Fig. 2). At the end of the experiment, the inhibitions were 39% and 33% for full strength plant leachate and soil-water respectively indicating lower effect by soil-water than plant leachate (Fig. 2a). Interactive effects (types of solution \times solution strength \times incubation time) were significant on germination percentage ($F_{6,48} = 4.31, P \leq 0.001$). The α -amylase activity was inhibited in test plant and the inhibition was increased with increasing concentration (Fig. 2b). The α -amylase activity in control was low at time 0 h, and increased as the process of germination occurred. At 45 h, only full strength plant leachate had significant negative effect on the activity and it was 73% of control. There was no interactive effect on α -amylase activity ($F_{6,48} = 1.16, P > 0.34$) but the activity was higher in relation to soil rather than plant solution ($F_{1,48} = 49.78, P \leq 0.001$). The concentration-response curves of both solutions indicate that percentage germination is positively correlated with the α -amylase activity in test seeds (Fig. 2c) and the relationships were linear with regression of determination for plant leachate ($R^2 = 0.789$) and soil-water ($R^2 = 0.751$).

ADVENTITIOUS ROOTING

Results showed that all types of solutions adversely affect the initiation of adventitious rooting of *M. pruriens* (Linn.) in terms of rooting percentage, number of roots and average root length and the effects were increased with increasing concentration (Fig. 3). Interactive effects (types of solution \times solution strength) were significant on rooting percentage ($F_{6,24} = 6.92, P < 0.0001$) and root length ($F_{6,24} = 12.11, P < 0.0001$) except number of roots ($F_{6,24} = 1.69, P > 0.16$). Moreover, numbers of roots were significantly

affected at all types of solution ($F_{2, 24} = 10.08, P < 0.001$) and the effects were significant as the solution strength increased ($F_{3, 24} = 48.61, P < 0.0001$).

SEASONAL EFFECTS

Phragmites australis invaded soil had significant interactive effect on test species in terms of root length ($F_{6, 24} = 3.48, P \leq 0.013$) and biomass ($F_{6, 24} = 3.01, P \leq 0.024$), except germination percentage ($F_{6, 24} = 2.46, P \leq 0.053$) (Fig. 4). Germination percentage was influenced significantly by the main effect of season ($F_{3, 24} = 13.92, P \leq 0.0001$) and species ($F_{2, 24} = 36.38, P \leq 0.0001$) whereas root length ($F_{3, 24} = 0.842, P \leq 0.484$) and biomass ($F_{3, 24} = 1.11, P \leq 0.364$) were not affected with seasons but varied significantly among species. The overall effects inconsistently varied with seasons and species (Fig. 4).

EFFECTS OF SOIL MICROBES

Phytotoxicity varied in non-sterile and sterile soil in relation to *L. sativa* and *P. labillardierei*. Germination percentage, root length and biomass of both species were more inhibited in sterile soil than non-sterile soil (Fig. 5 & Table 2). Germination percentage ($t = 5.53, df = 11, P \leq 0.0001$ for *L. sativa* and $t = 2.41, df = 11, P \leq 0.034$ for *P. labillardierei*), root length ($t = 5.24, df = 11, P \leq 0.0001$ for *L. sativa* and $t = 5.96, df = 11, P \leq 0.0001$ for *P. labillardierei*) and biomass ($t = 6.64, df = 11, P \leq 0.0001$ for *L. sativa* and $t = 3.97, df = 11, P \leq 0.002$ for *P. labillardierei*) significantly varied between non-sterile and sterile soil. In sterile soil, root length of *L. sativa* was inhibited by 69, 40 and 26% at full, half, and quarter-strength plant leachate respectively whereas they were 62, 39 and 3% for *P. labillardierei*. In non-sterile soil, the inhibition rate was 52, 38 and 5% for *L. sativa* and 42, 19 and 5% for *P. labillardierei*. Root growth and biomass of both species were suppressed significantly when grown in sterile soil treated

with leachate even at leachate levels of lower strength. There was significant interactive effect (leachate \times soil \times species) on root growth ($F_{3,32} = 3.51, P \leq 0.02$) but germination percentage and biomass were significantly affected by the individual effect of those factors (Table 2). Overall, *L. sativa* showed more sensitivity in effects than *P. labillardierei*.

Discussion

FIELD STUDIES

The decreased pH level in rhizosphere soil-water and soil surface water in invaded area indicates that *P. australis* lowers the pH level through root exudation and/or decomposition of residues. The findings are compatible with the results of Herr et al. (2007) who found that *Solidago gigantea*, a widespread invasive species in Europe, lowered soil pH compared with uninvaded area. Armstrong and Armstrong (1999) reported that decomposition of belowground organs of *P. australis* might increase the organic acids such as acetic, propionic, butyric and caproic, in sediments and thereby decrease the pH level. In turn, higher EC and OP level may occur in invaded areas, a fact supported by the studies of Atwell et al. (2013) who found higher EC in soil invaded by an invasive sedge in wetlands. WSP were higher during the vegetative stage (summer) suggesting that the plants are fully biologically active and suppress the surrounding plant community through direct allelopathy. The seasonal variation of WSP in soil is related to phenological cycles of the invasive plant, a point well documented in other studies (Carballeira and Cuervo, 1980) who suggested the persistence and accumulation of phenolic and other compounds in soil might vary with seasons and thereby, influence in shaping the structure of the plant community. In addition, phenolic compounds are highly correlated with DOC because a large part of phenolic compounds released into the environment are through organic matter decomposition

and they remain in the environment as DOC. Our results indicate that WSP accounted for about 6.5% of DOC but this varied seasonally. The occurrence and persistence of WSP in wetland rhizosphere soil-water and soil-surface water in *P. australis* invaded area suggest that they could be involved in complex and interactive processes occurring throughout organic matter decomposition and accumulation in wetland systems (Gallet and Pellissier, 1997; Rashid et al., 2010).

LABORATORY STUDIES

Accelerated respiratory metabolism in plant seeds during germination is essential in yielding metabolic energy and biosynthetic precursors (Perata et al., 1997). A constant supply of readily respiratory carbohydrates and soluble sugars plays a crucial role in maintaining respiratory metabolism and germination. Though, a limited amount of the readily utilizable soluble sugars in plant seeds is as usual whereas starch remains as the main reserve carbohydrate (Guglielminetti et al., 1995). The important role of α -amylase has been considered in conversion of reserve carbohydrate to soluble sugars during germination (Perata et al., 1997). Thus, induction of α -amylase plays an important role in maintaining the active respiratory metabolism, which helps germination of plant seeds. Our results showed that all solutions inhibited the germination and the induction of α -amylase activity in *L. sativa* seeds. The extent of germination in our study was positively correlated with the α -amylase activity in the seeds. These results suggest that solution may contain phenolic compounds and thereby reduce the germination of test seeds through inhibiting the α -amylase activity. This may be the possible mechanical action of phenolic compounds present in *P. australis* on the inhibition of seed germination identified in our previous study which reported the effects of aqueous extracts of *P. australis* on carbohydrate metabolism (Uddin et al., 2014a). Kato-Noguchi and Macías (2005) and Poonpaiboonpipat et al. (2013) also

reported a correlation between germination and the α -amylase activity of *L. sativa* and *Echinochloa crusgalli* in their phytotoxicity studies respectively.

Suppression of root initiation of hypocotyl cuttings in *M. pruriens* (Linn.) by all solutions indicates the presence of phytotoxins in those solutions which might be involved in activities that lead to interference on cell division (Batish et al., 2008a). Chou and Lin (1976) also reported phytotoxic effects by the extracts of decaying rice residue and soil from paddy field on root initiation of *M. pruriens* (Linn.). The findings of our study indicate that allelopathy in *P. australis* may potential interfere with the physiological process of root growth in plants during rhizogenesis as was found by Singh et al. (2013) who reported that phenolic acids are potent root inhibitors and impair rhizogenesis. As adventitious rooting has important ecological function for vegetative propagation of some wetland plants like *Melaleuca* and *Salix* so these might be affected through the release of phytotoxins by *P. australis* in their habitat. In addition, our results showed that suppression of germination, root length and biomass varied among seasons and species similar to that found by Inderjit (1996) who reported that the allelopathic effects of the same volume of soil from the same site may differ with season corresponding with the phenology of the plants invaded. Seasonal effects might be due to phenolic compounds present in the soil and/or altered soil nutrients that are themselves dependant on phenological cycle of the invaded plant. The availability of phenolic contents in soil depends on edaphic factors, plant phenology, climatic fluctuations and activities in soil (Dalton et al., 1983) thereby causing variable inhibition on associated plant species.

The soil sterilization experiment suggests that soil microorganisms might play an important role in degradation of phytotoxins present in the leachate of *P. australis* and thereby, result in less inhibition. The results found in this study align with the

results of Tongma et al. (1998) who suggested that the inhibitory effects of leaf water extract of *Tithonia diversifolia* was less in non-sterile than sterile soil. In addition, a large number of studies, for example, Kaur et al. (2009) and Zhu et al. (2011) have demonstrated that allelopathic effects could be reduced by the influence of soil biota but varied with the mode of allelochemical release and concentration in the soil. Recent studies have shown that *Centaurea maculosa* had allelopathic effects on native species in its invaded range but not in its native range a fact suggesting the net effects of native soil biota rather than adaptation by native plants (Thorpe et al., 2009). Our results might also suggest a greater role of soil biota in the invasion processes of *P. australis* through allelopathy.

Our studies found that *P. australis* can substantially modify plant growth characteristics in wetland ecosystem by the release of phytotoxins through root exudation and litter decomposition in soil and water. The allelochemicals in *P. australis* might have direct and indirect effects on soil and water chemistry which may in turn contribute to changes in ecosystem function. Our results suggest an overall relationship between the field chemistry of *P. australis* dominated wetland and the differential response of assayed species in terms of germination, growth, morphological features, and belowground mutualisms, but the strength and direction of relationship varied among species and seasons. However, this study along with our previous studies (Uddin et al., 2012; Uddin et al., 2014a; Uddin et al., 2014b; Uddin et al., 2014c) does go some way to explain *P. australis* gaining the competitive advantage over other species by the release of phytotoxins a fact supported by other studies (Rudrappa et al., 2007; Rudrappa et al., 2009). In addition to our studies, the community level effects through changing of field chemistry by *P. australis* were also reported in other studies (Chambers et al., 1999; Farnsworth and Meyerson, 1999). Thus, we can suggest

allelopathic effects of *P. australis* on competing plant species do exist in the invasion processes. Additionally, we found a significant effect of soil biota in altering the allelopathic effects on the competing plant species but to what extent such allelopathic effects are being influenced, and the indirect effects of allelochemicals secreted by *P. australis* on belowground mutualisms needs to be further investigated. The invasion success of *P. australis* may in part be attributable to the release of phenolics compounds and the subsequent effects on the neighbouring plants. In the context of global invasion of *P. australis*, our findings might have important ecological implications for understanding the effects of invasive species and mechanism of its invasion. Furthermore, these results may forward to future study in understanding the realistic evidence for allelopathy of *P. australis* through separation of resource effects from chemical effects.

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Table 1. ANOVA of seasonal variation for soil surface water and soil water chemistry such as pH, electrical conductivity (EC), osmotic potential (OP), water soluble phenolics (WSP), and dissolved organic carbon (DOC) in invaded and uninvaded area by *Phragmites australis*.

| Source | df | pH | | EC | | OP | | WSP | | DOC | |
|------------------|-------|--------|----------|-------|----------|-------|----------|--------|----------|--------|----------|
| | | F | P | F | P | F | P | F | P | F | P |
| Season (S) | 3, 24 | 106.84 | < 0.0001 | 71.68 | < 0.0001 | 72.04 | < 0.0001 | 6.51 | 0.002 | 14.38 | < 0.0001 |
| Sample type (ST) | 1, 24 | 270.00 | < 0.0001 | 45.12 | < 0.0001 | 44.99 | < 0.0001 | 373.72 | < 0.0001 | 77.62 | < 0.0001 |
| Invasion (I) | 1, 24 | 12.21 | 0.002 | 8.24 | 0.0001 | 8.32 | 0.008 | 698.42 | < 0.0001 | 592.01 | < 0.0001 |
| S × ST | 1, 24 | 48.13 | < 0.0001 | 0.37 | 0.34 | 0.38 | 0.544 | 20.23 | < 0.0001 | 4.13 | 0.053 |
| S × I | 3, 24 | 1.46 | 0.249 | 0.82 | < 0.0001 | 0.83 | 0.487 | 7.33 | 0.001 | 13.60 | < 0.0001 |
| ST × I | 1, 24 | 0.13 | 0.718 | 0.06 | 0.50 | 0.06 | 0.795 | 0.002 | 0.962 | 11.18 | 0.003 |
| S × ST × I | 1, 24 | 3.33 | 0.080 | 1.61 | 0.02 | 1.60 | 0.217 | 1.009 | 0.325 | 0.08 | 0.780 |

Table 2. ANOVA for the effects of *Phragmites australis* whole plant leachate on germination, root length and biomass of *Lactuca sativa* and *Poa labillardierei* when grown in non-sterile or sterile soil.

| Source | <i>df</i> | Germination | | Root length | | Biomass | |
|-------------------|-----------|-------------|----------|-------------|----------|----------|----------|
| | | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> |
| Leachate (L) | 3, 32 | 138.13 | < 0.0001 | 126.71 | < 0.0001 | 86.62 | < 0.0001 |
| Soil (S) | 1, 32 | 70.56 | < 0.0001 | 101.08 | < 0.0001 | 64.07 | < 0.0001 |
| Plant species (P) | 1, 32 | 2.56 | 0.119 | 88.24 | < 0.0001 | 169.64 | < 0.0001 |
| L × S | 3, 32 | 6.88 | < 0.001 | 1.14 | 0.34 | 1.97 | 0.13 |
| L × P | 3, 32 | 1.38 | 0.256 | 8.64 | < 0.0001 | 11.20 | < 0.0001 |
| S × P | 1, 32 | 19.36 | < 0.0001 | 0.46 | 0.50 | 18.05 | < 0.0001 |
| L × S × P | 3, 32 | 2.18 | 0.109 | 3.51 | 0.02 | 1.55 | 0.22 |

Fig. 1. Seasonal variation for soil water chemistry such as pH, water soluble phenolics (WSP), and dissolved organic carbon (DOC) in invaded and uninvaded area by *Phragmites australis*. Data are mean \pm SE, $n = 3$.

Fig. 2. Effects of whole plant leachate (open symbols) and soil extract (closed symbols) on (a) germination, (b) α -amylase activity, and (c) relationship between germination and α -amylase activity of *Lactuca sativa* with three concentrations: full-strength (triangle), half-strength (circle) and quarter-strength (square). Data are mean \pm SE, $n = 3$.

Fig. 3. Effects of whole plant leachate; soil extract and soil surface water on (a) rooting percentage, (b) number of roots/explant, and (c) average root length of *Phaseolus aureus* with three concentrations: full-strength, half-strength and quarter-strength. Data are mean \pm SE, $n = 3$.

Fig. 4. Seasonal effects of *Phragmites australis* infested soil on (a) germination, (b) root length, and (c) biomass of *Lactuca sativa*, *Poa labillardierei* and *Melaleuca ericifolia*. Data are mean \pm SE, $n = 3$.

Fig. 5. Differences in (a) germination, (b) root length, and (c) biomass of *Lactuca sativa* and *Poa labillardierei* treated by *Phragmites australis* leachate with three concentrations (full-strength, half-strength and quarter-strength) in non-sterile and sterile soil. Data are mean \pm SE, $n = 3$.

Fig. 1.

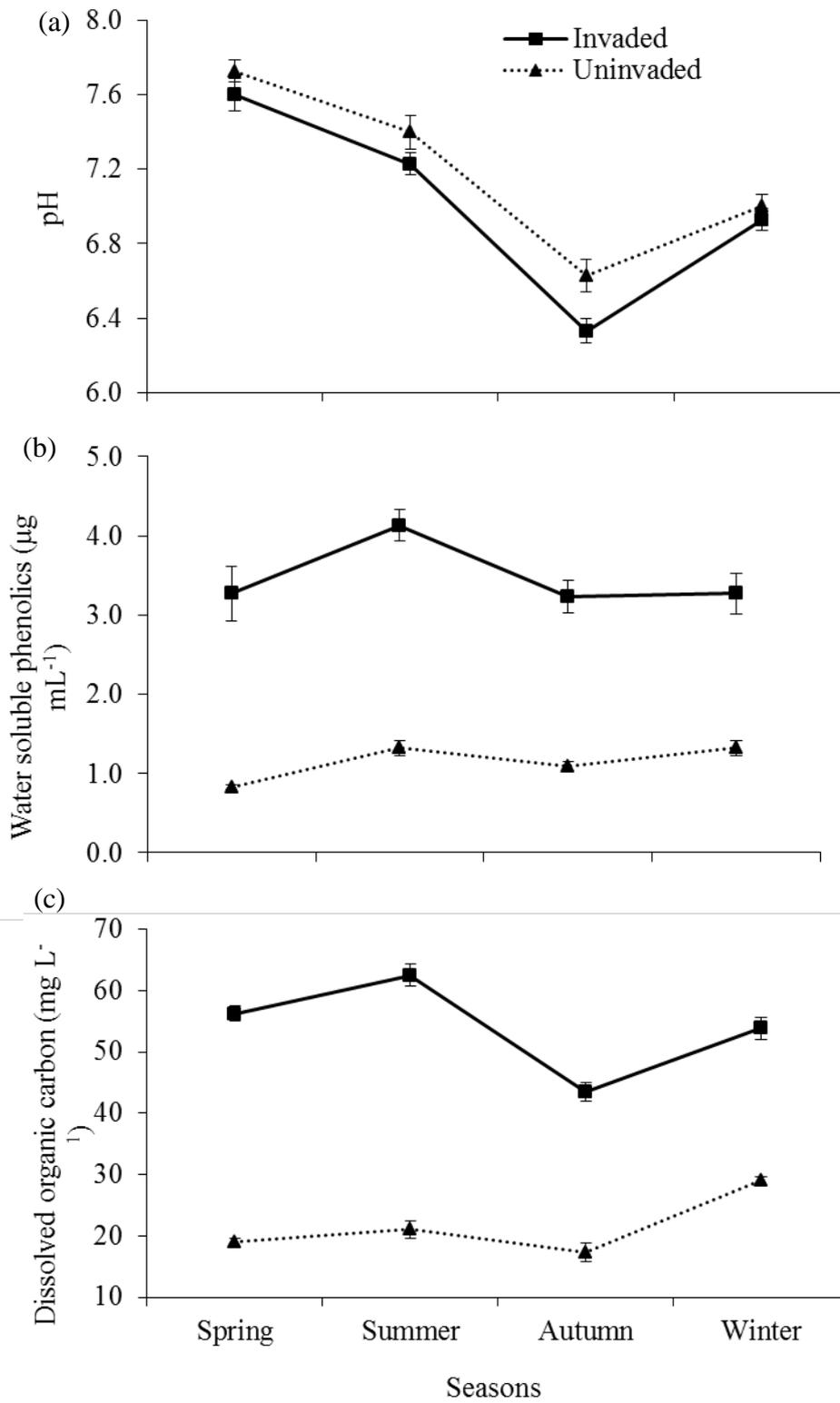


Fig. 2.

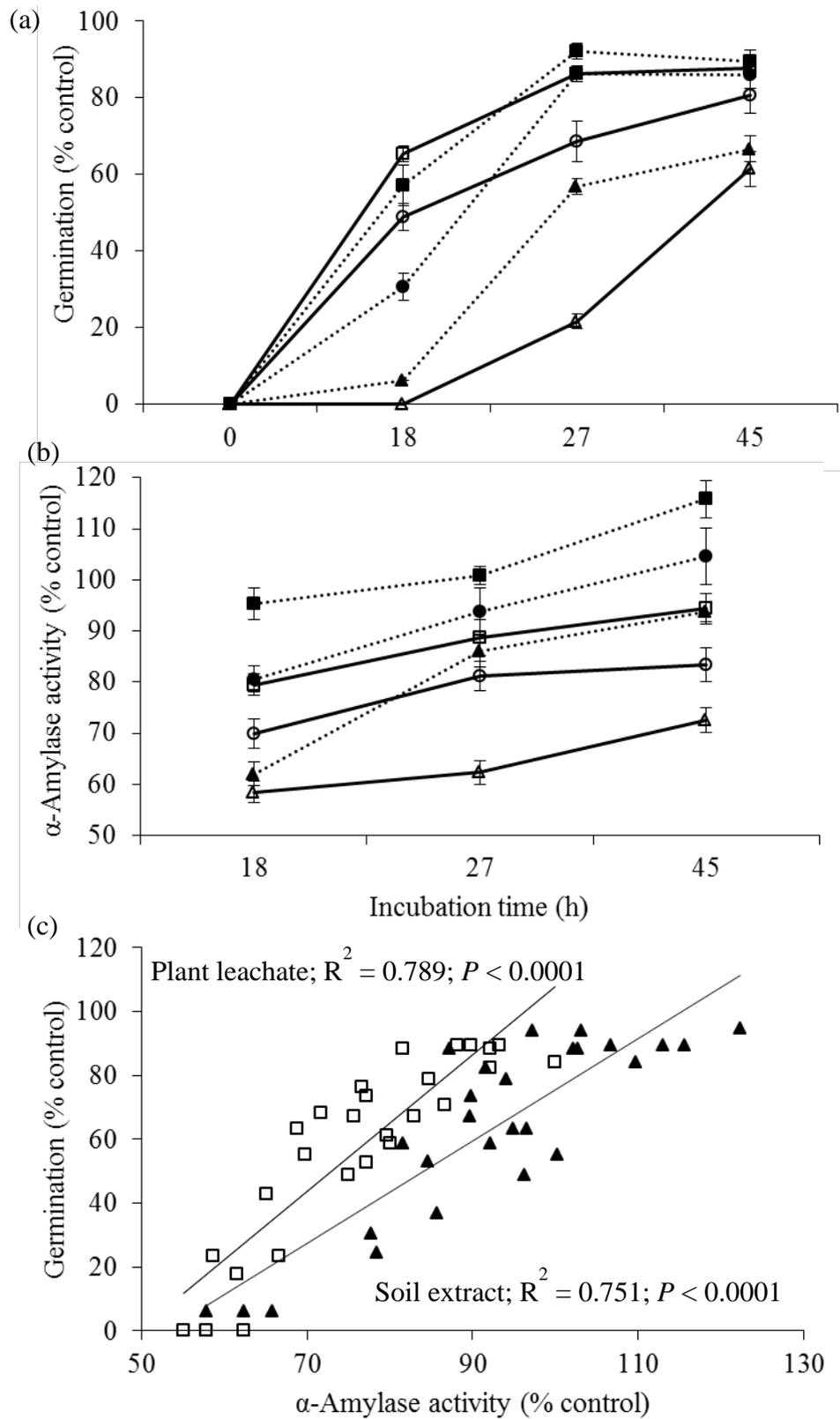


Fig. 3.

