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# Allelopathic Potential of the Invasive Alien Himalayan Balsam (*Impatiens glandulifera* Royle)

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**Allelopathic Potential of the Invasive Alien  
Himalayan Balsam (*Impatiens glandulifera* Royle)**

by

OWEN PETER SMITH

A thesis submitted to Plymouth University  
in partial fulfilment for the degree of

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University of Plymouth  
School of Biomedical and Biological Sciences  
Faculty of Science and Technology

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# Allelopathic Potential of the Invasive Alien Himalayan

## Balsam (*Impatiens glandulifera* Royle)

By Owen Peter Smith

### Abstract

Investigations were carried out into the allelopathic potential of the invasive alien annual Himalayan Balsam (*Impatiens glandulifera* Royle) using a series of bioassays, including ones developed or adapted for this study. They were evaluated for their suitability to detect three of the four main modes of allelochemical release, namely leaching, exudation and decomposition. Assays which involved the measurement of lettuce radicles and hypocotyls gave reliable results and allowed a range of different *Impatiens* material, both living and dead, to be assessed and ranked according to the allelopathic effects demonstrated. Attempts were made to isolate resource competition from allelopathy using separately grown but connected donor and receiver plants and a density dependent design where single *I. glandulifera* plants were grown in pots with variable numbers of receiver plants. Results proved inconclusive.

Initial experiments showed that the allelopathic potential of *I. glandulifera* varied according to the organ from which the material was derived. Pods, leaves and stems produced the greatest inhibition of lettuce seedlings. Effects on germination were not significant at most of the concentrations tested.

Live roots of *I. glandulifera* plants produced pronounced orange staining of the agar into which they were placed and showed clear evidence of distance dependent inhibition of lettuce radicles. Effects were limited to growth rather than germination of the test plants.

Germinating *I. glandulifera* seeds caused a significant inhibition of lettuce radicle elongation when the two species were grown together in an agar medium. The inhibitory effects increased significantly with increasing exposure time. Increasing *I. glandulifera* seedling number also produced significant reductions in lettuce radicle length. Dormant seeds, by contrast, stimulated growth. Dead seeds did not produce significant changes to the growth of the test plants.

When rhizosphere soil was gathered from pot grown *I. glandulifera* plants, the results were mixed. Initial samples inhibited growth, whereas those collected from dying plants over a period of weeks stimulated growth.

Further experimentation is required before the indications of allelopathic interactions demonstrated here can be applied to the behaviour of wild populations of *I. glandulifera*.

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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## **List of abbreviations**

2MNQ	2-methoxy-1,4-naphthoquinone
AC	Activated Charcoal
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
CABI	Centre for Agricultural Bioscience International
DEFRA	Department for Environment, Food and Rural Affairs
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
ECAM	Equal Compartment Agar Method
EPPO	European Plant Protection Organisation
ERCA	Evolutionary Reduced Competitive Ability
GC-MS	Gas Chromatography-Mass Spectroscopy
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
IAS	Invasive Alien Species

IPANE	Invasive Plant Atlas of New England
ITS	Internal Transcribed Spacer
MECAM	Modified Equal Compartment Agar Method
NEC	No Effect Concentration
OAP	Overall Allelopathic Potential
PAR	Photosynthetically Active Radiation
PCR-DGGE	Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis
PDA	Potato Dextrose Agar
PDMS	Polydimethylsiloxane
PLFA	Phospholipid-derived Fatty Acids
ppm	Parts per million
PSII	Photosystem II
PSM	Plant Secondary Metabolites
RH	Relative Humidity

ROS                      Reactive Oxygen Species

TTC                      Tetrazolium Chloride





# 1 General Introduction

“The global spread of plant species by humans is both a fascinating large-scale experiment and, in many cases, a major perturbation to native plant communities.”

from *Ecology and Control of Introduced Plants*, by Judith H. Myers and Dawn R. Bazely, Cambridge University Press 2003

This study aims to investigate the potential of allelopathy as a factor in the spread of the invasive alien plant Himalayan balsam (*Impatiens glandulifera*), which was introduced in the 19<sup>th</sup> century and is now widespread and abundant in suitable habitats throughout the United Kingdom. The focus of this introductory chapter is a general overview of invasion biology, the definitions of terms associated with the topic and a short account of the autecology of *Impatiens glandulifera*. Chapter 2 discusses the possible mechanisms by which *I. glandulifera* achieves its dominance in suitable habitats, including evidence for the role of allelopathy.

## **1.1 Introduction**

The human species has been involved in the transportation of plants and animals around the globe for thousands of years. This biotic transfer can be seen as an integral part of our history and a crucial factor in societal and economic development (di Castri, 1989). In many, if not most areas, the staple crops and domestic animals utilised for food and other purposes are introductions (Pimentel, 2000). As well as crops, many plant species have been transported deliberately for use as garden ornamentals, or inadvertently as contaminants in seed mixtures (Cronk & Fuller, 1996). Some of these plants may then go on to establish self-sustaining populations and a few can become serious weeds (Williamson & Fitter, 1996).

## **1.2 Definitions of the terms alien and invasive species**

The literature concerning the definitions of what constitutes an invasion and the terminology necessary to describe them has increased over recent decades in response to increasing alarm about their effects on ecosystems (Bingelli, 1994; Carlton, 1996; Colautti et al., 2004; Richardson et al., 2000; Usher, 1986). For the sake of clarity, it is worth considering some of the definitions for the terms alien and invasive.

### **1.2.1 Alien Species**

Alien or introduced species are those which have colonised an area previously unoccupied during recorded history, whether this spread has been natural or human-mediated (Myers & Bazely, 2003). In Japan, alien plants are defined as those which have been introduced to the Japanese archipelago since the beginning of the Meiji era (1868), when the self-imposed isolation of the Tokugawa Shogunate was ended by liberalisation and international trade. This excludes the many species introduced to Japan in association with rice cultivation in preceding centuries Fujii (2008) personal communication.

It is important to recognise that biological invasions are continually occurring, whether as a result of human activity or as spontaneous events whose causes are not necessarily linked to direct or indirect human influence. One classic example is the spread of the collared dove (*Stertopelia decaocto*) throughout Europe during the 20<sup>th</sup> Century (Williamson & Fitter, 1996).

### **1.2.2 Invasive species**

Many definitions of what constitutes an invasive plant have been proposed. The simplest is probably the “ability of a plant species to increase when rare” (Crawley, 1997). All plant species, can, according to

Crawley (1997), be invasive when conditions are suitable, according to the formula

$$\frac{\delta N}{\delta t} > 0$$

where  $N$  is population size and  $t$  is time, without consideration as to the origins of the invader. A more comprehensive definition is “an alien plant spreading naturally (without the direct assistance of people) in natural or semi natural habitats, to produce a significant change in terms of composition, structure or ecosystem processes” (Cronk & Fuller, 1996). This