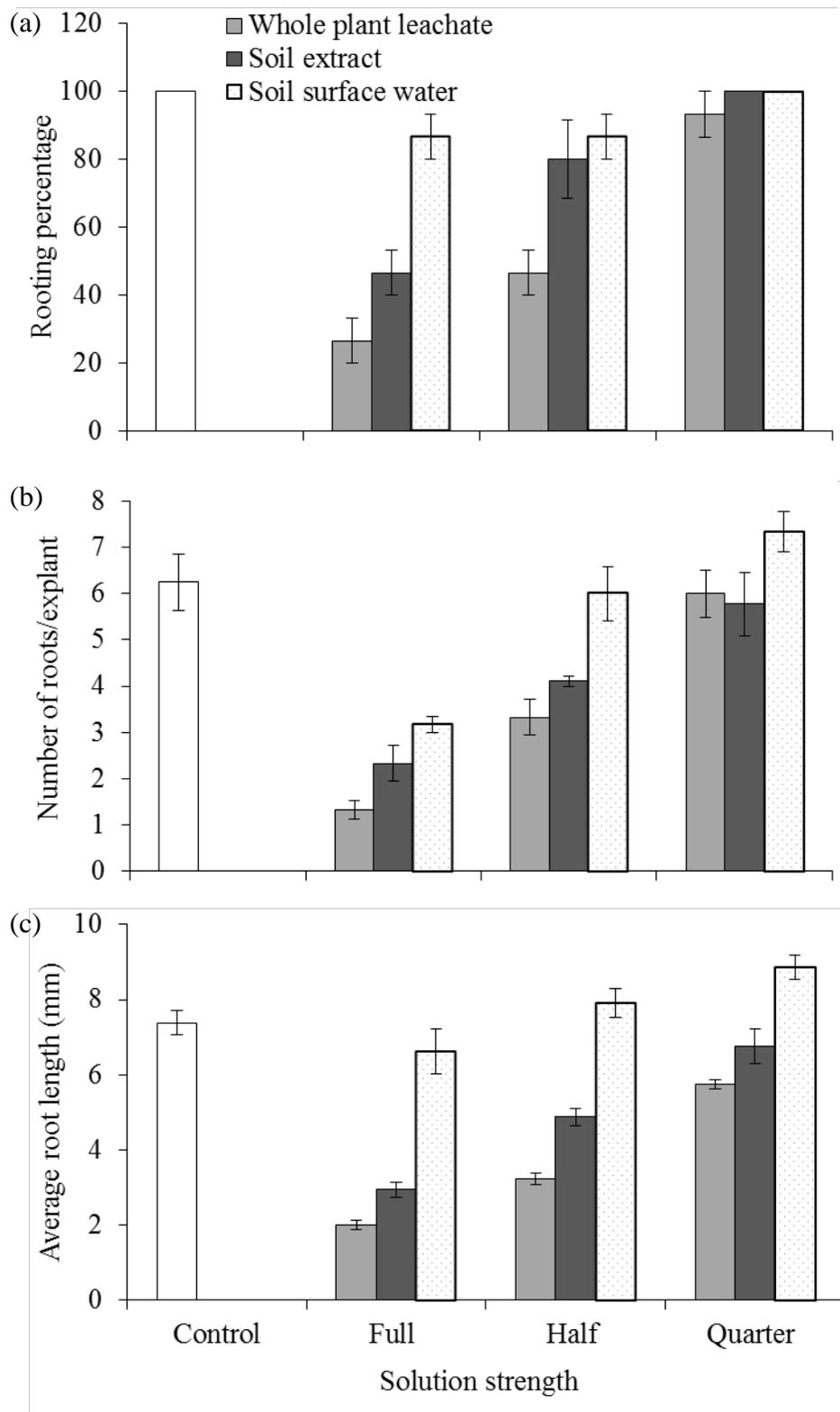


Fig. 4.

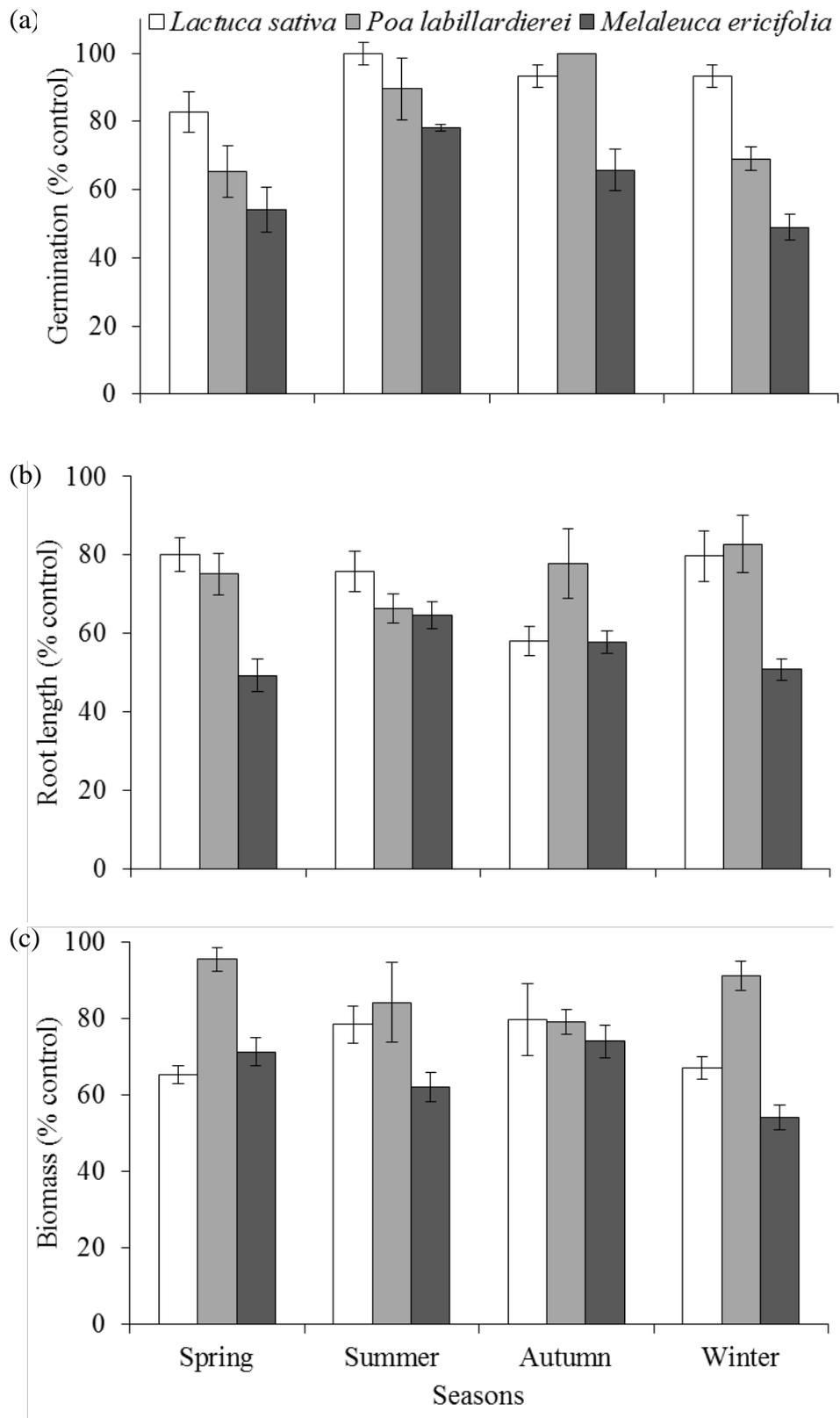
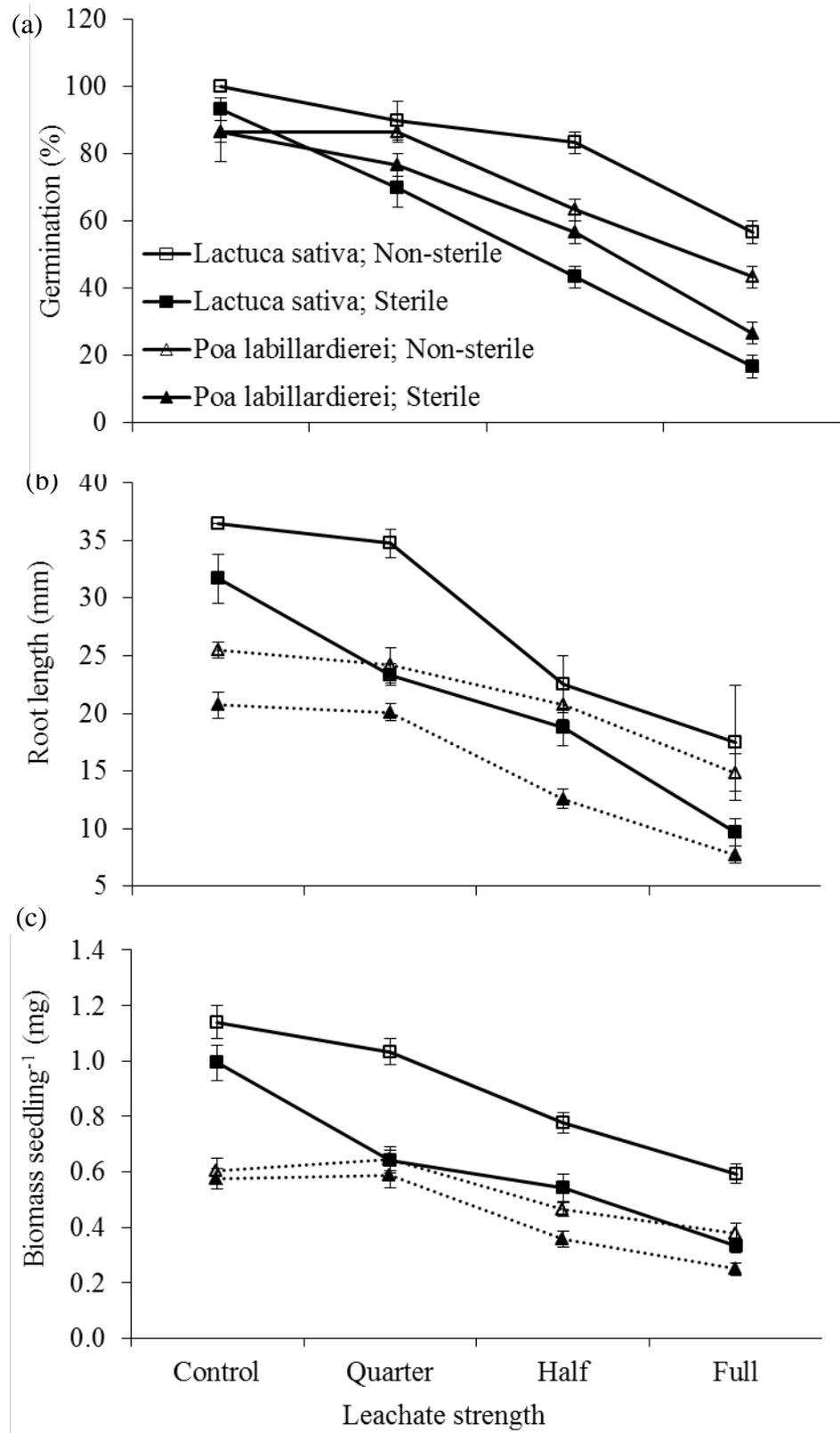


Fig. 5.



Chapter Six

Assessment of Root and Litter Mediated Potential Allelopathic Interference of Phragmites australis through Density-Dependent Approach

Introduction

This chapter set out to differentiate between allelopathy and resource competition. The difficulty of distinguishing allelopathy from resource competition among plants has hindered investigations of the role of phytotoxic allelochemicals in plant communities. Considering the complexity we addressed a series of ecological realistic experiments in the greenhouse and laboratory.

The following manuscript entitled '**Assessment of root and litter mediated potential allelopathic interference of *Phragmites australis* through density-dependent approach**' by Md. N. Uddin and Randall W. Robinson has been submitted for publication in *Australian Journal of Botany*, received revision and it is again submitted.

PART B:

**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS
 INCORPORATED IN THESIS BY PUBLICATION**

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Md. Nazim Uddin

Signature: 

Date: 24/07/2014

Paper Title: Assessment of root and litter mediated potential allelopathic interference of *Phragmites australis* through density-dependent approach

In the case of the above publication, the following authors contributed to the work as follows:

| Name | Contribution % | Nature of contribution |
|---------------------|----------------|--|
| Md. Nazim Uddin | 85 | Concept development; plant, soil and seed collection; conducting experiments and chemical analysis; data collection, statistical analysis and interpretation; and manuscript writing, editing and submitting for publication |
| Randall W. Robinson | 15 | Concept development and manuscript editing |

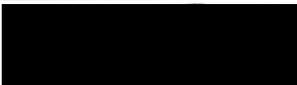
DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
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|-------------|---|------------|
| Signature 1 |  | 18/07/2014 |
| Signature 2 |  | 18/07/2014 |

Assessment of root and litter mediated potential allelopathic interference of *Phragmites australis* through density-dependent approach

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Abstract. The response of plants to phytotoxins varies from stimulatory to inhibitory as the concentration of the phytotoxin increases. In this study, a biological response of exposed plants was measured to estimate the responses to changes in phytotoxin concentrations as plant densities increased. The difficulty of distinguishing allelopathy from resource competition among plants has hindered investigations of the role of phytotoxic allelochemicals in plant communities. To address this complex issue we conducted a series of ecologically realistic experiments in the greenhouse and laboratory. Experimental plants (*Melaleuca ericifolia*, *Rumex conglomeratus*, and *Lactuca sativa*) were grown at varying densities with the allelopathic plant, *Phragmites australis* and varying concentrations of aqueous leachate and extracts of *P. australis* litter to investigate the potential interacting influences of allelopathy and resource competition on plant growth-density relationships. Results showed that phytotoxicity decreased as plant density increased and maximum individual plant biomass and some other positive effects on plant traits occurs at an intermediate density. This was attributed to plant dilution of phytotoxins, i.e. the sharing of the available phytotoxin among plants at high densities. The results demonstrate that either decreasing phytotoxicity with increasing plant density or a reversal in slope of the growth-density

relationship is proposed as an indication of the allelopathic interference of *P. australis* rather than resource competition.

Additional keywords: density, dilution, ecosystems, growth, invasive species, phytotoxins, regressions.

Introduction

Allelopathic interference by invasive plant species has the potential to impact seed germination, seedling growth and development and establishment of neighbouring plant species, as well as of the same species, in both natural and agricultural systems (Dorning and Cipollini 2006; Lara-Núñez *et al.* 2006). Allelopathy has been considered an important attribute to the success of an invasive species in natural ecosystems (Callaway and Ridenour 2004; Lorenzo *et al.* 2010). The sources of allelochemicals released into the rhizosphere include leaching from leaves and other aerial parts, volatilization, root exudation and litter decomposition (Hussain and Reigosa 2012; Uddin *et al.* 2012; Weir *et al.* 2004).

Phragmites australis, a ubiquitous wetland plant, has been considered one of the most invasive species in the world (Uddin *et al.* 2012), however, the origin of the species is still unclear (Plut *et al.* 2011). It is a perennial graminaceous plant, to 3 m tall, which reproduces mainly through rhizomes and, at low frequency, through seeds. It grows in all temperate zones of the world, especially North America, most of the countries in Europe, some part of Canada and Australia (Hocking *et al.* 1983; Kulmatiski *et al.* 2011), being especially common in south-eastern Australia (Kettenring *et al.* 2011; Morris *et al.* 2008). The worldwide, regional and local distribution and abundance of *P. australis* has expanded over the last 150 years and in most areas it forms dense monocultures (Saltonstall *et al.* 2005). Due to the impacts of *P. australis*

invasions, habitats have been diminished or altered significantly for other flora and fauna causing loss of biodiversity and ecosystem functions (Mack *et al.* 2000). Several studies have identified chemicals within *P. australis* organs which have antialgal, antifungal or antibacterial effects (Li and Hu 2005). Some chemicals produced by decomposition of belowground organs of *P. australis* may be responsible for die-back of *P. australis* itself (Armstrong and Armstrong 1999) and photo-degradation of secreted chemicals from *P. australis* produces severe phytotoxicity to other plant species (Rudrappa *et al.* 2009). Previous allelopathic studies, including ours, have shown that water extracts, decomposed materials, root exudates and specific identified chemicals of *P. australis* organs have strong phytotoxic effects on germination, growth, and establishment of other plant species (Kettenring *et al.* 2011; Rudrappa *et al.* 2007; Uddin *et al.* 2012; Uddin *et al.* 2014a; Uddin *et al.* 2014b; Uddin *et al.* 2014c) and thus, achieves its inference success into invasion process in wetlands (Bains *et al.* 2009; Rudrappa *et al.* 2007).

While *Phragmites australis* clearly has shown phytotoxic potential, the effects should be considered in more ecologically realistic ways and in so differentiating allelopathic interactions from resource competition because the allelopathic effects might be masked by resource competition among target plants in some case, and a better understanding of the dose-response relationship of allelochemicals of *P. australis* would help clarify this issue. Toxin dilution is thought to occur because plants share and compete not only for resources but also for toxin. Thus, the dose of a phytotoxin received by a plant is inversely related to plant density and this toxin dilution study can be performed using density-dependent experiments which might be a potential tool for exploring the effects of toxins on plant growth as well as for differentiating the resource competition from allelopathy. As the study of allelopathic interactions may be hindered

by the lack of proper experimental methods it may be more productive to first demonstrate explicit interference by allelochemicals rather than rely solely on explanations that involve resource competition or other mechanisms (Barto and Cipollini 2009; Thijs *et al.* 1994).

The maximum seasonal biomass of living *P. australis* shoots and rhizomes may reach more than 200 t ha⁻¹ (Engloner 2009) that makes *P. australis* one of the largest biomass producers in aquatic ecosystems. The worldwide distribution and the extremely large areas covered by *P. australis* stands may have a considerable effect on the accumulation of phytotoxins through litter decomposition in wetlands (Uddin *et al.* 2014c). Litter management has been identified as an important issue in the control of invasive plant species. Prescribed burning has been used as a management strategy to reduce biomass in some ecosystems of invasive species such as *P. australis* but no studies have been carried out on the fate of allelochemicals in *P. australis* after burning.

The use of activated carbon (AC) as a soil amendment, may be useful for determining distinct differences between allelopathy and resource competition (Inderjit and Callaway 2003) although AC may change the availability of soil nutrients (Weißhuhn and Prati 2009). Weidenhamer (2006) recently proposed that allelopathic effects might be differentiated experimentally using the density-dependent nature of phytotoxic effects, which cause deviations from predicted growth-density relationships. The effects by allelochemicals depends on density of neighbouring target plant species and might be masked by resource competition at high density (Weidenhamer 1996). Phytotoxicity modifies population density to alter plant response through dilution of available toxins among plants i.e. phytotoxicity decreases as plant density increases (Thijs *et al.* 1994). Density-dependent models suggest that yield decreases with increasing density due to resource competition acting as a dominating factor.

Alternatively, allelopathy might be strong but that results show a slow decrease of yield or even increase in yield as density increases, until density reaches a point where resource competition among neighbouring target plant becomes the dominating factor. Results of previous studies have shown that plants exposed to pure chemicals (Andersen 1981), ground tissue (Tseng *et al.* 2003) and soil (Weidenhamer *et al.* 1989) mediated by allelopathic plants operate in accordance with the above model. Because phytotoxic affects follow this density and phytotoxicity interactions that may cause deviations from expected growth-density relationships (Weidenhamer 2006).

On the basis of methodological complexity and other issue, this study has been designed to determine the occurrence and magnitude of potential allelopathic effects mediated by *P. australis* root and litter for a wide range of doses using a density-dependent approach. We hypothesized that allelochemical phytotoxicity depends on the neighbouring plant density due to phytotoxins dilution among individual plant. This method might be effective in distinguishing the allelopathic interactions of *P. australis* with neighbouring plant species from resource competition.

Materials and methods

Study site, plant litter and soil sample collection

We collected fallen leaves of *P. australis* in June 2011 from natural stands adjacent to Cherry lake (37°51' 30"S, 144°50' 5"E), a coastal wetland in Altona, Melbourne, Australia. All samples were placed into sealable plastic bags for transportation to the laboratory. Plant samples were sorted from other plant residue and debris then kept at room temperature to air dry until constant dry weight. After desiccation, the sorted samples were cut into small pieces (< 2 cm) and preserved in plastic ziplock bag until use. Soil samples were collected from the top layer of *P. australis* free areas of the same

study site, separated from other organic materials, dried in room temperature and kept into ziplock bag after passing through 2 mm sieve.

Choice of target species

Seeds of several species were used to determine any differential response in native, introduced and model species to allelopathy. Seed capsules of the native species, *Melaleuca ericifolia* and introduced species, *Rumex conglomeratus* were collected in May 2011 from Cherry Lake and stored in paper bags at room temperature for one week. Seeds were shaken from the capsules and sieved to remove empty capsules and other detritus. Associated species were used because of ecological relevance. ‘Seeds of a model species’, *Lactuca sativa* were purchased from a commercial source (DT Brown Seeds, South Windsor, NSW, Australia). *Lactuca sativa* was selected as it is widely used in phyto-toxicity bioassays. Model species are easily grown, minimizing the risk of observed growth differences being due to factors other than the treatments applied in the experiments. Additionally, it is not possible to affirm that growth differences in *Melaleuca ericifolia* and *Rumex conglomeratus* are totally due to treatments applied in the experiments, but also factors other than treatments.

Greenhouse experiments

Root mediated effects on M. ericifolia seedlings

Spring buds of *P. australis* with rhizome attached were collected on 8 September 2011 from Cherry Lake. Each live rhizome was cut to contain exactly one active node, weighed and planted within 6 h of collection in 7 L plastic pots lined with watertight plastic bags filled with 4 L substrate [a 1 : 7 mixture of unsterilized river sand and potting mix soil respectively (Earth-wise Growing Essential, Australian Prime Fibre Pty Ltd, Queensland- 4660)]. Potting mix contained organic materials (pine bark), living

organisms (bacteria, fungi and protozoa), minerals and fertilizers additives (details on potting mix in Appendix S1). The control pots contained only substrates were kept with other treated pots in the greenhouse with same condition until *M. ericifolia* juvenile plants were replanted. To all of the pots, 1 g/L of mixed pelletised fertilizer (Pivot fertilizer-900; N-P-K: 16-8-9) was incorporated into the tilled topsoil bimonthly. Pots were kept in a natural lit greenhouse at $23 \pm 3^{\circ}\text{C}$ and $12 \pm 2^{\circ}\text{C}$ day/night temperature and watered regularly with an auto irrigation system equipped by micro sprinklers at the soil surface to keep soil moist at a level of $55 \pm 5\%$ compatible with field soil. Soil moisture was monitored weekly by measuring the water content in soil randomly collected from the pots. Pots were randomly shuffled every week to minimise the spatial effects and unwanted germinant (weeds) were removed. Tube stock of *M. ericifolia* (6 mo old), grown in potting mix, were purchased from 'Go Native Landscapes Pty Ltd' (Inverloch, Victoria, 3996, Australia) which were grown from seeds collected from *M. ericifolia* stands at Dowd Morass wetland in Inverloch, Victoria, Australia. On 10 November 2011, the purchased *M. ericifolia* juvenile plants were planted into the prepared pots with and without *P. australis* by three neighbour densities (one, two, and four plants per pot) with five replicates of each treatment. After six months of *M. ericifolia* growth, all plants (*P. australis* and *M. ericifolia*) were harvested and data collected on above-ground biomass (AGB), below-ground biomass (BGB), root-shoot length, plant height, stem diameter, and number of growth points.

Litter mediated effects on M. ericifolia seedlings

Phragmites australis free soil (200 g) was set in 1.5 L pot, moistened to approximately field saturation level and kept for two days in the greenhouse. Then, equal size two-month old *M. ericifolia* seedlings grown in natural lit greenhouse replanted in each pot with a density of one, two, and four plants at three replicates. After 3 weeks of plant

acclimation, equal size litter (≤ 2 cm) was put on soil surface in the pot with a concentration of 4 g/100 g of soil but no litter for control and kept them as above conditions. The amount of litter given on the soil surface was chosen on our previous field observations on the amount of litter fall in a 1-m² quadrat. After 4 months, all plants were harvested and measured for the various phenotypic characteristics.

Laboratory experiments

*Litter extract mediated effects on *R. conglomeratus* seedlings*

Litter extract was made soaking litter in distilled water with a concentration of 10% (100 g litter in 1 L water). After 24 h it was filtered with cheese cloth, centrifuged with 3000 rpm and the filtrate was used for the experiment with pH adjustment at 6.5. Fifty grams of *P. australis* free soil was moistened with 30 mL litter extract at four different concentrations (0, 2.5, 5.0, and 10.0 %) in 250 mL container, and incubated for 24 hours. In general, the most of the bioassays are conducted in the allelopathy study at concentrations of 1-5% (the weight or mass of the plant matter per volume of solvent) (Reigosa *et al.* 2013). In addition, we used a wide range of doses to ensure that they would encompass the lowest dose for an observable effect, as well as the highest dose for maximal effect (Belz *et al.* 2007). Equal numbers (15 seeds with three replicates) of *R. conglomeratus* seeds were sown in each pot to ensure emergence of adequate numbers of even-age seedlings. All pots were then placed in a growth chamber (Westinghouse, Electrolux home products, Australia) set to 25/15°C day/night temperature and a 12 h photoperiod with illumination of 84 $\mu\text{mol s}^{-1} \text{m}^{-2}$. One week after emergence, seedlings were thinned to a density of one, two, four, and eight in each pot. Water loss by evaporation was measured by weight and compensated with extract twice in a week. Plants were harvested and measured the phenotypic and physiological characteristics after 6 weeks of treatments.

Unburnt versus burnt litter extracts mediated effects on Lactuca sativa seedlings

Phragmites australis litter was placed in a furnace at 300°C for 1 h to produce burnt litter and a phyto-toxicity test was conducted with extracts of both type of litter (unburnt and burnt). Extracts were made by mixing unburnt and burnt litter powder passed through 0.5 mm sieve with distilled water to a concentration of 10%. After 24 h it was filtered with cheese cloth and centrifuged at 3000 rpm with the resultant filtrate being used as the extract for the experiment with pH adjustment at 6.5. Extract (5 mL) at four different concentrations (0, 2.5, 5.0, and 10.0 %) was placed into a sterile 9 cm Petri dish containing two sterile sheets of filter paper (Whatman No. 1). At least, three replicates were used for each treatment with density of one, two, four and eight pre-germinated *L. sativa* seedlings. Petri dishes were sealed with parafilm (Pechiney, Plastic Packaging Company, Menasha, WI 54952) then placed in polyethylene bags to prevent water loss by evaporation and to avoid contamination by fungi and bacteria. The prepared dishes were arranged in a completely randomized design (CRD) and placed in a growth chamber according to above mentioned conditions. The Petri dishes were randomized each day to minimize the spatial effect. After 7 days of experiment, phenotypic characteristics of the grown of the plants were measured.

Litter mediated effects on L. sativa seed germination

Seed germination bioassay was conducted using the 'sandwich' method adopted from Fujii et al (2004). Four different concentrations of air dried litter by weight (0, 2.5, 5.0, and 10%) were placed in between two layers of 0.5% agarose (total 10 ml) in a container of 4.5 by 5.5 cm. Agarose was autoclaved at 121°C for 15 min and subsequently cooled at room temperature. Then seeds of *L. sativa* at a density of four, eight, and 16 were placed on each container and incubated for 7 days as above condition

with three replicates. The germination and biometric parameters were measured at the end of the experiment.

Phenolics determination

Total phenolics (TP) and water soluble phenolic (WSP) content were measured in unburnt and burnt litter with three replicates. Approximately 100 mg portion of powder (burnt and unburnt litter) was weighed out and transferred to Eppendorf tube. After addition of 5 ml of 70% acetone for TP and distilled water for WSP, they were incubated at 4°C for 1 h to extract phenolics followed by centrifuging at 15,000 rpm for 10 min at 4°C. The 0.5 ml of the supernatant was taken and made up to 1 ml with distilled water. Then 5 ml of 2% Na₂CO₃ in 0.1 N NaOH was added and mixed using vortex mixer (Vortex Mixer, VOU1, Ritek Instruments Pty Ltd., Australia). To the mixture obtained by the above process 0.5 ml Folin-Ciocalteu reagent was added and mixed. After 2 h, absorbance was read at 760 nm. Based on the standard curve, phenolic was determined as gallic acid equivalents of the sample (mg TP and WSP per g sample).

Chlorophyll measurement

Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll were determined by placing about 15 mg of fresh leaf of *R. conglomeratus* in 7 ml of N-N dimethylformamide (DMF) for 24 h in the dark at 4°C for extraction (Moran and Porath 1980). The absorbance of the extracts was measured spectrophotometrically at 664 and 647 nm and chlorophyll was determined following the equation proposed by Inskeep and Bloom (1985).

Statistical analysis

All the experiments were conducted in a completely randomized design with at least three replicates. All data were referred as % of the values of control treatments at each density, analysed and presented according to Hansi et al. (2014). Briefly, data were analysed by ANOVA (one, two and three way depending on the data specifically mentioned in the table and figure) using IBM SPSS statistics 21.0. Variance homogeneity was tested using Levenes's test and transformed if necessary using SPSS to compensate for variation within population. Significance tests were performed using univariate analysis of variance with 2-sided Duncan's tests at the 0.05 probability level. Furthermore, results were subjected to linear regression procedures and the correlation coefficient values, evaluating the dependence of each measured variables on the concentration of the residues and residue extracts as well as plant and seed density, were calculated using Microsoft Excel 2010 according to Singh et al. (2002). Again, an effort has been made to determine whether the relationship is statistically significant through regression analysis by Microsoft Excel 2010.

Results

Greenhouse experiments

Root exudates of *P. australis* affected the biomass and other morphological characters of *M. ericifolia* at various densities (Table 1). Most of the growth parameters declined with increasing plant density indicating that plant density was the most significant factor manipulating the growth of *M. ericifolia* such as AGB ($F_{2,6} = 26.96, P < 0.01$), BGB ($F_{2,6} = 52.91, P < 0.001$), total biomass ($F_{2,6} = 29.04, P < 0.001$), root length ($F_{2,6} = 39.95, P < 0.001$), plant height ($F_{2,6} = 14.65, P < 0.005$), and growth points ($F_{2,6} = 111.83, P < 0.001$).

Treated soil mixed with or without the leaf litter of *P. australis* reduced growth of *M. ericifolia* by inhibiting biomass, root length, plant height, stem diameter and number of growth points at various densities (Table 2). A differential in reduction was observed in the whole experiment but medium density treatments had increased growth compared to low and high density treatments. Total dry biomass per plant was reduced by 94%, 77% and 90% at low, medium and high density respectively. The reduction of root length (65%), plant height (44%), stem diameter (61%) and number of growth points (89%) was more severe at low density than high density but comparatively less inhibited in the medium density. Growth parameters like AGB ($F_{2,12} = 20.90$, $P < 0.001$), BGB ($F_{2,12} = 16.39$, $P < 0.001$), total biomass ($F_{2,12} = 20.71$, $P < 0.001$), root length ($F_{2,12} = 18.22$, $P < 0.001$), plant height ($F_{2,12} = 6.65$, $P < 0.01$), stem diameter ($F_{2,12} = 28.83$, $P < 0.001$), and number of growth points ($F_{2,12} = 6.41$, $P < 0.01$) varied significantly across the densities. Regression analyses among densities and growth parameters of *M. ericifolia* showed that most of the slopes were reduced in the pots to *P. australis* treatments compared to the control (not shown in figure).

Laboratory experiments

Plants grown in *P. australis* litter leachate treated soil showed that the growth, biomass and other morphological and physiological parameters of *R. conglomeratus* were affected by both leachate concentration and density of test plant (Fig. 1 and Table 3). The degree of inhibition and stimulation by leachates was density-dependent. Generally, all growth parameters (AGB, BGB, total biomass, root length, plant height, number of leaves and chlorophyll content) increased with increasing plant density at the lower and medium leachate concentration but this phenomenon sometimes occurred even in higher level. The highest biomass reduction was observed at the low plant density (one seedling/pot) treatment by 51% at 2.5% leachate amended soil compared to control soil

whereas 49% and 35% were in medium (two seedlings/pot) and high (eight seedlings/pot) density respectively but stimulated at intermediate (four seedlings/pot) density by 49%. All results showed a reduced leachate effect in relation to plant density and there was a clear trend to stimulation of growth as density increases. The variation in the magnitude of stimulation with density demonstrates dose-dependency of the stimulatory response whereas no stimulatory effects were observed in the control. Deviations in the relationships between plant growth parameters and density were pronounced but comparisons of the regression lines showed that the differences in observed slopes were not significant with the exception of total number of leaves and chlorophyll content (not shown in figure).

The effects of unburnt and burnt litter extracts of *P. australis* on the growth of *L. sativa* seedlings indicated significant reduction in terms of AGB, BGB, total biomass, root and shoot length (Table 4 and Figs. 2 & 3). Greater inhibition was observed with increasing concentration of the aqueous extracts. Materials (unburnt versus burnt), concentrations and densities all showed differential effects individually and interactively on biomass and root length of *L. sativa* (Table 4). BGB was inhibited more than AGB but AGB-BGB ratio showed significant individual and interactive effects (Table 4). Inhibition was higher in unburnt residue than burnt extracts (Figs. 2 & 3) but the degree and magnitude of inhibition was not strongly correlated with the TP and WSP of the extracts. TP in unburnt versus burnt was 17.77 ± 0.64 versus 4.15 ± 0.24 mg g⁻¹ whereas WSP was 4.59 ± 0.1 versus 2.3 ± 0.3 mg g⁻¹ (Fig. 4). Although phenolics were significantly lower in burnt residue extracts, the inhibition shown by burnt extracts was not significantly lower than unburnt residue (Figs. 2 & 3). The observed deviations (inhibitory and stimulatory) from the growth-density relationships were measured by the comparison of the slopes among treatments (Figs. 2 & 3). Medium density had

larger biomass and root length compared to low and higher density (Figs. 2 & 3). Total biomass of *L. sativa* was significantly higher in burnt extracts than unburnt (Fig. 2). The total biomass increased with increasing density as compared to the control, except for density one in both unburnt and burnt litter extract.

Density-dependent germination bioassay using the 'sandwich' method showed differential effects on germination and growth parameters (Table 5 and Fig. 5). Germination percentage was significantly affected by both interactive and individual effect of residue concentration and density. All measured parameters (AGB, BGB, total biomass, root and shoot length) varied significantly across the residue concentrations and density individually but not interactively (Table 5). BGB was the lowest at lower density and decreased (13%, 31% and 68%) with increasing concentration (1.25%, 2.5% and 5.0% respectively) compared to control while highest in the intermediate density. Root and shoot length was always higher in low densities and concentrations but decreased with increasing densities and concentrations (Fig. 5).

Discussion

Analysis of growth-density relationships may be useful as a tool for understanding the resource competition and allelopathic interference between plants prior to costly phytochemical investigations of the suspected invasive species (Weidenhamer *et al.* 1989). Though the issue 'separating/distinguishing allelopathy from resource competition' is controversial in natural ecosystems (Inderjit and del Moral 1997; Weidenhamer 2006) it is important in plant-plant interactions to identify the mechanisms involved in biological invasion processes. In general, the yield (in terms of growth and development) of plants decreases with increasing density; in contrast, density-dependent phytotoxicity studies revealed that plant growth may be positively influenced up to the point where resource competition acts as the dominant factor.

Density-dependent phytotoxicity studies imply a positive feedback between population density and phytotoxins present in a system, as the toxin is shared among increased plant biomass with each plant receiving a proportionately smaller amount of toxin. Density-dependant phytotoxicity stands in contrast to resource competition as increased growth of plants at low density is dependent on large part to the amount of resources available. Despite the allelopathic potential of *P. australis* on associated and model plant species, as shown by the growth of *M. ericifolia*, *R. conglomeratus* and *L. sativa* observed in this study was masked by the resource competition but the allelopathic effects are well supported by our previous studies (Uddin *et al.* 2012; Uddin *et al.* 2014a; Uddin *et al.* 2014b; Uddin *et al.* 2014c) as well as the study of Rudrappa *et al.* (2007) of *P. australis*. Those studies showed that water extracts of different organs, residue decomposition and root secreted phytotoxins had negative effect on germination, growth, and development of plant species.

In our greenhouse experiment, the root exudates of *P. australis* exhibited a differential pattern of growth of *M. ericifolia*. There are obvious effects by root exudates on the neighbouring plants, but there is also a possibility of competitive effects this is possible to be separated by density-dependent phytotoxicity concept. The strongest growth inhibition was observed in high density treatments when compared to low and medium density demonstrating that resource competition is the dominating factor, as was found in our previous studies (Uddin *et al.* 2014b) as well as in Inderjit and del Moral (1997). Our studies demonstrated that allelopathy through root exudates of *P. australis* had relatively low contribution in suppression of *M. ericifolia* in comparison to other competitive effects. Again the *P. australis* litter mediated soil experiment showed the highest root suppression of *M. ericifolia* that could be due to allelochemicals leached from the litter mediated soil as the intermediate density of *M. ericifolia* showed

increased growth compared to low and high density. The findings are well supported by the density-dependent phytotoxicity concept that demonstrates either of the consequences which are contrary to the expected outcomes of resource competition and support the presence of phytotoxins in soil system. The finding is also well-aligned with the results of Barto and Cipollini (2009) where inhibition was observed as the density increases due to resource completion at higher density. The total assumptions of the density-dependent phytotoxicity concept proposed by Weidenhamer (2008) state that growth is reduced at low but diminished at high density compared to control; and plant growth is highest at intermediate density due to a reversal in slope of the predicted growth-density line. Moreover, the density-dependent theory in phytotoxicity states that if allelopathy is the dominating factor, then plant yield will decrease more slowly, or even increase, as density increase, until a density is reached where resource competition among target plants become the dominating factor (Weidenhamer *et al.* 1989).

In addition to the greenhouse experiments, the laboratory experiments showed a clear density-dependent phytotoxic effect, a result well aligned with other studies where both allelochemicals and herbicides studies showed that phytotoxicity is density-dependent (Weidenhamer *et al.* 1987). In addition, recently Hansi et al (2014) showed that the phenomenon of density dependence of toxin interactions is also applicable to inorganic compound such as copper. Our results suggest that high concentration of phytotoxin may cause a reverse slope of predicted growth-density relationships at low plant densities, in contrast to the expected consequences of increased density and resource competition. The relationship between growth, in terms of biomass, root length, and plant height and plant density of *R. conglomeratus* exhibited a reversal in

slope (not shown in figure) that indicates the presence of phytotoxins in the litter leachate mediated soil used in the bioassay.

Prescribed burning is a management strategy used to control invasive plants (Lavoie *et al.* 2005), in particular for the control of *P. australis* (Mal and Narine 2004) and, to restore fire to the ecosystem and recreate natural disturbance dynamics (Cannac *et al.* 2011). But we have limited knowledge about the burning effects on the chemical compounds of *P. australis* including phenolics and their associate effects on plant species. In our studies, the significant variation in phenolic content of unburnt versus burnt residues did not reflect the associate effects on plant growth suggest that heat induced transformation of phenolic compounds might be effective in suppression of plant growth even though these residues may contain small amount of phenolic compounds. Zhang *et al.* (2011) also found that there was no significant difference between unburnt versus burnt residues of *Flaveria bidentis* (L) Kuntze on the growth of wheat (*Triticum aestivum* L.) seedlings. This study also commented that burning might not be a good way to eliminate the allelopathic effects of *Flaveria bidentis* (L). Furthermore, prescribed burning in natural wetlands might reduce phenolic content but the biological effects of burnt residue may be similar to unburnt residue. Overall, the effects of phytotoxins on the target plants depends on the bioactive concentration, which in turn depends on the quality of the phenolics and their renewal rate from the donor plant (Tan *et al.* 2008). A continuous influx of phytotoxins released into soil is therefore essential to induce the phytotoxic effects on the associated plant species which might be a reasonable explanation for *P. australis* because the wetlands dominated by *P. australis* achieved a large biomass in each life cycle. This huge biomass contributes the influx of phytotoxins through decomposition especially in anaerobic decomposition and

other biological activities in the wetlands. Thus the accumulated phytotoxins might harm to the germination and growth of the associated species.

The density-dependent seed germination study showed that germination percentage and root-shoot length of *L. sativa* are a function of both concentration and the amount of phytotoxin available per seed (Table 5 & Fig. 5). The inhibition increased as the concentration increased at lower density but stimulation was observed with intermediate density in most of the cases. This suggests that lower seed density increases the availability of phytotoxin per seed. Weidenhamer et al. (1987) found that even lower phytotoxin concentration may cause similar or greater inhibitory effects than higher concentrations when the amount of phytotoxin per seed is greater.

In general, allelopathy research is more concerned about the concentrations used in the bioassays by the question whether the doses involved are ecologically relevant. Despite it may be difficult to determine what concentrations are closest to those occurring naturally in the field which is important in determining casual relationships and minimizes effects due simply to unnaturally high doses of putative allelochemicals. An effort has been made to overcome the concern through measuring the concentration of allelochemicals in the *P. australis* rhizosphere soil (Uddin *et al.* 2012), considering the osmotic potential of higher concentrations (Uddin *et al.* 2014a), adjusting pH, as well as measuring the quantity of litter biomass produced per unit of soil or covered area in our previous studies that is considered in this study.

Moreover, separation of allelopathic effects from resource competition is a vital point in allelopathy research which was addressed in this project and indicated that phytotoxins secreted by different means from *P. australis* are responsible for invasion process except root exudation. Despite the results indicate less inhibition by root exudates by *P. australis* on *M. ericifolia* transplanted plants (Uddin *et al.* 2014b) but

toxin may arise from other sources like residue decomposition into soil that inhibit the germination processes and other growth parameters (Uddin *et al.* 2014c). These results are well aligned with other allelopathy studies of *Agropyron repens* in which Welbank (1960) found that decaying roots and rhizomes of *Agropyron* markedly inhibit the root and shoot growth of rape seedlings but no significant inhibition by root secretion. On the other hand, plant-plant allelopathic interactions may be explained by species-specific (Hierro and Callaway 2003; Prati and Bossdorf 2004) and contextual relationships (Bauer *et al.* 2012) that proves the consistency of whole results of our studies.

The overall observation of growth reductions in test plant species at low densities was inconsistent with the standard resource competition hypothesis and provides support for the hypothesis of chemical interference by *P. australis*. Although, the growth response of test species did not follow the same pattern in all experiments, in most of the cases the data demonstrate the density-dependent phytotoxicity concept. Therefore, these studies may provide an understanding of plant-plant allelopathy interactions and may distinguish the mechanisms involved in plant interference i.e. resource competition and allelopathy. Our findings may be useful to evaluate the response of agricultural plants such as *L. sativa* to weed residues, and it may also provide insight evidence of allelopathic potential in *P. australis* invaded wetlands. The density-dependent phytotoxicity phenomenon might have important ecological implications as a methodological approach in allelopathy (Weidenhamer and Romeo 1989).

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Table 1.

| Plant density/pot | Aboveground mass | Belowground mass | Total biomass | Root length | Plant height | Growth point |
|-------------------|------------------|------------------|------------------|------------------|-----------------|-----------------|
| Low | 94.47 ± 3.09 a | 88.03 ± 6.16 a | 93.87 ± 2.54 a | 100.00 ± 7.22 a | 90.56 ± 3.09 a | 98.27 ± 5.38 a |
| Medium | 62.98 ± 4.26 a | 67.90 ± 10.55 a | 63.42 ± 4.77 a | 85.86 ± 7.89 a | 93.74 ± 8.28 a | 71.72 ± 8.27 b |
| High | 165.45 ± 16.70 b | 273.33 ± 24.04 b | 171.65 ± 17.10 b | 195.56 ± 12.37 b | 126.92 ± 2.21 b | 194.42 ± 3.80 c |

Table 2.

| Plant density/pot | Aboveground mass | Belowground mass | Total biomass | Root length | Plant height | Stem diameter | Growth point |
|-------------------|------------------|------------------|----------------|----------------|-----------------|----------------|----------------|
| Low | 6.18 ± 0.84 a | 7.06 ± 1.13 a | 6.35 ± 0.86 a | 34.02 ± 2.71 a | 55.93 ± 4.08 a | 38.71 ± 2.33 a | 11.38 ± 1.47 a |
| Medium | 22.85 ± 4.40 b | 25.15 ± 4.80 b | 23.27 ± 4.26 b | 61.75 ± 3.49 b | 82.74 ± 1.49 b | 68.77 ± 2.32 b | 17.94 ± 2.18 b |
| High | 10.81 ± 1.24 a | 10.02 ± 2.15 a | 10.63 ± 1.35 c | 66.50 ± 8.80 b | 66.51 ± 6.42 ab | 48.27 ± 2.61 c | 8.22 ± 0.71 a |

Table 3.

| Plant growth parameters | Concentration (C) | Density (D) | C × D |
|-------------------------|--------------------------|---------------------------|--------------------------|
| Above-ground biomass | $F_{2,24} = 68.15^{***}$ | $F_{3,24} = 316.71^{***}$ | $F_{6,24} = 74.66^{***}$ |
| Below-ground biomass | $F_{2,24} = 40.02^{***}$ | $F_{3,24} = 267.80^{***}$ | $F_{6,24} = 28.92^{***}$ |
| Total biomass | $F_{2,24} = 71.26^{***}$ | $F_{3,24} = 313.54^{***}$ | $F_{6,24} = 70.88^{***}$ |
| Root length | $F_{2,24} = 23.49^{***}$ | $F_{3,24} = 51.72^{***}$ | $F_{6,24} = 17.87^{***}$ |
| Plant height | $F_{2,24} = 3.11^{ns}$ | $F_{3,24} = 39.01^{***}$ | $F_{6,24} = 9.12^{***}$ |
| Number of leaf/plant | $F_{2,24} = 9.00^{***}$ | $F_{3,24} = 5.50^{**}$ | $F_{6,24} = 5.72^{***}$ |
| Total chlorophyll | $F_{2,24} = 40.39^{***}$ | $F_{3,24} = 49.22^{***}$ | $F_{6,24} = 3.99^{**}$ |

Table 4.

| Source | <i>df</i> | Above-ground biomass (AGB) | Below-ground biomass (BGB) | Total biomass | AGB-BGB ratio | Root length |
|-------------------|-----------|-------------------------------|-------------------------------|--------------------------|--------------------------|---------------------------|
| Materials (M) | 1, 48 | F = 64.76 ^{***} | F = 1.92 ^{ns} | F = 25.55 ^{***} | F = 64.27 ^{***} | F = 246.88 ^{***} |
| Concentration (C) | 2, 48 | F = 10.54 ^{***} | F = 50.81 ^{***} | F = 9.71 ^{***} | F = 93.17 ^{***} | F = 180.34 ^{***} |
| Density (D) | 3, 48 | F = 37.02 ^{***} | F = 23.85 ^{***} | F = 40.55 ^{***} | F = 5.20 ^{**} | F = 154.91 ^{***} |
| M × C | 2, 48 | F = 11.03 ^{***} | F = 4.44 [*] | F = 4.55 [*] | F = 27.20 ^{***} | F = 19.55 ^{***} |
| M × D | 3, 48 | F = 7.41 ^{***} | F = 1.57 ^{ns} | F = 3.41 [*] | F = 11.98 ^{***} | F = 16.25 ^{***} |
| C × D | 6, 48 | F = 6.91 ^{***} | F = 3.78 ^{**} | F = 6.10 ^{***} | F = 2.06 ^{ns} | F = 6.19 ^{***} |
| M × C × D | 6, 48 | F = 1.00 ^{ns} | F = 1.34 ^{ns} | F = 1.01 ^{ns} | F = 2.96 [*] | F = 2.29 [*] |

Table 5.

| Plant growth parameters | Concentration (C) | Density (D) | C × D |
|-------------------------|--------------------------|--------------------------|------------------------|
| Germination percentage | $F_{2,18} = 24.17^{***}$ | $F_{2,18} = 7.61^{***}$ | $F_{4,18} = 2.76^*$ |
| Above-ground biomass | $F_{2,18} = 71.35^{***}$ | $F_{2,18} = 7.95^{**}$ | $F_{4,18} = 4.51^*$ |
| Below-ground biomass | $F_{2,18} = 30.54^{***}$ | $F_{2,18} = 7.19^{**}$ | $F_{4,18} = 0.43^{ns}$ |
| Total biomass | $F_{2,18} = 53.59^{***}$ | $F_{2,18} = 6.19^{**}$ | $F_{4,18} = 2.05^{ns}$ |
| Root length | $F_{2,18} = 91.53^{***}$ | $F_{2,18} = 14.99^{***}$ | $F_{4,18} = 2.40^{ns}$ |
| Shoot length | $F_{2,18} = 29.67^{***}$ | $F_{2,18} = 20.82^{***}$ | $F_{4,18} = 1.95^{ns}$ |

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Fig. 1. Effects of *Phragmites australis* litter leachate on (a) above-ground biomass, (b) below-ground biomass, (c) total biomass, (d) root length, (e) plant height and (f) total chlorophyll of *Rumex conglomeratus* seedlings at different densities. Values (weight per plant) are means as % of control treatments at each density \pm standard errors ($n = 3$). Letters indicate homogenous subgroups ($P \leq 0.05$) at each density in Duncan's test.

Fig. 2. Relationship of above-ground biomass, below-ground biomass and total mass and seedling densities of *Lactuca sativa* grown at different concentrations of *Phragmites australis* litter [unburnt (a, b, c) versus burnt (d, e, f)] extract. Values (weight per plant) are means as % of control treatments at each density \pm standard errors ($n = 3$). The figures include regression slope, coefficient of determination (R^2), and the significant level (P) according to extract concentration.

Fig. 3. Relationship of root length and seedling densities of *Lactuca sativa* grown at different concentrations of *Phragmites australis* litter [unburnt (a) versus burnt (b)] extract. Values (length per plant) are means as % of control treatments at each density \pm standard errors ($n = 3$). The figures include regression slope, coefficient of determination (R^2), and the significant level (P) according to extract concentration.

Fig. 4. Total phenolics (TP) and water soluble phenolics (WSP) *Phragmites australis* litter (unburnt versus burnt). Values are means \pm standard errors ($n = 3$).

Fig. 5. (a) Germination percentage, (b) above-ground biomass, (c) below-ground biomass, (d) total mass, (e) root length, and (f) shoot length in different concentrations of *Phragmites australis* litter mediated agarose at different seed densities of *Lactuca sativa*. Values (per plant) are means as % of control treatments at each density \pm

standard errors ($n = 3$). Letters indicate homogenous subgroups ($P \leq 0.05$) at each density in Duncan's test.

Fig. 1.

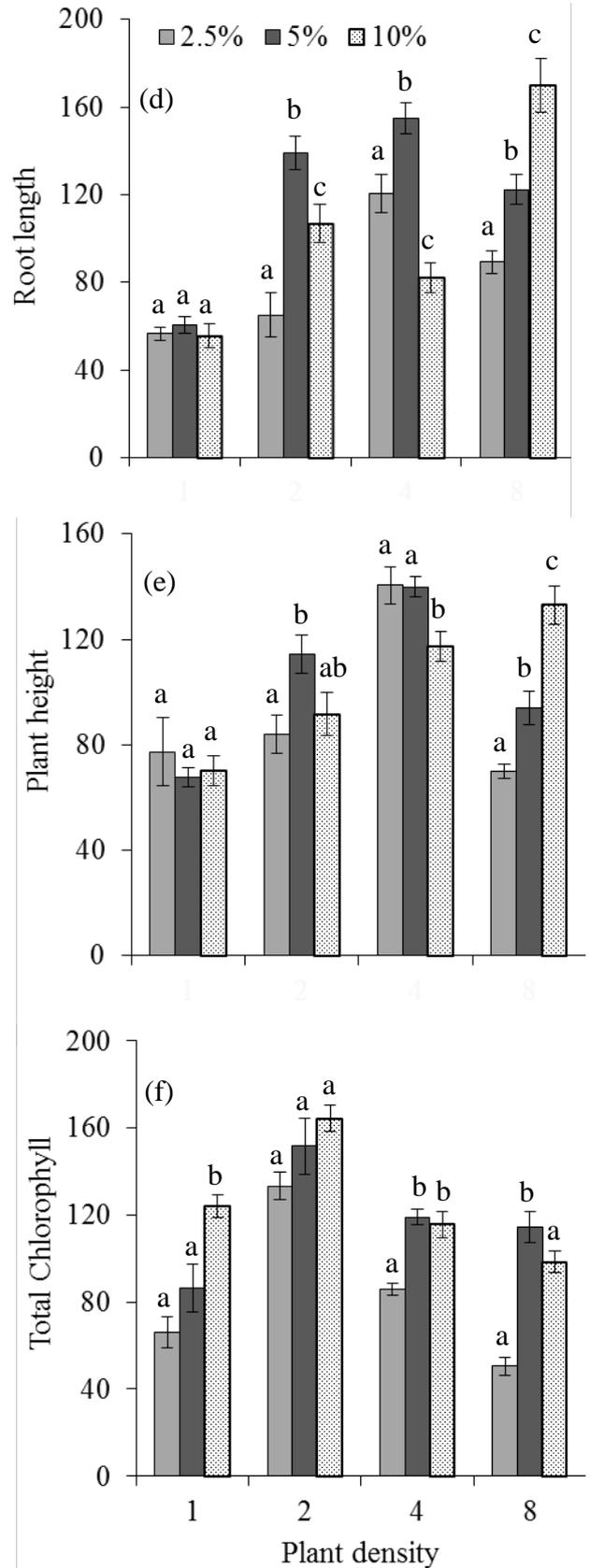
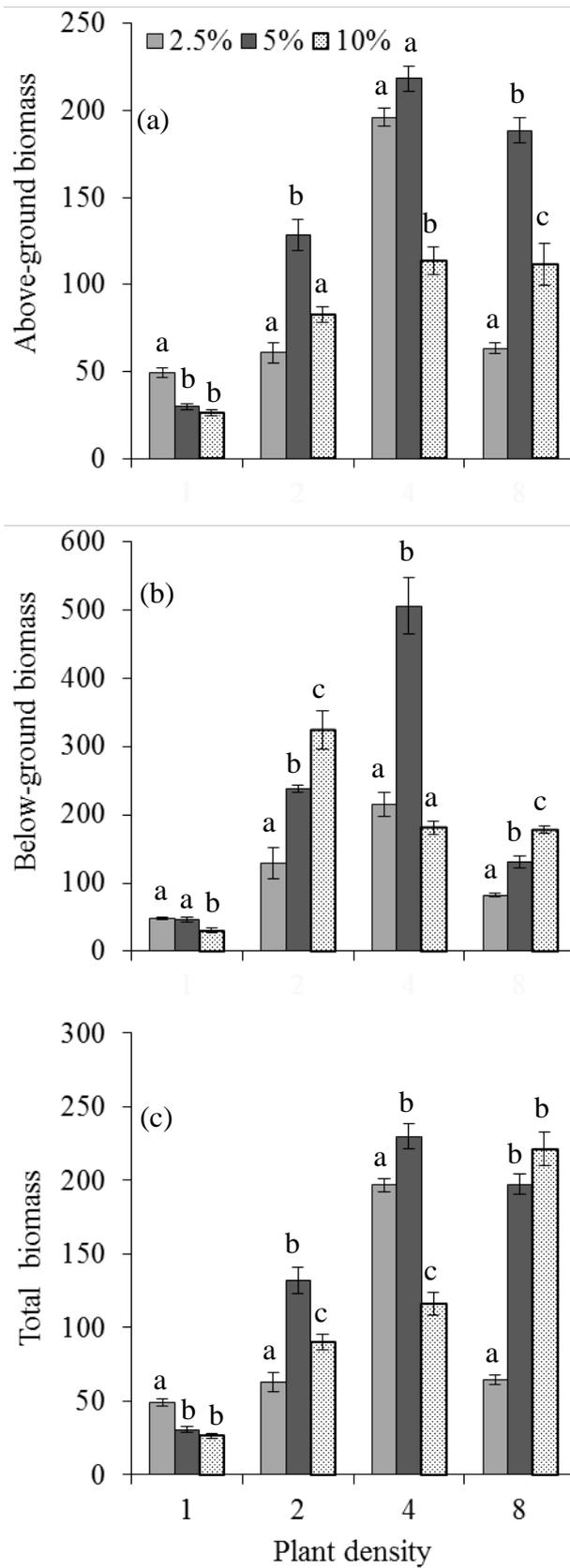


Fig. 2.

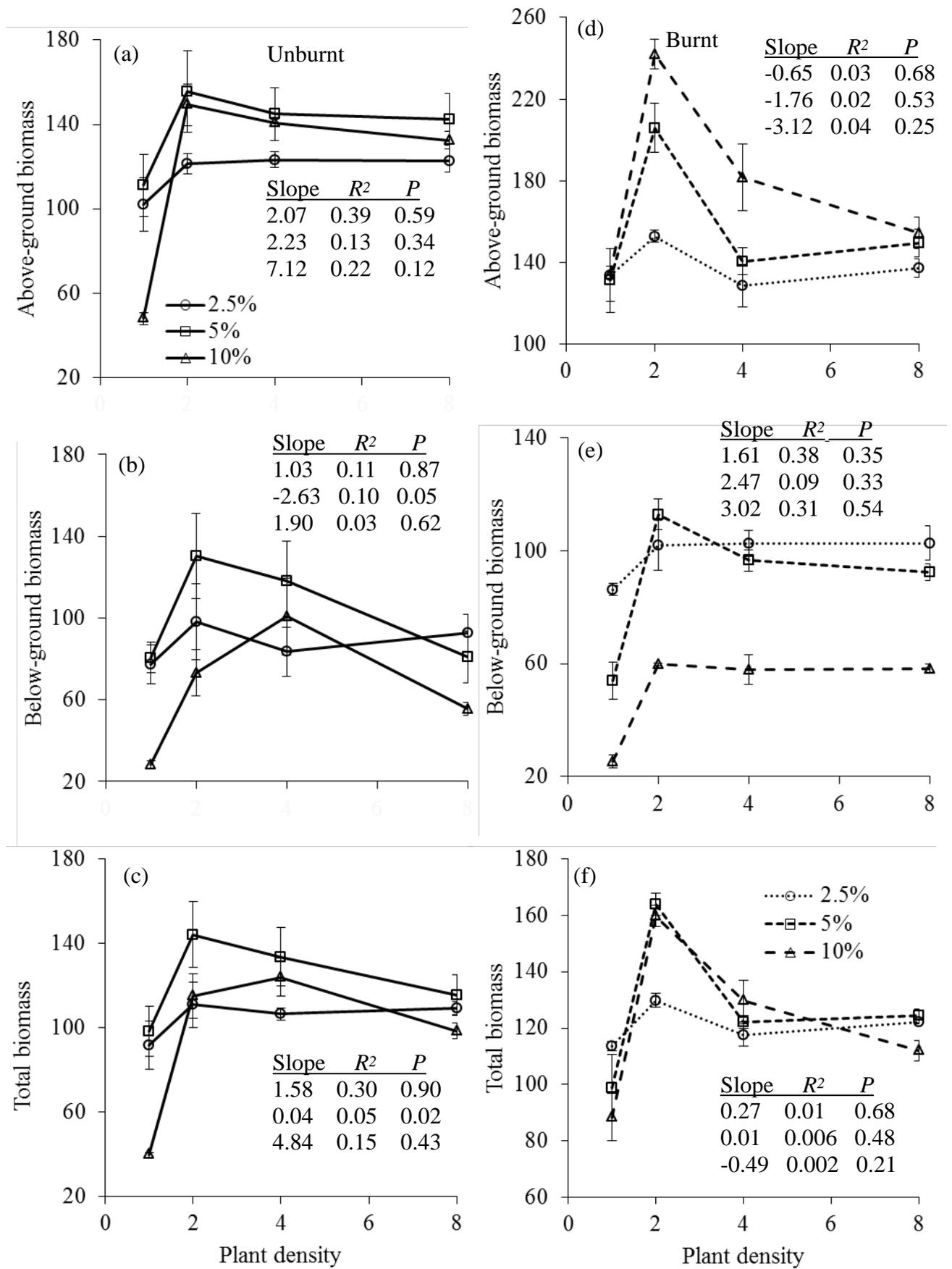


Fig. 3.

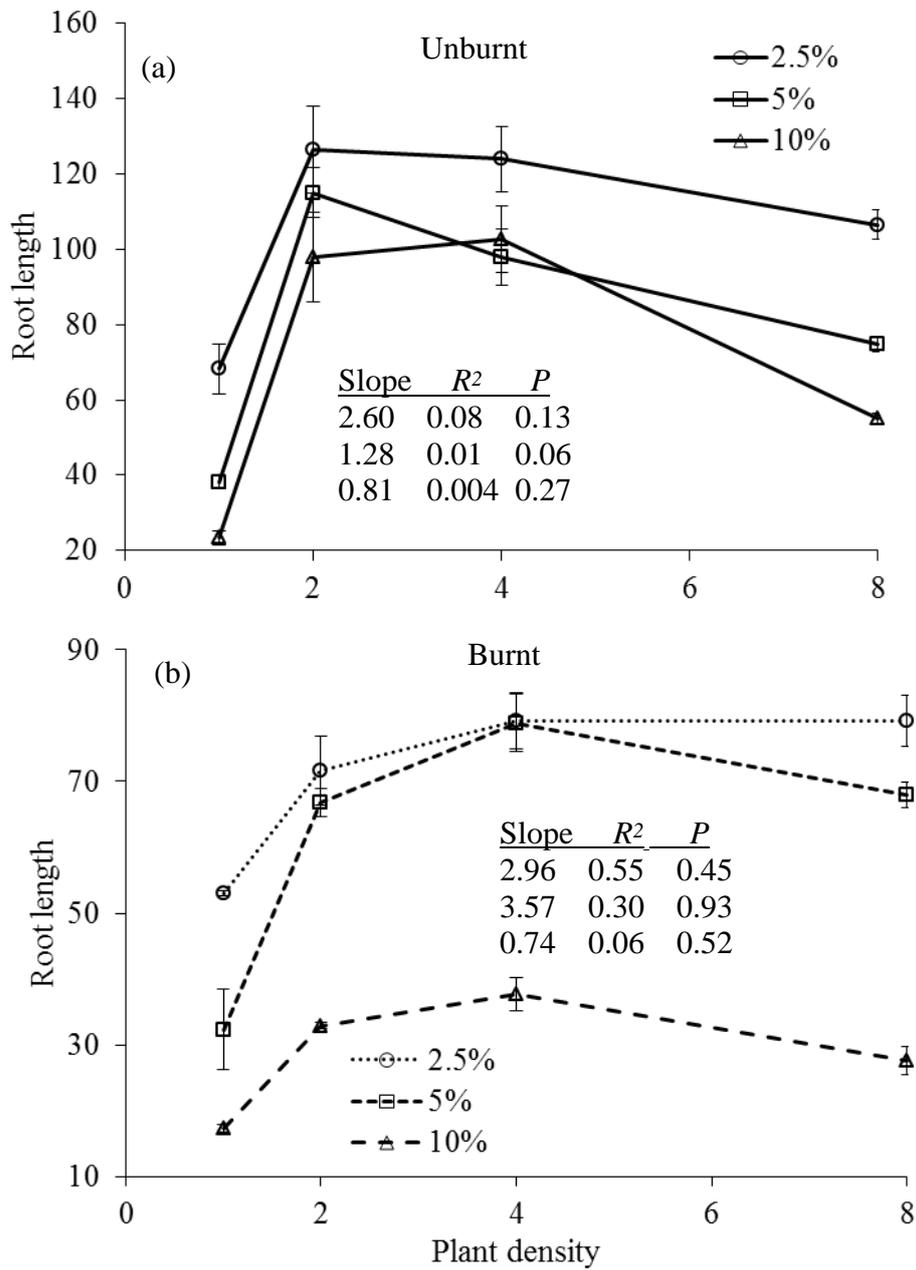


Fig. 4.

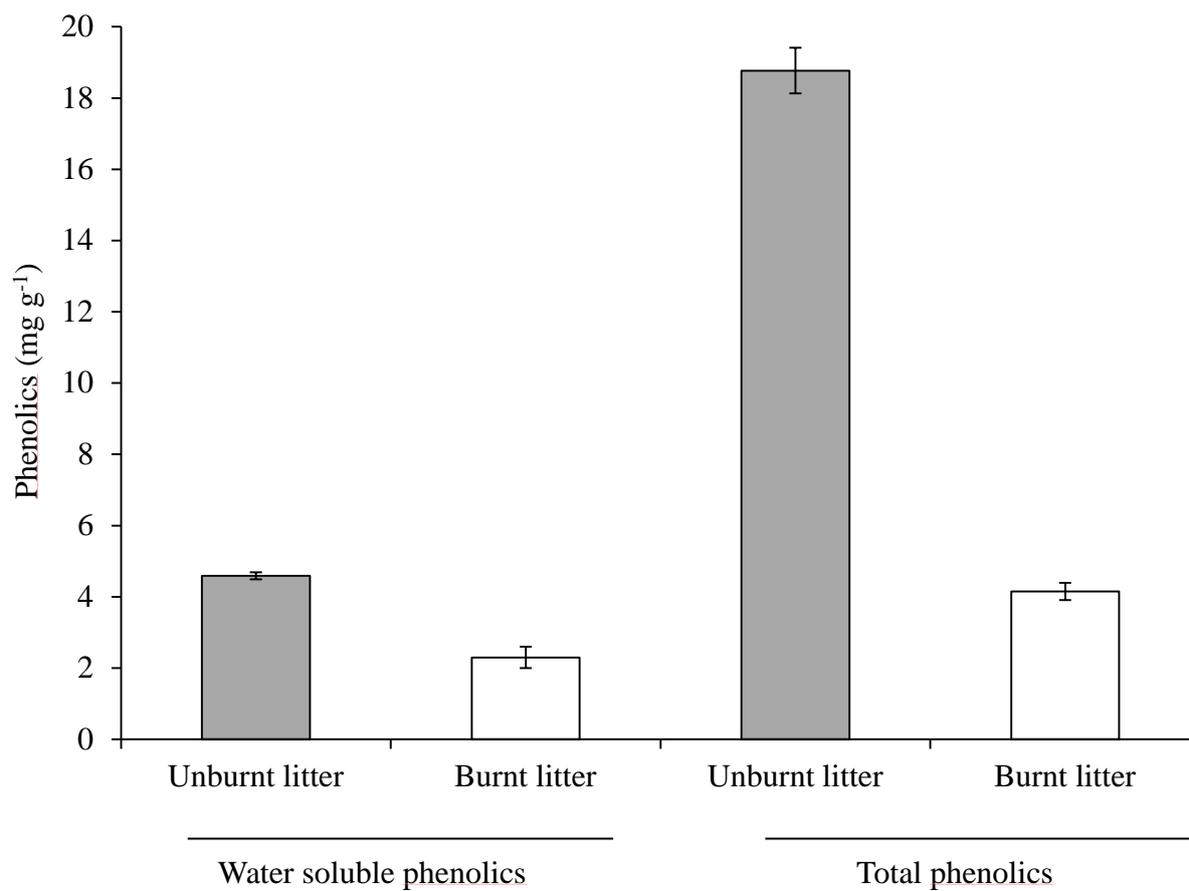
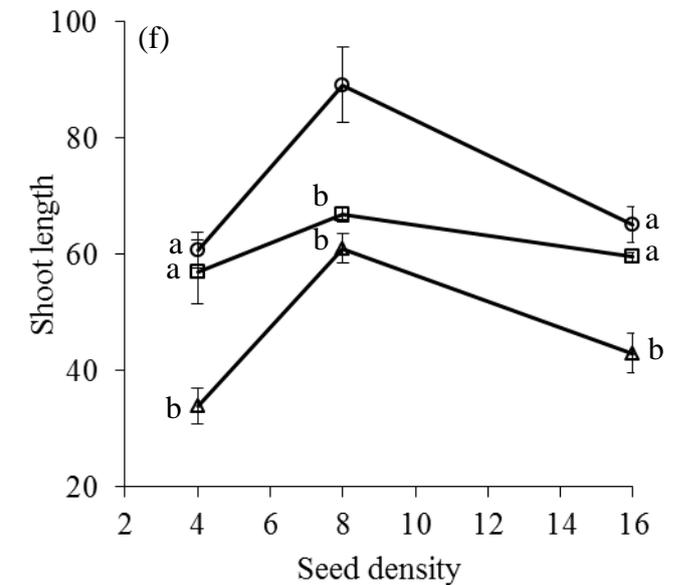
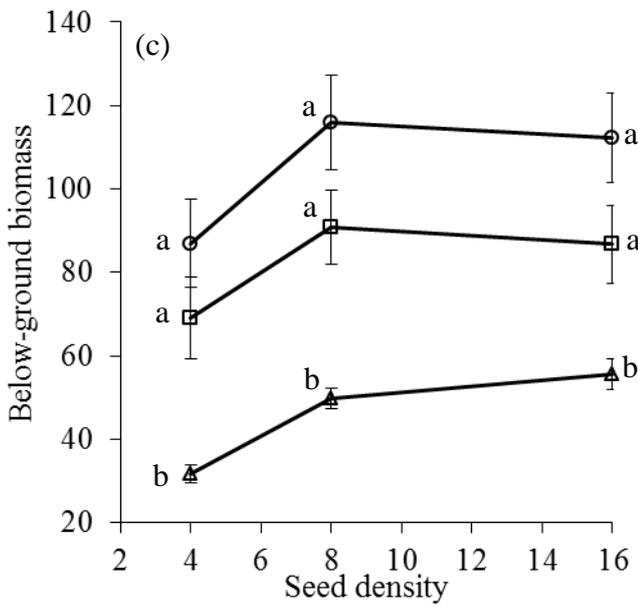
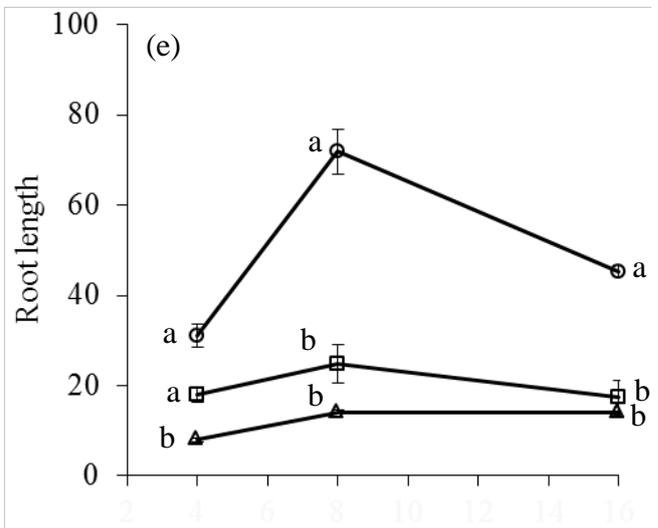
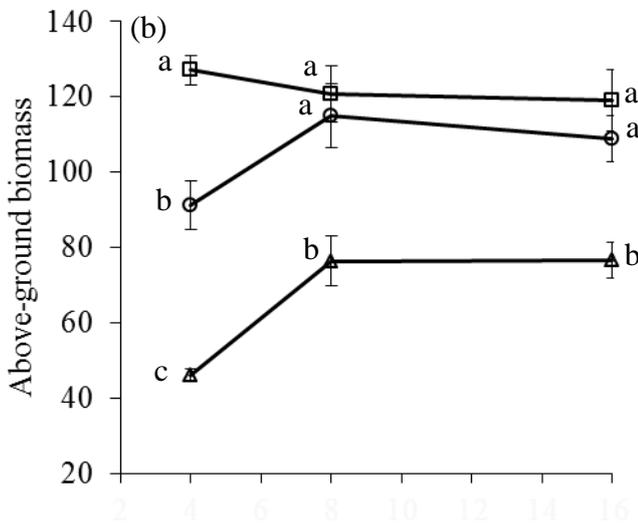
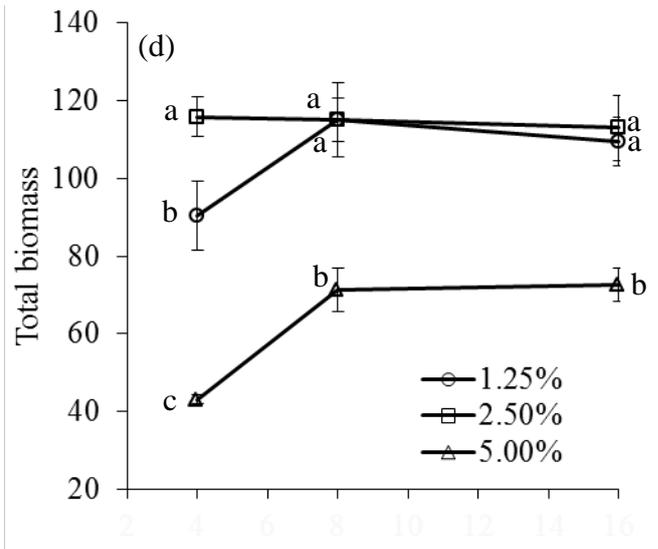
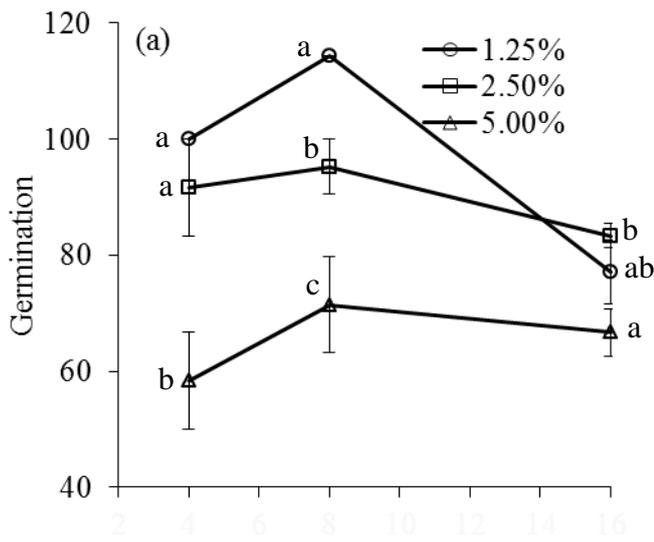


Fig. 5.



Supplementary Material

Appendix S1. The condition of “earth-wise growing essentials potting mix” used in the experiments.

| Potting mix components | Value (unit) |
|------------------------|--------------------------|
| Composted pine bark | 5 (%) |
| Earthwise compost* | 95 (%) |
| Ferrous sulphate | 1 (kg/m ³) |
| Gypsum | 0.6 (kg/m ³) |

*Earth-wise compost is made from sawdust, cane dust and mill-mud. Mill-mud is a residue from the processing of sugar cane.

Typical analysis of potting-mix soil used in the experiments

| Potting mix properties | Value (unit) |
|-------------------------|----------------|
| Air filled porosity. | 18 (%) |
| Water holding capacity | 56 (%) |
| pH | 5.8-6.2 |
| EC | 1.6-2.0 (dS/m) |
| Nitrogen drawdown index | > 0.5 |
| Toxicity | > 0.7 (%) |
| Nitrogen | 10 (mg/L) |
| Phosphate | 2.7 (mg/L) |
| Potassium | 30 (mg/L) |
| Calcium | 150 (mg/L) |
| Magnesium | 55 (mg/L) |
| Iron | 25 (mg/L) |

Source: Australian Prime Fibre Pty. Ltd, 186 Glenmount Road, Tanawah, QLD- 4556, Australia

Chapter Seven

Suppression of native Melaleuca ericifolia by the invasive Phragmites australis through allelopathic root exudates

Introduction

This study was aimed at evaluating the allelopathic impact of *P. australis* root exudates on *M. ericifolia* germination, survival, establishment, and growth. Additionally, the effectiveness of two possible controlling methods (AC addition versus shoot cutting) was assessed for the ability to reduce the impacts or production of allelochemicals by *P. australis*.



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PART B:

**DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS
 INCORPORATED IN THESIS BY PUBLICATION**

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by: Md. Nazim Uddin

Signature: 

Date: 24/07/2014

Paper Title: Suppression of native *Melaleuca ericifolia* by the invasive *Phragmites australis* through allelopathic root exudates

In the case of the above publication, the following authors contributed to the work as follows:

| Name | Contribution % | Nature of contribution |
|---------------------|----------------|--|
| Md. Nazim Uddin | 77 | Concept development; plant, soil and seed collection; conducting experiments and chemical analysis; data collection, statistical analysis and interpretation; and manuscript writing, editing and submitting for publication |
| Randall W. Robinson | 15 | Concept development; sample collection; and manuscript writing and editing |
| Domenico Caridi | 3 | Manuscript editing |
| Md A Y Harun | 5 | Sample collection and manuscript editing |

DECLARATION BY CO-AUTHORS

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
5. The original data is stored at the following location(s):

Location(s): College of Science & Engineering, Victoria University, Melbourne, Victoria, Australia

and will be held for at least five years from the date indicated below:

| | | Date |
|-------------|------------|------------|
| Signature 1 | [Redacted] | 18/07/2014 |
| Signature 2 | [Redacted] | 18/07/2014 |
| Signature 3 | [Redacted] | 21/7/2014 |
| Signature 4 | [Redacted] | 18/07/2014 |

Suppression of native *Melaleuca ericifolia* by the invasive *Phragmites australis* through allelopathic root exudates

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Premise of the study: Invasive plants are a great threat to the conservation of natural ecosystems and biodiversity. Allelopathy as a mechanism for invasion of plant such as *Phragmites australis*, one of the most aggressive invaders, has potential to suppress neighbouring plant species. Allelopathic interference through root exudates of *P. australis* on native *Melaleuca ericifolia* has been investigated to find out the underlying invasion mechanisms.

Methods: Germination and growth effects of *P. australis* on *M. ericifolia* were studied in greenhouse using potting mix with or without activated carbon and a combination of single and repeated cutting of *P. australis* as management tool.

Key Results: *P. australis* had significant negative effects on germination and growth of *M. ericifolia* by inhibiting germination percentage, maximum root length and plant height, biomass, stem diameter, and number of growth points with a little effect on leaf physiology. Activated carbon (AC) in turn counteracted these effects modestly. The cutting of *P. australis* shoots significantly reduced the suppressive effects on *M. ericifolia* compared to the addition of AC to soil. Furthermore, significant changes in soil such as pH, electrical conductivity, osmotic potential, phenolics and dehydrogenase

activity were identified among cutting treatments with little variation between AC treatments.

Conclusion: The results demonstrated that allelopathy through root exudates of *P. australis* had relatively low contribution in suppression of *M. ericifolia* in comparison to other competitive effects. Management combining repeated cutting of *P. australis* shoots with AC treatments may assist partly in restoration of native ecosystems invaded by *P. australis*.

Key words: activated carbon; allelopathy; biological invasion; ecological restoration; *Melaleuca ericifolia*; *Phragmites australis*; soil chemistry.

Biological invasions are a great threat to local and global biodiversity (Zayed et al., 2007). These invasions may be effective in transforming native ecosystems by changing fundamental ecosystem processes, structure and function (Mack et al., 2000) as well as plant community composition and diversity, nutrient dynamics and associated food webs (Siemens and Blossey, 2007). Loss of biodiversity, native species extinctions and increasing global economic expenditure on environmental remediation are major concerns to conservation biologists, plant community ecologists, and land managers (Pimentel et al., 2000; Silliman and Bertness, 2004; Pisula and Meiners, 2010). Many studies have been conducted to identify the mechanisms of plant invasiveness such as life history characteristics, physiological properties, changes in genetics, resource competition and in more recent years allelopathy (Pisula and Meiners, 2010). In many highly visible and aggressive species such as *Phragmites australis* it is assumed by many land managers and ecologists that physical competition for space and resources is the major driving factor of invasion even when allelopathy is now increasing evidence as an alternative mechanism playing a role in the success of those plant invaders (Catford et al., 2009). Allelopathy may be defined as the inhibition of any aspect of

growth and development of one species by another species through the release of secondary metabolites (allelochemicals) to the environment (Inderjit et al., 2005). Allelopathy as an invasion strategy have the effects on seed germination, seedling growth, development and establishment of neighbouring plant species, as well as the same species, in both natural and agricultural systems (Dorning and Cipollini, 2006; Lara-Núñez et al., 2006). Due to the less visually obvious effects of allelopathy on plant species recruitment, land managers place more emphasis on the more directly observable competition for resources. The mechanisms involved in invasiveness are difficult to determine and are often species-specific and context dependent. Allelopathic potential is, however, an important attribute to the success of many invasive species in ecosystems like grassland, forest and wetlands (Callaway and Ridenour, 2004). Allelochemicals are released from plants into the rhizosphere through leaching from leaves and other aerial parts, volatilization, root exudation and residue decomposition (Weir et al., 2004; Uddin et al., 2012). Most allelopathy studies are limited to seed germination and growth interference bioassays using aqueous extracts of invasive plant in the laboratory with very few of these studies carried out in greenhouses and the field (Rice, 1984; Inderjit et al., 2001). In addition, various authors speculate that seed germination bioassay may not be the primary site for allelopathic interactions (Stowe, 1979; Lorenzo et al., 2010).

Phragmites australis, a ubiquitous wetland plant, has been considered one of the most invasive species in the world (Uddin et al., 2012; Weidenhamer et al., 2013), however, the origin of the species is still unclear (Plut et al., 2011). It is a perennial graminaceous plant, 3 - 4 m tall, which reproduces mainly through rhizomes, and at low frequency, through seeds. It grows in all temperate zones of the world, and is especially common in North America, Europe and Australia (Hocking et al., 1983; Morris et al.,

2008; Kulmatiski et al., 2011). The distribution and abundance of *P. australis* has expanded over the last 150 yr and in most areas it forms dense monocultures (Saltonstall et al., 2005). Due to the impacts of *P. australis* invasions, habitats have been diminished or altered significantly for other flora and fauna causing loss of biodiversity and ecosystem functions (Warren et al., 2001; Silliman and Bertness, 2004). Most recent studies have shown that the lateral expansion of *P. australis* occurs at a rate of about 1.5 m per yr (Minchinton and Bertness, 2003). In addition to ecological impacts, *P. australis* may cause substantial economic damage. An estimated cost of \$25 billion is spent each year in the USA for management of invasive species (Pimentel et al., 2005). Many multi-million dollar projects have been undertaken in wetland restoration initiatives with particular reference to *P. australis* invasion in wetlands throughout the world, including the USA, Europe, Canada and Australia (Streever, 1997; Van der Putten, 1997; Silliman et al., 2009). It is generally acknowledged that those restoration projects that involve control of invasive plant such as *P. australis* might be limited by specific knowledge of the species with regard to allelopathy (Walker et al., 2007). Restoration of ecosystems after the removal of invasive species, particularly allelopathic species, relies heavily on a sound knowledge of biochemical processes (Siemens and Blossey, 2007).

Melaleuca ericifolia, a native wetland shrub (< 7 m), is commonly found in fresh and brackish water swamps across south-eastern Australia (Robinson et al., 2008) and it is a critically important species as nesting and roosting habitat for colonially breeding water birds. *M. ericifolia* dominated wetlands face a wide range of disturbances that include salinization, contamination with nutrients and other toxic substances, some derived from other wetland plants (Chee et al., 2009). Recent studies postulated that invasions of *P. australis* in *M. ericifolia* dominated wetland might

directly disadvantage *M. ericifolia* by root allelopathy or indirectly by altering interactions with soil microbes (Morris et al., 2008). We used *M. ericifolia* as a test plant and considered the juvenile stage since transplanting juvenile plant is a common practice in wetland and riparian restoration. It is critical that the relationship between *P. australis* and *M. ericifolia* be established if already depleted *M. ericifolia* wetlands are to be conserved and restored.

Several studies have identified bioactive compounds within *P. australis* organs which have antialgal, antifungal or antibacterial effects (Li and Hu, 2005; Hong et al., 2008). Again, some compounds produced by decomposition of belowground organs of *P. australis* may be responsible for die-back of *P. australis* itself (Armstrong and Armstrong, 1999). Photo-degradation of phytotoxins produced by *P. australis* may induce severe phytotoxicity on other plant species like *Arabidopsis thaliana* and *Spartina patens* (Rudrappa et al., 2009). Previous studies of *P. australis* regarding water extracts of organs (Uddin et al., 2012), root exudation (Rudrappa et al., 2007), and residue decomposition (Uddin et al., 2013b) have shown strong phytotoxic effects on germination, growth and physiology of other plant species. The phenolic compound gallic acid has been identified in *P. australis* organs (Uddin et al., 2013a) and root exudates (Rudrappa et al., 2007), and it showed inhibitory effects on germination and growth of various species (Rudrappa et al., 2007). The findings of Rudrappa et al. (2007) and Bains et al. (2009) regarding the persistence of gallic acid in *P. australis* infested soil and of high concentration of gallic acid and gallotannins in *P. australis* rhizome are now being questioned (Weidenhamer et al., 2013) but the concerns have been critically addressed in terms of environmental, edaphic and bio-geographical concept in our other studies (Uddin et al., 2013a). However, while assays with plant extracts are suggestive of allelopathy, more ecologically realistic experiments are

essential to ascertain the true significance of allelochemical interferences. Inderjit et al. (2011) reported root exudates as a potential source of allelochemicals into the environment but to date, no detailed study has been conducted addressing allelopathy of *P. australis* in situations that closely approximated natural settings. Again, effectively reducing or preventing the impacts of allelochemicals depends upon the ability to distinguish the mechanisms involved in ecological dominance but little is known about their relative importance with empirical evidence (Nilsson, 1994; Inderjit and Callaway, 2003).

Thus, this study was aimed at evaluating the allelopathic impact of *P. australis* root exudates on *M. ericifolia* in terms of germination, survival, and growth. Activated carbon (AC), an effective neutralizing compound for allelopathic effects (Callaway and Aschehoug, 2000; Prati and Bossdorf, 2004), was used to differentiate between allelopathy and resource competition (Nilsson, 1994; Hierro and Callaway, 2003), and we combined it with mechanical control treatments (shoot cutting) of *P. australis*. Additionally, the effectiveness of two possible methods (AC addition versus shoot cutting) was assessed for the ability to reduce the impacts or production of allelochemicals by *P. australis*. More specifically we asked the following questions: What are the total effects of *P. australis* on *M. ericifolia* in terms of germination, growth and leaf physiology? Does *P. australis* interfere with *M. ericifolia* via allelopathic root exudates? Are AC and cutting methods effective in alleviating root-mediated allelopathy? Does AC show any undesirable effects by directly affecting the plant growth and soil chemistry?

MATERIALS AND METHODS

Plant materials—Spring buds of *P. australis* (rhizome attached) were collected on 8 September 2011 from Cherry Lake (37° 51' 30"S, 144° 50' 5"E), a coastal wetland

in Altona, Melbourne, Victoria, Australia. Each live rhizome was cut to contain exactly one active node. Tube stock of *M. ericifolia* (6 mo old), grown in potting mix, were purchased from 'Go Native Landscapes Pty Ltd' (Inverloch, Victoria, 3996, Australia) which were grown from seeds collected from *M. ericifolia* stands at Dowd Morass wetland in Inverloch, Victoria, Australia.

Experimental design—Live rhizomes were weighed and planted within 6 h of collection in 7 L plastic pots lined with watertight plastic bags filled with 4 L substrate [1 : 7 mixture of unsterilized river sand and potting mix soil respectively (Earth-wise Growing Essential, Australian Prime Fibre Pty Ltd, Queensland 4660, Australia)]. Potting mix contained organic materials (pine bark), living organisms (bacteria, fungi and protozoa) and minerals and fertilizers additives (details on potting mix in Appendix S1). Half of all pots contained AC (Steps to Life (Aust) Ltd, Victoria 3140, Australia) at a concentration of 40 mL/L substrate to reduce the allelopathic effect following the method of Jarchow and Cook (2009). Potting mix and AC were mixed properly in a separate container by hand, and then put into the experimental pots for transplantation of *P. australis* rhizome cuts but no transplantation for control. The control pots were kept with other treated pots in the greenhouse with same condition until *M. ericifolia* seeds and juvenile plants were used. To all of the pots, 1 g/L of mixed pelletized fertilizer (Pivot fertilizer-900, Pivot. Ltd., Victoria 3006, Australia; N-P-K: 16-8-9) was incorporated into the tilled topsoil bimonthly. Pots were kept in a natural lit greenhouse at $23 \pm 3^\circ\text{C}$ and $12 \pm 2^\circ\text{C}$ day/night temperature and watered regularly with an auto irrigation system equipped by micro sprinklers at the soil surface to keep soil moist at a level of $55 \pm 5\%$ compatible with field soil. Soil moisture was monitored weekly by measuring the water content in soil randomly collected from the pots. Pots were

randomly shuffled every wk to minimize the spatial effects, and unwanted germinant (weeds) were removed regularly.

Experiment 1—This experiment evaluated the effects of *P. australis* root exudates on germination and growth of *M. ericifolia*. Ten replicates with (treatment) and without (control) *P. australis* were maintained, half of which contained AC. Field collected *M. ericifolia* seeds (450 individuals) were distributed evenly in slightly tilled soil around the base of plant stem in each pot during the vegetative stage of *P. australis*. Germinated plants were counted after 3 wk and all plants were harvested for biometric (maximum root length, maximum plant height and biomass) measurements after 5 mo. In addition, rhizosphere soil from 2 cm below the surface was collected to check the differences between with and without AC amendment in soil chemistry such as pH, electrical conductivity (EC), osmotic potential and total phenolic content. The size of the containers used and with overhead watering there was little likelihood of variation (gradients) in the pot soil column.

Experiment 2—On 10 November 2011, the *M. ericifolia* juvenile plants were planted into the pots (one per pot) to test the differential effects of mechanical control. The pots were subjected to four management strategies: (a) no *P. australis* plants, (b) *P. australis* but no mechanical control (no shoot cutting), (c) *P. australis* with single shoot cutting (once after 6 wk in 6 mo growing period), and (d) *P. australis* with repeated shoot cutting (twice in 6 mo growing period at 6 wk intervals at the beginning and then left to grow for the remainder of the time). Shoots of *P. australis* were cut at 30 cm above from the soil surface with sharp knife. Sixteen replicates of each treatment were used, half of which contained AC. Before planting of *M. ericifolia*, the biometric parameters like above-ground biomass, below-ground biomass, maximum root length and maximum plant height were measured.

Measurement of variables in plants—After 6 mo of growth all plants were harvested (*P. australis* and *M. ericifolia*) and data were collected for above-ground biomass, below-ground biomass, maximum root length, maximum plant height, stem diameter and number of growth points. Two wk before harvesting, a small quantity of leaves of *M. ericifolia* were collected for measurement of leaf chlorophyll [chlorophyll *a*, chlorophyll *b*, and total chlorophyll] using the method of Inskeep and Bloom (1985) as well as leaf moisture, leaf dry matter content, and leaf relative water content according to the method proposed by Saura-Mas and Lloret (2007). These parameters were measured for assessment of leaf physiology of *M. ericifolia* growing with *P. australis*.

Measurement of variables in soil—Rhizosphere soil was collected from every pot as mentioned above to check the soil chemistry. The results might be helpful in ratifying the undesired effects due to AC addition as some previous studies have shown that AC may alter the soil chemistry such as pH, EC and nutrient availability that may influence plant growth (Berglund et al., 2004; Weißhuhn and Prati, 2009). Total phenolic content of soil was measured according to Singleton and Rossi (1965) with little modification and gallic acid was used as the standard. Briefly, 100 mg air dried sieved soil (0.5 mm) was transferred to Eppendorf tube with addition of 5 mL 70% acetone and incubated at 4°C for 1 h to extract phenolics. Extracts was centrifuged at 250 Hz for 10 min at 4°C followed by transferring 0.5 mL of the supernatant into a test tube to make 1 mL with distilled water. Then 5 mL of 2% Na₂CO₃ in 0.1 N NaOH was added and mixed using vortex mixer (Vortex Mixer, VOU1, Ratek Instruments Pty. Ltd., Melbourne, Victoria, Australia). After that 0.5 mL Folin-Ciocalteu reagent was added and mixed. After 2 h, absorbance was read at 760 nm with a UV/ Visible spectrophotometer (Biochrom Libra S12, Cambridge, UK). Soil pH was measured with

pH meter (Pocket digital pH meter, 99559, Dick-smith electronics, Australia) and EC with a conductivity meter (TPS Digital conductivity meter, 2100, TPS Pty Ltd., Brisbane, Queensland, Australia). Osmotic potential was calculated using the equation (Osmotic potential = EC * - 0.36) according to McIntyre (1980). Soil dehydrogenase activity was determined using the method of Gu et al. (2009) with little modification. Briefly, 2 g fresh sieved soil (2 mm) and 4 mL of 2 g/L 2, 3, 5-triphenyltetrazolium chloride were mixed thoroughly using mixer and incubated at 37°C in dark for 24 h. After that 0.5 mL of 1 M sulfuric acid was added to stop the reaction and centrifuged at 4°C and 67 Hz for 10 min with addition of 4 mL of ethyl acetate. The colour intensity of the supernatant was measured at 485 nm in spectrophotometer calibrated with ethyl acetate as a blank.

Experiment 3—A separate experiment was conducted to test the direct effect of AC on rhizomes of *P. australis* (one rhizome/pot), and *M. ericifolia* juvenile (one juvenile plant/pot) with the same substrate and AC concentration as mentioned above. There were five replicates per treatment (with and without AC) for a total of 20 pots. Though AC has been considered as adsorber of organic compounds with allelopathic properties and thus, reduce the negative effects on plants but it has been recently claimed that inference of allelopathy might be complicated by effects of AC on plant growth (Lau et al., 2008). Therefore, this experiment was conducted to check the direct effects of AC on our experimental plants' growth. After 6 mo of growing, plants were harvested and measured the above mentioned biometric parameters.

Statistical analyses—Nested analysis of variance (ANOVA) designs were used to test the effects of treatments (*P. australis*, AC and cutting of *P. australis* shoots) on biometric parameters such as total and the partitioning of biomass (above-ground biomass and below-ground biomass), maximum root length, maximum plant height,

stem diameter, number of growth points, in *M. ericifolia*. In addition, leaf physiology like leaf moisture, leaf dry matter content, and leaf relative water content, chlorophyll *a*, chlorophyll *b*, and total chlorophyll of *M. ericifolia* were analysed using nested ANOVA design. Each model included the main effects of *P. australis*, AC, and their interactions, as well as the effects of cutting (nested within *P. australis*), cutting frequency (nested within cutting), and their interactions with AC. Two sample t-test was used to evaluate the differences in soil chemistry (pH, EC, osmotic potential, total phenolic content, and dehydrogenase activity) between treatments of with and without AC. Normality and homogeneity of variance were examined using Levene's test of equality. Data were square root transformed if necessary. Regression procedures were performed to determine the correlations in evaluating the dependence of *M. ericifolia* growth pattern with total phenolic content of experimental soil. A significant value of $p < 0.05$ was used for all analyses. All statistical procedures were performed using IBM SPSS statistics 21.0 (IBM Corporation, NY, USA).

RESULTS

Experiment 1—*P. australis* had strong negative effects on germination and growth of *M. ericifolia* (Fig. 1). When grown with *P. australis*, the germination percentage, maximum root length, maximum plant height, number of leaves and total biomass were inhibited by 56%, 74%, 84%, 58% and 73% respectively (Fig. 1). AC treatment significantly increased germination percentage (Fig. 1) but no significant effect found on other measured parameters (maximum root length, maximum plant height, number of leaves and total biomass) (Fig. 1). There was no interactive effect of *P. australis* and AC on other parameters except germination percentage ($F_{1,8} = 10.37$, $P < 0.01$). The overall effect of AC on the germination and growth of *M. ericifolia* in competition with *P. australis* was positive (Fig. 1).

Experiment 2—The initial rhizome biomass of *P. australis* ($t = 0.409$, $df = 8$, $P = 0.69$) as well as the initial maximum plant height ($t = 1.90$, $df = 8$, $P = 0.09$), maximum root length ($t = 1.2$, $df = 8$, $P = 0.26$), above-ground biomass ($t = 1.81$, $df = 8$, $P = 0.1$), and below-ground biomass ($t = 1.27$, $df = 8$, $P = 0.23$) of *M. ericifolia* did not vary significantly between with and without AC treatments. The data sets for initial rhizome biomass of *P. australis* and *M. ericifolia* have been provided on Appendix S2. All *P. australis* rhizomes survived to the end of the experiment and had positive growth whereas 10% and 15% juvenile of *M. ericifolia* with AC and without AC treatments died respectively but they were replaced within 1 wk of adjustment period.

Above-ground biomass, below-ground biomass, maximum root length, maximum plant height, stem diameter, and number of growth points of *M. ericifolia* were suppressed when grown with *P. australis* either with or without AC (Table 1, Figs. 2 & 3). Total biomass (94%), above-ground biomass (94%), below-ground biomass (93%), maximum root length (64%), maximum plant height (43%), stem diameter (43%), and number of growth points (88%) of *M. ericifolia* were reduced when growing with *P. australis* without AC (Figs. 2 & 3). AC addition had contrasting effects on the interactions between *P. australis* and *M. ericifolia* but in most of the cases it reduced the negative effects of *P. australis* on *M. ericifolia* growth parameters (Figs. 2 & 3). The interactions of AC and *P. australis* were not significant on other measured growth parameters except stem diameter (Table 1). AC increased the growth parameters of *M. ericifolia* such as above-ground biomass (33%), below-ground biomass (27%), total biomass (33%), maximum root length (23%), stem diameter (20%), and number of growth points (50%) except maximum plant height. Mechanical control significantly reduced the above-ground biomass, below-ground biomass, number of shoots, and maximum plant height of *P. australis*, and consequently, increased all measured

parameters of *M. ericifolia* (Tables 1, 2, & 3; Figs. 1, 2, & 3). Single cutting of *P. australis* suppressed above-ground biomass (27%), below-ground biomass (18%), number of shoots (30%), and maximum plant height (7%) of *P. australis* and increased above-ground biomass (27%), below-ground biomass (7%), maximum root length (22%), stem diameter (9%), and number of growth points (24%) of *M. ericifolia*. Whereas, double cutting reduced above-ground biomass (43%), below-ground biomass (39%), number of shoots (39%), and maximum plant height (10%) of *P. australis* and increased above-ground biomass (86%), below-ground biomass (57%), maximum root length (42%), maximum plant height (36%), stem diameter (50%), and number of growth points (182%) of *M. ericifolia*. In case of *P. australis* growth parameters (above-ground biomass, below-ground biomass and number of shoots), no significant differences were found between the same cutting treatment with and without AC (Fig. 4) whereas most of the measured parameters of *M. ericifolia* were varied significantly (Figs. 2 & 3). Again, among leaf physiology variables of *M. ericifolia*, leaf dry matter content, and leaf relative water content were significantly affected when grown together with *P. australis* without AC (Table 2). AC addition and cutting treatments had no significant changes on the measured variables (Table 2).

Soil chemistry—Values of pH ($t = 1.631$, $df = 22$, $P = 0.11$), EC ($t = 0.168$, $df = 22$, $P = 0.86$), and osmotic potential ($t = 0.168$, $df = 22$, $P = 0.86$) did not differ between with and without AC treatments except total phenolic content ($t = 6.19$, $df = 22$, $P < 0.001$), and dehydrogenase activity ($t = 2.48$, $df = 22$, $P = 0.02$) (Fig. 5). Total phenolic content was significantly higher in all treatments without AC compared to with AC ($F_{1, 14} = 92.95$, $P < 0.001$). Cutting treatments significantly decreased the total phenolic content in soil ($F_{1, 14} = 12.90$, $P = 0.003$) but there was no interactive effects (AC \times mechanical control) ($F_{1, 14} = 0.01$, $P = 0.924$). There were no significant correlations

between total phenolic content of soil and the growth parameters of *M. ericifolia* except chlorophyll *a* ($F_{1,6} = 7.41$, $P = 0.034$; $r^2 = 0.55$) and total chlorophyll content ($F_{1,6} = 6.21$, $P = 0.046$; $r^2 = 0.51$). Dehydrogenase activity was significantly changed in AC ($F_{1,6} = 61.35$, $P < 0.001$) and cutting treatments ($F_{1,14} = 22.63$, $P < 0.001$) but no significant interaction was observed between AC and cutting treatments ($F_{1,14} = 0.73$, $P = 0.405$). Strong positive correlation between total phenolic content and dehydrogenase activity ($r = 0.54$, $n = 18$, $P < 0.05$) was found.

Experiment 3—The direct effects of AC on experimental plants showed that there was no significant effect on the measured parameters of *P. australis* (above-ground biomass, below-ground biomass, number of shoots and maximum plant height) and *M. ericifolia* (above-ground biomass, below-ground biomass, total biomass, maximum root length, maximum plant height except stem diameter and number of growth points) (all $p > 0.5$). The actual data have been provided in Appendix S3.

DISCUSSION

Plant-plant interactions have been explained not only with resource competition, but also allelopathic potential has been considered, in particular for invasive plant species (Rice, 1984; Nilsson, 1994). Though, considerable doubt continues to separate resource competition from allelopathy (Inderjit and del Moral, 1997) but it is important to determine the relative contribution of those mechanisms in invasion process in ecosystems (Nilsson, 1994). Whilst it is established that *P. australis*, through direct competition for light, space and nutrients, can greatly inhibit germination and suppress the growth of *M. ericifolia* seedlings (Robinson et al., 2006; Morris et al., 2008), this study shows that allelopathy may also contribute in suppression of *M. ericifolia* though the influence was relatively lower. Our findings of substantial negative impact by *P. australis* on most measured germination and growth parameters of *M. ericifolia* (Fig. 1)

are consistent with other studies of Rudrappa et al (2007) who found similar impacts of *P. australis* on *Arabidosis thaliana* with a positive effect in AC treatments.

M. ericifolia juvenile plants were sensitive to root exudates of *P. australis* in terms of mortality observed on pots with *P. australis* during early stage of its plantation. The contrasting effects of AC on competition of *M. ericifolia* against *P. australis* indicate that *M. ericifolia* might be suppressed through root mediated allelochemicals produced by *P. australis* along with other interactive mechanisms. Morris et al. (2008) who carried out competitive trials in soil, suggested that the growth of *M. ericifolia* might be influenced through *P. australis* root exudates and alteration of soil microbes although they did not examine either. In addition, Rudrappa et al., (2007) reported that the root secreted allelochemical, gallic acid, may be responsible for the invasion of *P. australis* but recently, Weidenhamer et al. (2013) contradicted the findings and demonstrated that gallic acid is not the primary explanation for invasion of *P. australis*.

In our study the statistical effects of AC addition on the germination and growth of *M. ericifolia* (experiment 1 and 2) were significant for many variables, but the magnitude of the effect did not seem to support a dominant role for allelopathy compared to other mechanisms. Our statement is based on two facts: first, the reductions in germination, root length, shoot length, and biomass of *M. ericifolia* (Fig. 1 A, B & C) were more noticeable in presence of *P. australis*, in contrast, the modest increases in those variables due to AC addition suggest a dominant role of resource competition by *P. australis*. Second, Figure 2 and 3 (Experiment 2) showed a similar negative impact of growth of *P. australis* on *M. ericifolia*. For example, there was a huge biomass reduction (> 80%) in *M. ericifolia* growth in the presence of *P. australis* both above (Fig. 2A) and below (Fig. 2B) ground, and these effects were modestly improved by the use of AC, particularly when *P. australis* was cut twice, but even there

the biomass was still reduced by more than 75% compared to the control. On the other hand, biomass of *M. ericifolia* doubled due to addition of AC in soil substrates and twice cut of *P. australis* (Fig. 2B) which supports an involvement of allelopathy in the growth effects of *M. ericifolia*. However, on the basis of these whole comparisons between direct competitive and allelopathic effects, it still seems that the direct competitive effects were much greater than the allelopathic effects.

The addition of AC in such type of study could help to differentiate allelopathy from resource competition by focusing solely on presence or near-absence of allelochemicals. Despite some evidence of undesired effects in using AC for testing allelopathy (Weißhuhn and Prati, 2009) it has been successfully applied to examine the allelopathic interactions in a number of studies (Hierro and Callaway, 2003; Gómez-Aparicio and Canham, 2008), and has been recommended as an effective approach for allelopathy studies (Inderjit and Callaway, 2003). We used single dose of AC like other previous studies for testing allelopathy interactions (Callaway and Aschehoug, 2000; Siemens and Blossey, 2007; Murrell et al., 2011) but a wide range of dosages might provide more precise indication of allelopathic effects. Furthermore, the quantity of AC we used in our experiments might be insufficient to neutralize the allelopathic effects of *P. australis* as we found in some cases insignificant positive impacts of AC addition on the growth of *M. ericifolia*.

P. australis had significant negative effects on leaf dry matter content and leaf relative water content of *M. ericifolia* with a variation between treatments, a finding supported by other studies that found a negative effects of natural phytochemicals on leaf physiology (Wilson et al., 1999; Kittelson et al., 2008; Hussain and Reigosa, 2012). Our findings also support the results of Callaway et al. (2005) who reported similar effects of invasive plant on neighbouring plant species. Leaf physiology may respond to

resource availability as well as surrounding plant diversity and presence of invader (Kittelson et al., 2008). Our results revealed the patterns of resource uptake and they also might be linked with phenolic compounds produced by *P. australis*. The results might be useful for quantification of the overall impacts including allelopathic effects by an invader through measuring the variation of plant function and resource availability.

Soil dehydrogenase activity was amplified with an increase in soil total phenolic content and potentially led to fostering the soil microbial activity (Wu et al., 2009; Zhou and Wu, 2012). Conversely, AC addition reduced total phenolic content in soil and consequently, dehydrogenase activity was decreased that are in agreement with other studies (Zackrisson et al., 1996; Berglund et al., 2004; Weißhuhn and Prati, 2009). In our studies, soil total phenolic content reduction coupled with increasing cutting frequency suggests that above-ground biomass may play an important role for changing the plant chemistry and consequently, soil biological activity. In addition, we found no direct effects of AC on the growth of *P. australis* and *M. ericifolia* but soil total phenolic content and dehydrogenase activity varied significantly between treatments. This variation between with and without AC treatments were attributable to adsorption of phenolics by AC. Finally, higher phenolic content was observed in soil with presence of *P. australis*, and that AC effectively reduced soil phenolic levels to concentrations at or below levels without presence of *P. australis* and AC in the soil respectively (Fig. 5B). However, even with levels of phenolics "controlled" in this manner by the addition of AC, the impact of *P. australis* on *M. ericifolia* growth was substantial suggesting that allelopathy did not play a dominant role for the suppressing effects by *P. australis*. Moreover, it has been suggested that soil microorganisms might play an important role in influencing the bioavailability of allelochemicals in soil (Inderjit, 2005; Ehlers, 2011)

that could be achieved by addition of microbial inoculum in experimental soil substrate collected from test plant grown field which could be recommended for further study. In addition, Inderjit and Callaway (2003) recommend fertilizing pots to minimize the trace concentration of nutrients contributed by AC, and thereby, we used nutrient in addition with non-sterile mixture of sand and potting mix in our experiments to minimize the other direct competing factors following the studies of Ridenour and Callaway (2001).

In general, established *P. australis* stands are very dense with persistent canopies that reduce the access of light and therefore inhibit the germination (Robinson et al., 2006) and growth of associated plant species, *M. ericifolia* (Thompson and Shay, 1985). In our experiment, the direct cutting effect of above-ground biomass in *P. australis* increased the growth and biomass of *M. ericifolia* suggesting increased access of light coupled with reduced ability to produce allelochemicals may change the overall plant-plant biochemical interactions. Furthermore, reduced below-ground biomass of *P. australis* with cutting frequency indicates that both above and belowground competition by *P. australis* limits the growth of associated plant species, is supported by Murrell et al. (2011) who found that *Fallopia bohemica* was suppressed by shoot cutting of the species that significantly reduced allelopathic effects on its neighbouring species. Although, some studies showed higher amounts of allelochemical production by similar treatments on invasive species (Bernstein and McLean, 1980; Warren et al., 2001), but we could not find any increase in allelochemical production in relation to cutting in the case of *P. australis*, demonstrate that cutting may be suggestive to manage *P. australis* from a ecological point of view.

In summary, our results suggest that *P. australis* had less but distinct allelopathic effects on germination, and growth of *M. ericifolia* compared with other direct competitive effects such as resource competition. These effects might confer a

portion of the competitive success of *P. australis*. Mechanical control of *P. australis*, an ecologically safe technique, reduced a little bit more phenolics content in soil compared with AC addition demonstrating that biomass removal would be more effective for reducing allelopathic potential than addition of AC. Based on overall results, we can conclude that above and below ground competitions are not the sole mechanism responsible for invasion of *P. australis* or suppression of *M. ericifolia* but a relatively low involvement of allelopathy has been acknowledged to its invasion success in comparison to other competitive effects. In addition, this study demonstrated that the integrated approach (regular cutting and potential soil amendments with AC) might be useful as an ecological restoration technique for management of wetlands invaded by *P. australis*. More sophisticated and multifactorial approaches such as chemical identification of root exudates and their effects, microbial activities associated with allelopathic effects in soil and separation of allelopathic effects from other competitions in natural settings are essential for understanding and predicting invasion of *P. australis* in wetlands.

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TABLE 1. The effects of *Phragmites australis*, activated carbon, and mechanical control of *P. australis* (cutting of shoots) on above-ground biomass (AGB), belowground biomass (BGB), maximum root length (MRL), maximum plant height (MPH), stem diameter (SD) and number of growth points (NGP) in *Melaleuca ericifolia*.

| Source ^a | AGB | | BGB | | MRL | | MPH | | SD | | NGP | |
|-------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | <i>F</i> | <i>P</i> |
| <i>P. australis</i> | 813.11 | <0.001 | 657.98 | <0.001 | 131.76 | <0.001 | 40.75 | <0.001 | 219.67 | <0.001 | 223.14 | <0.001 |
| Activated carbon | 0.635 | 0.038 | 0.69 | <0.001 | 1.072 | 0.305 | 0.51 | 0.478 | 16.55 | <0.001 | 16.27 | <0.001 |
| P × AC | 0.03 | 0.666 | 0.04 | 0.370 | 1.978 | 0.165 | 0.06 | 0.808 | 4.65 | 0.035 | .846 | 0.362 |
| Cutting (P) | 16.06 | <0.001 | 11.67 | <0.001 | 13.30 | <0.001 | 7.95 | 0.007 | 10.36 | 0.002 | 27.56 | <0.001 |
| C frequency (C, P) | 19.94 | <0.001 | 14.13 | <0.001 | 5.36 | 0.024 | 19.26 | <0.001 | 13.50 | <0.001 | 22.63 | <0.001 |
| C (P) × AC | 0.63 | 0.536 | 1.60 | 0.211 | 1.23 | 0.292 | 0.97 | 0.386 | 2.91 | 0.063 | 0.51 | 0.601 |
| C frequency (C, P) × AC | 1.88 | 0.144 | 2.22 | 0.096 | 0.90 | 0.441 | 1.68 | 0.186 | 3.03 | 0.038 | 1.95 | 0.132 |

^a AC = activated carbon, C = cutting, P = *P. australis*

TABLE 2. The effects of *Phragmites australis*, activated carbon, and mechanical control of *P. australis* (cutting of shoots) on leaf moisture (LM), leaf dry matter content (LDMC), leaf relative water content (LRWC), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (TChl) in *Melaleuca ericifolia*.

| Source ^a | LM | | LDMC | | LRWC | | Chl <i>a</i> | | Chl <i>b</i> | | TChl | |
|-------------------------|----------|----------|----------|----------|----------|----------|--------------|----------|--------------|----------|----------|----------|
| | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> |
| <i>P. australis</i> | 2.49 | 0.120 | 4.12 | 0.047 | 4.23 | 0.044 | 1.41 | 0.243 | 1.97 | 0.169 | 1.57 | 0.217 |
| Activated carbon | 2.64 | 0.110 | 3.51 | 0.066 | 0.43 | 0.515 | 0.04 | 0.843 | 0.004 | 0.952 | 0.03 | 0.871 |
| P × AC | 0.155 | 0.696 | 0.33 | 0.569 | 0.06 | 0.803 | 1.56 | 0.220 | 2.14 | 0.151 | 1.72 | 0.197 |
| Cutting (P) | 2.13 | 0.150 | 1.47 | 0.232 | 2.61 | 0.112 | 0.06 | 0.814 | 0.073 | 0.788 | 0.01 | 0.922 |
| C frequency (C, P) | 3.48 | 0.068 | 2.55 | 0.113 | 2.11 | 0.152 | 0.05 | 0.817 | 0.011 | 0.918 | 0.02 | 0.891 |
| C (P) × AC | 0.08 | 0.923 | 0.28 | 0.760 | 0.58 | 0.565 | 0.79 | 0.461 | 1.12 | 0.338 | 0.88 | 0.422 |
| C frequency (C, P) × AC | 0.32 | 0.809 | 0.37 | 0.776 | 0.51 | 0.674 | 0.50 | 0.684 | 0.71 | 0.551 | 0.56 | 0.648 |

^a AC = activated carbon, C = cutting, P = *P. australis*

TABLE 3. The effects of adding activated carbon, and of mechanical control of *Phragmites australis* (cutting of shoots) on above-ground biomass (AGB), belowground biomass (BGB), total biomass, number of shoots and maximum plant height (MPH) of *P. australis* growing in experimental communities with *Melaleuca ericifolia*.

| Source ^a | AGB | | BGB | | Total biomass | | Number of shoots | | MPH | |
|----------------------|----------|----------|----------|----------|---------------|----------|------------------|----------|----------|----------|
| | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> | <i>F</i> | <i>P</i> |
| Activated carbon | 3.16 | 0.081 | 0.26 | 0.609 | 1.28 | 0.262 | 1.568 | 0.216 | 0.12 | 0.733 |
| Cutting | 50.32 | <0.001 | 30.22 | <0.001 | 45.47 | <0.001 | 45.31 | <0.001 | 1.52 | 0.227 |
| AC * C | 0.04 | 0.961 | 0.10 | 0.901 | 0.03 | 0.970 | 0.07 | 0.930 | 0.16 | 0.850 |
| C frequency (C) | 25.40 | <0.001 | 35.31 | <0.001 | 42.71 | <0.001 | 3.23 | 0.078 | 0.96 | 0.332 |
| C frequency (C) * AC | 0.58 | 0.630 | 0.464 | 0.709 | 0.63 | 0.601 | 0.05 | 0.983 | 0.75 | 0.529 |

^a AC = activated carbon, C = cutting

Appendices

Appendix S1. The condition of “earth-wise growing essentials potting mix” used in the experiments.

| Potting mix components | Value (unit) |
|------------------------|--------------------------|
| Composted pine bark | 5 (%) |
| Earthwise compost* | 95 (%) |
| Ferrous sulphate | 1 (kg/m ³) |
| Gypsum | 0.6 (kg/m ³) |

*Earth-wise compost is made from sawdust, cane dust and mill-mud. Mill-mud is a residue from the processing of sugar cane.

Typical analysis of potting-mix soil used in the experiments

| Potting mix properties | Value (unit) |
|-------------------------|----------------|
| Air filled porosity. | 18 (%) |
| Water holding capacity | 56 (%) |
| pH | 5.8-6.2 |
| EC | 1.6-2.0 (dS/m) |
| Nitrogen drawdown index | > 0.5 |
| Toxicity | > 0.7 (%) |
| Nitrogen | 10 (mg/L) |
| Phosphate | 2.7 (mg/L) |
| Potassium | 30 (mg/L) |
| Calcium | 150 (mg/L) |
| Magnesium | 55 (mg/L) |
| Iron | 25 (mg/L) |

Source: Australian Prime Fibre Pty. Ltd, 186 Glenmount Road, Tanawah, QLD- 4556, Australia

Appendix S2. The initial condition of *Phragmites australis* and *Melaleuca ericifolia* used in the experiments between treatments of with and without activated carbon (AC) ($n = 5$). Before plantation, rhizome of *P. australis* and juvenile of *M. ericifolia* were divided into two groups (with and without AC). Then 5 random samples were taken for measurement of initial condition of these two experimental plants.

| Plant materials | With AC | Without AC |
|-----------------------------|-----------------|-----------------|
| <u><i>P. australis</i></u> | | |
| Rhizome biomass (g) | 2.67 ± 0.20 | 2.79 ± 0.21 |
| <u><i>M. ericifolia</i></u> | | |
| Aboveground biomass (g) | 0.37 ± 0.03 | 0.35 ± 0.01 |
| Belowground biomass (g) | 0.37 ± 0.02 | 0.36 ± 0.04 |
| Maximum root length (cm) | 13.4 ± 0.81 | 12.2 ± 0.58 |
| Maximum plant height (cm) | 19.2 ± 0.37 | 18.6 ± 0.40 |

Appendix S3. Direct effects of AC on *Phragmites australis* and *Melaleuca ericifolia* used in the experiments between treatments of with and without activated carbon (AC) ($n = 5$).

| Plant materials | With AC | Without AC |
|-----------------------------|----------------|---------------|
| <u><i>P. australis</i></u> | | |
| Above ground biomass (g) | 65.55 ± 3.26 | 69.34 ± 4.33 |
| Belowground biomass (g) | 88.75 ± 5.05 | 93.41 ± 2.93 |
| Number of shoots | 41.20 ± 1.98 | 42.40 ± 1.43 |
| Maximum plant height (cm) | 123.40 ± 7.11 | 118.80 ± 4.09 |
| <u><i>M. ericifolia</i></u> | | |
| Aboveground biomass (g) | 30.53 ± 2.21 | 28.12 ± 1.88 |
| Belowground biomass (g) | 7.66 ± 0.36 | 6.90 ± 0.82 |
| Total biomass (g) | 38.20 ± 2.07 | 35.03 ± 2.03 |
| Maximum root length (cm) | 48.4 ± 2.67 | 49.60 ± 4.11 |
| Maximum plant height (cm) | 80.6 ± 3.24 | 78.00 ± 3.25 |
| Stem diameter (mm) | 10.00 ± 0.54 | 7.46 ± 0.52 |
| Number of growth points | 494.40 ± 32.97 | 342.6 ± 30.71 |

Figure Legends

Fig. 1. (A) Germination, (B) root and shoot length, and (C) biomass or number of leaves in *Melaleuca ericifolia* grown alone, or together with *Phragmites australis* either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 3$).

Fig. 2. (A) Above-ground biomass, (B) below-ground biomass, and (C) maximum root length of *Melaleuca ericifolia* grown alone or together with *Phragmites australis* subjected to mechanical control (single or repeated cutting of shoots) either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 8$).

Fig. 3. (A) Maximum plant height, (B) stem diameter, and (C) number of growth points per plant of *Melaleuca ericifolia* grown alone, or together with *Phragmites australis* subjected to mechanical control (single or repeated cutting of shoots) either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 8$).

Fig. 4. (A) Above-ground biomass, (B) below-ground biomass, and (C) number of shoots per pot of *Phragmites australis* grown alone, or together with *Melaleuca ericifolia* whether *P. australis* were subjected to mechanical control (single or repeated cutting of shoots) either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 8$).

Fig. 5. Soil (A) pH, (B) total phenolic content, and (C) dehydrogenase enzyme activity of *Melaleuca ericifolia* grown alone or together with *Phragmites australis* subjected to mechanical control (single or repeated cutting of shoots) either with or without activated carbon (AC) in the soil. Values are mean \pm standard error ($n = 5$).

Fig. 1

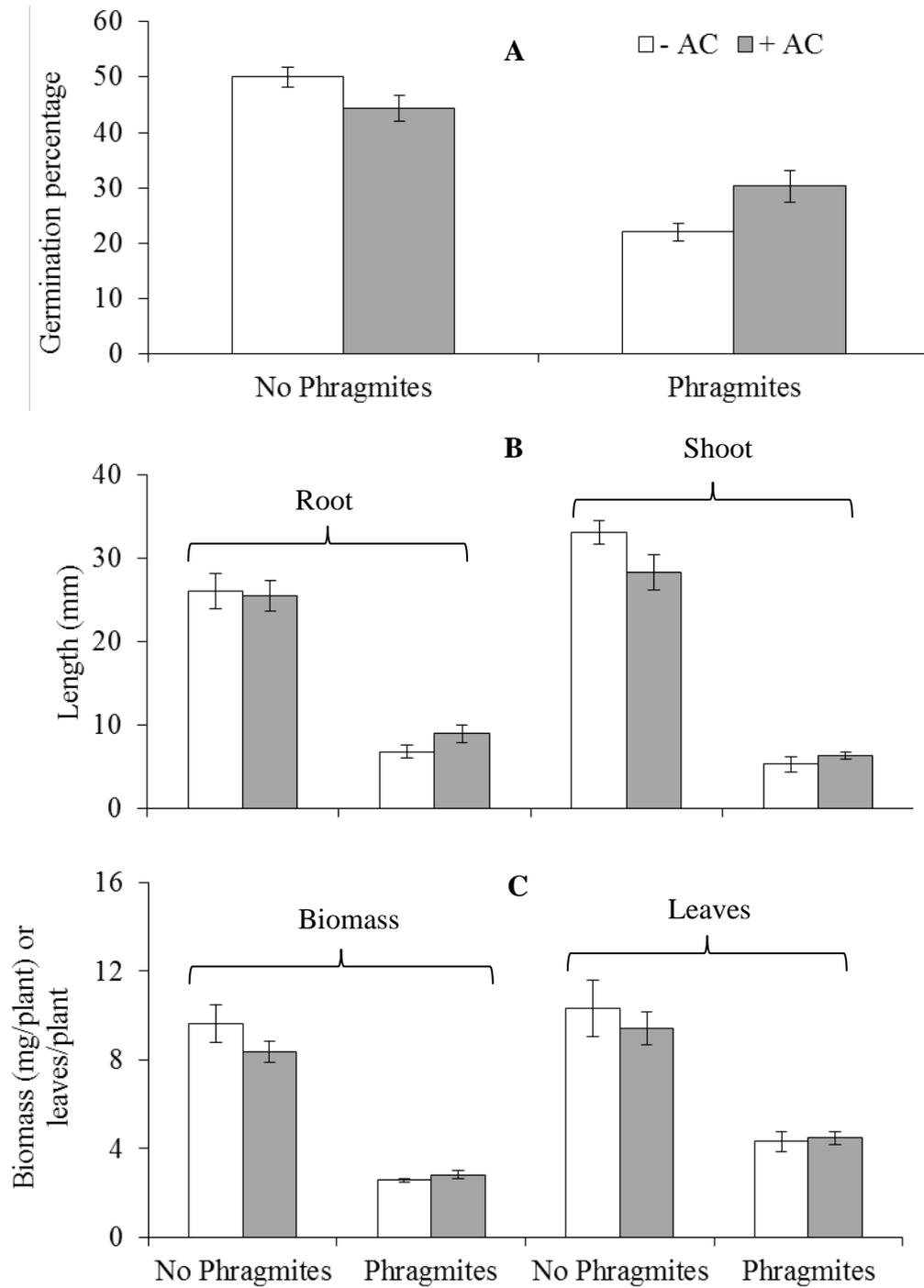


Fig. 2.

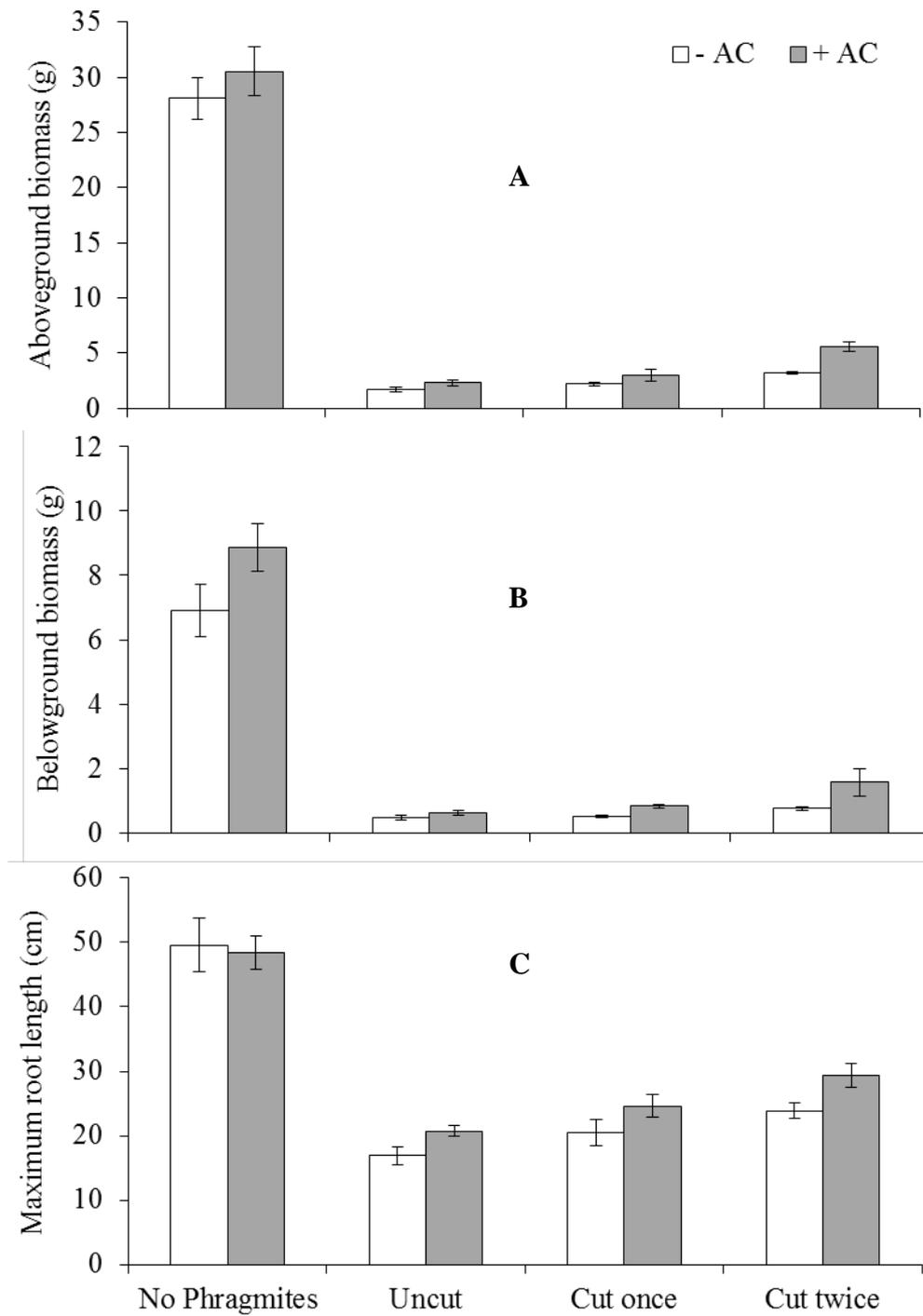


Fig. 3.

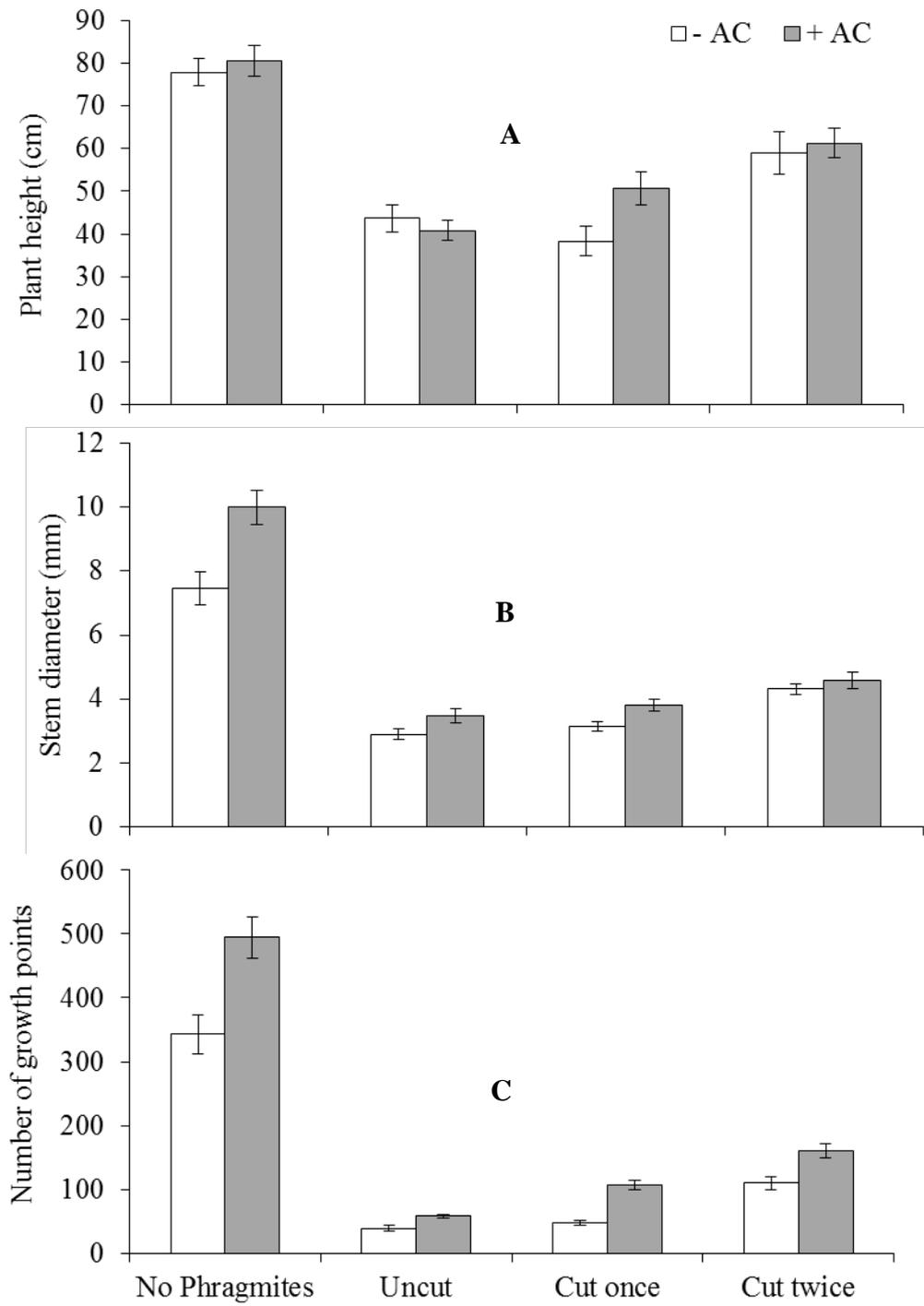


Fig. 4.

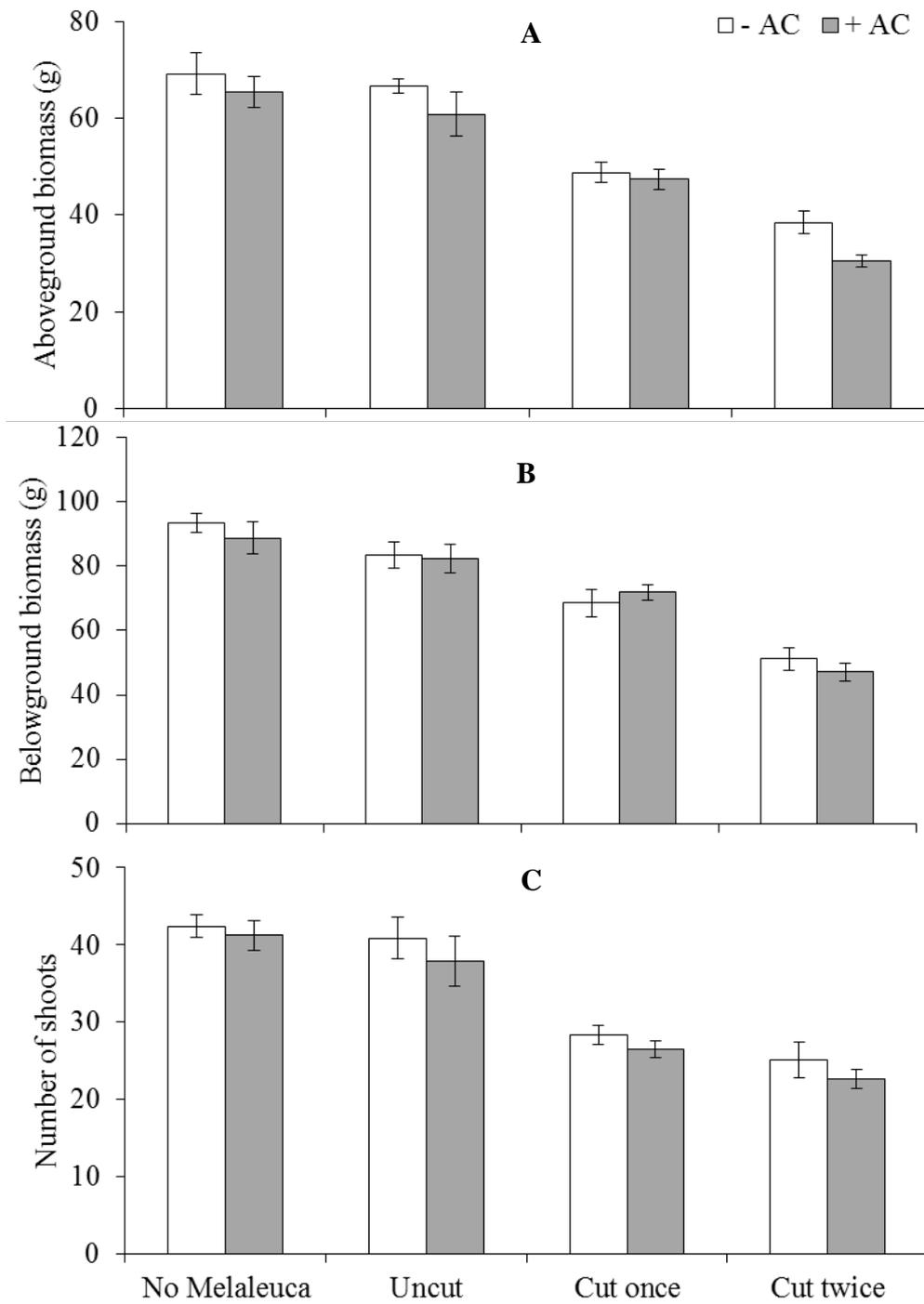
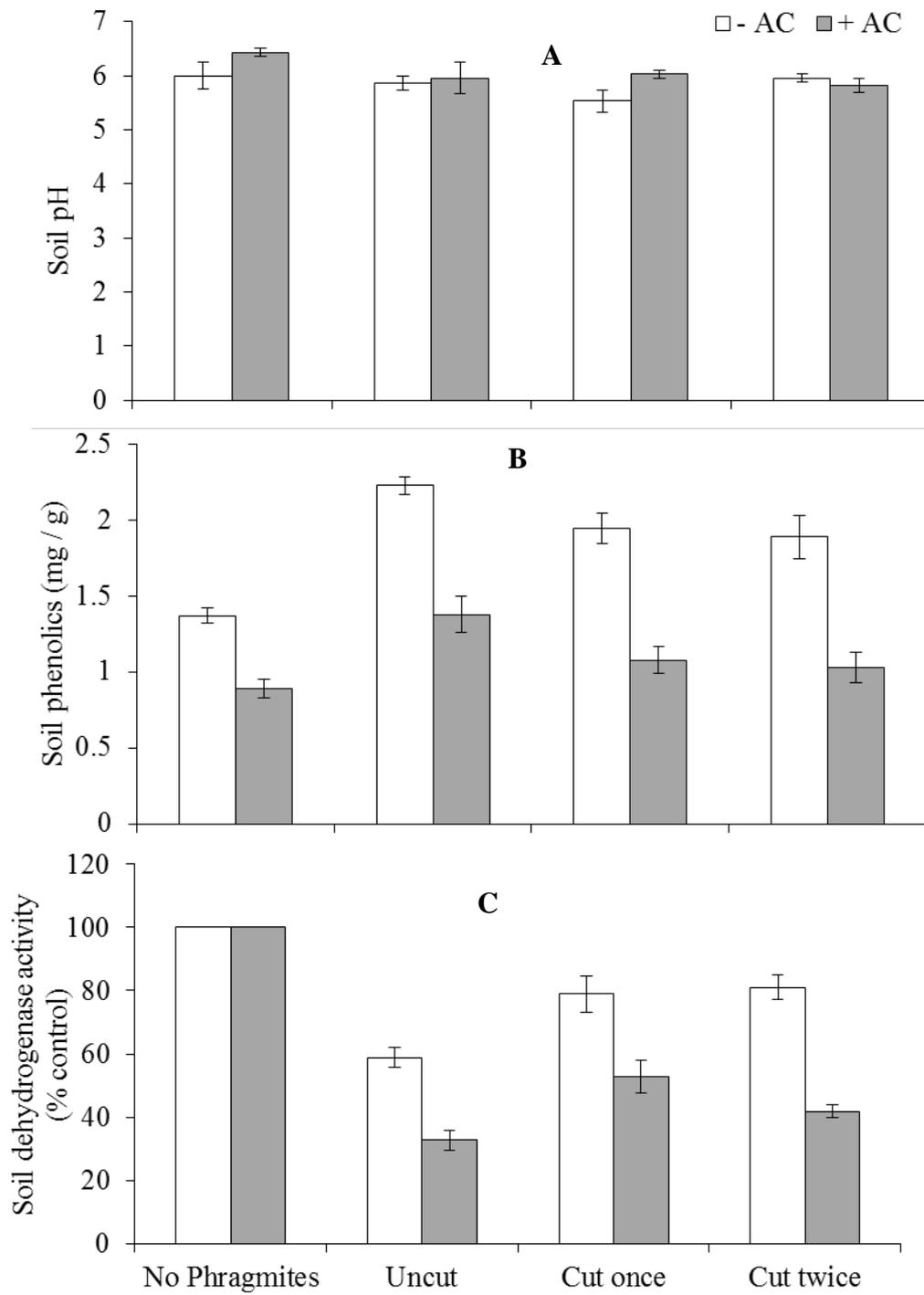


Fig. 5.



Chapter Eight

Conclusions and future directions

Conclusions

This research investigated allelopathy as a mechanism for the invasion of *Phragmites australis* through a series of ecologically realistic experiments, and chemical analyses in the laboratory, greenhouse and field. During the process of investigations, the methods used in these studies were assessed to verify the ambiguity between allelopathy and resource competition and what parameters are useful indicators of 'mode of action' for allelopathy in one of the world's most invasive plants. In addition, we examined, in a limited way, the present control and management options and what role they may play in allelopathy.

The results of the studies carried out here demonstrated that extracts and leachates of leaves and rhizomes of *P. australis* have more potential to produce phytotoxic chemicals than those of other organs of the species. Germination inhibition and reduction of growth with consequent lowering of chlorophyll content and maximum quantum yield provided direct evidence of phytotoxicity and the persistence of allelochemicals in soil. Sequentially, the physiological studies clearly determined that *P. australis* has phytotoxic effects on the germination processes and seedling development of other plant species. The impacts were partially influenced by osmotic effects of aqueous plant extracts. The correlated and combined effects on water imbibition, alterations in reserve carbohydrate mobilization and increasing reactive oxygen species (ROS) in germination and growth processes caused oxidative stress in cells of impacted seedlings. Thus, these phenomena can result in cell death and ultimately inhibit the growth of plants and may, in sensitive species, lead to plant death. Notably, this study confirms the presence of gallic acid, an important phytotoxin, in different organs of *P. australis* with varying concentrations.

Consistent with previous works (Uddin *et al.*, 2012; Uddin *et al.*, 2014a), the decomposition experiment in this present work demonstrated that physicochemical variables of the aqueous extracts of decomposed materials in soil showed a consistent pattern of

degradation in aerobic conditions with a high level of inconsistency in anaerobic condition. Pattern consistency among variables was largely dependent on the condition and residues, revealing that the factors influencing variables across decomposition types over time may play a crucial role in natural wetland ecosystems and thereby enhancing the phytotoxic effects on associated plant species (Uddin et al., 2014b) . Subsequently, the field studies (as evidence of allelopathy in the field) with supportive laboratory experiments specified that *P. australis* can substantially modify the wetland ecosystem through litter decomposition, root exudation, and other plausible reasons. We found an overall relationship between the field chemistry of *P. australis*-dominated wetland and the differential response of assay species in terms of germination, growth, and morphological features but the strength and direction of relationship varied among species and seasons. However, this study explained that *P. australis* might gain competitive advantage through the release of phytotoxins through different means in soil and water while soil biota may play an important role by altering the allelopathic effects, and that competing species may have strongly differential responses.

Furthermore, the series of density-dependent experiments made clear that the mechanisms of invasion were associated with phytotoxins of *P. australis* and not simply related to resource competition. The observation of growth reduction in test plant species at low population densities was inconsistent with the standard resource competition hypothesis and provides support for the hypothesis of chemical interference by *P. australis*. Although, the growth response of test species did not follow the similar pattern in all experiments, in most of the cases the data clearly demonstrated the density-dependent reverse slope phytotoxicity concept. The studies that disentangle allelopathy and resource competition may provide a sound methodological approach for understanding plant-plant interactions in allelopathy and clearly distinguish the mechanisms involved in interference.

Likewise, the final studies demonstrated that root exudates of *P. australis* had less but distinct allelopathic effects on germination, and growth of *Melaleuca ericifolia* compared with other direct competitive effects such as resource competition (Uddin et al., 2014c). These effects confer a portion of the competitive success of *P. australis*. Mechanical control of *P. australis*, an ecologically safe technique, reduced more phenolics content in soil substrates compared with activated carbon (AC) addition which demonstrates that biomass removal would be more effective for reducing allelopathic potential than addition of AC. Based on our results, we can conclude that above and below ground competition is not the sole mechanism responsible for invasion of *P. australis* or suppression of *M. ericifolia*, but the relatively low involvement of allelopathy has contributed to its invasion success compared to other competitive effects. In addition, this study establishes that the integrated approach (regular cutting and potential soil amendments with AC) might be useful as an ecological restoration technique for successful management of wetlands invaded by *P. australis*.

In general, the findings obtained from our studies suggest that *P. australis* is able to compete with other plant species in wetland ecosystem through allelopathy. In addition to 'chemical warfare', above and below ground competition for resources are involved in the invasion process. Methodologically, these studies demonstrate that an integrated approach (laboratory, greenhouse and field) is necessary in establishing the conclusive proof of allelopathy because laboratory or greenhouse study alone is not sufficient to provide a definite insight into allelopathy of a plant. Moreover, separation of allelopathic effects from resource competition is a vital point in allelopathy research which was addressed in this project and indicated that phytotoxins secreted by different means from *P. australis* are responsible for invasion process except root exudation. Despite the results indicate less inhibition by root exudates by *P. australis* on *M. ericifolia* transplanted plants (Uddin et al.,

2014c) but toxin may arise from other sources like residue decomposition into soil that inhibit the germination processes and other growth parameters (Uddin et al., 2014b). These results are well aligned with other allelopathy studies of *Agropyron repens* in which Welbank (1960) found that decaying roots and rhizomes of *Agropyron* markedly inhibit the root and shoot growth of rape seedlings but no significant inhibition by root secretion. On the other hand, plant-plant allelopathic interactions may be explained by species-specific (Hierro and Callaway, 2003; Prati and Bossdorf, 2004) and contextual relationships (Bauer et al., 2012) that proves the consistency of whole results of our studies.

From ecological point of view, the integrated experimental design is crucial for understanding the chemically mediated plant-plant interactions in natural ecosystems. Our studies evaluated the most probable processes of the allelopathy mechanism of *P. australis* and noted that residue decomposition is the most reliable source for liberating the phytotoxins in the wetland water and soil especially under anaerobic condition. Thereby, *P. australis* phytotoxins exert detrimental effects on a plant community by inhibiting the germination process and seedling growth, development and establishment and having other adverse effects on plant physiology. Consequently, *P. australis* receive an advantage over the associated plant species that allows it to invade. Historically, a division in the literature between allelopathy and resource competition of many invasive plant species which lead many attempts to prove that either one or the other is dominant in ecological invasion processes. In reality, both are aspects of the same occurrence so it needs to incorporate both that may prove more fruitful than focusing on either one independently in allelopathy study. This study confirmed that mechanical control might be an ecologically safe method to reduce allelochemical contribution into wetlands in preference to amendment of the soil with AC. From a purely mechanistic point of view, this study may provide valuable information regarding the underlying mechanism of invasion of *P. australis* for land managers, plant

ecologists and conservationists that may be of assistance to restore wetlands invaded by *P. australis*.

Ecological Implications

In light of all observations, it appears that *P. australis* exhibits its allelopathic potential in many ways, especially through the accumulation and persistence of allelochemicals in soil systems. Our results imply that residue decomposition of *P. australis* significantly contributes phytotoxins to the environment and induces associated physicochemical changes in soil and water systems and thereby, causes suppression of germination and growth of associated plant species. In addition, the density-dependent phytotoxicity phenomenon may have important ecological implications as a methodological approach that might provide clear ideas about the allelopathic effects (Weidenhamer and Romeo, 1989). The results of this study may go some way to explaining the long-term effects of *P. australis*-dominated wetlands on community ecology and more specifically allelochemical suppression of competing species. This study may provide land managers with a mechanistic understanding of the *P. australis* invasion process that strongly implies that successful wetland restoration may involve limiting the invasion and expansion of *P. australis*.

The present study may also influence sustainable management practices in wetlands through the knowledge of allelochemical activities in wetland ecosystems more generally. It is through understanding the potential impacts of allelochemicals on recruitment of associated plants in wetlands that contain *P. australis* or indeed other allelopathic species, which the present study contributes to explaining the long-term effects, particularly the floristic simplifications, on wetlands resulting from the invasion of *P. australis*. The results may also be applicable to agriculture practices in assessing the suppressive effects of organic matter used as crop cover and crop residue. In the context of global invasion of *P. australis*, the findings may have important ecological implications for understanding the effects of

invasive allelopathic species and their mechanisms of invasion. The findings may be useful to evaluate the response of agricultural plants to weed residues, and it may provide insight and evidence of a more general allelopathic effect than is presently understood.

Future directions

Though the study confirms the presence of gallic acid in different organs of *P. australis*, further studies are required to identify more details about the known and unknown compounds contained in the species and their phytotoxicity. In addition, whether the induction of oxidative stress associated with the increased defence mechanisms by antioxidant enzyme activity in the cells of the plant persists is a question to be studied in further research. Future studies of allelochemicals, particularly identification and associated phytotoxicity in different phases of decomposition as well as field-based observations are needed to more fully understand the role of these allelochemicals in the environment. A multidirectional approach is necessary for a better understanding of phytotoxicity dynamics through decomposition processes in natural ecosystems, especially the specific role of soil microorganisms and limiting environmental conditions.

Most importantly, the question remains as to what extent allelopathic effects are influenced by the soil biota and the indirect effects of allelochemicals secreted by *P. australis* on below ground mutualisms. Preliminary work (unpublished) in this regard and studies carried out by other researchers indicate that allelochemicals secreted by *P. australis* may, in fact, have direct impacts on rhizosphere organisms and, therefore, may impact associated species in an indirect manner. Moreover, a more sophisticated approach such as chemical identification of root exudates and their effects, as well as examination of microbial activities associated with allelopathic effects in soil and the separation of allelopathic effect from competition in natural settings is essential for understanding and predicting invasion of *P. australis* in wetlands. In contrast to phytotoxic effects, some other biotic factors such as

herbivores and diseases, which may reduce plant growth and might be expected to increase as plant density increases, also need to be addressed (Harper, 1977). Along with direct interactions between phytotoxins and plant density in soil, the indirect relationships between phytotoxins and rhizospheric processes (mycorrhizal activities and nitrification), which might be also density-dependent, is a field that is prospective for study.

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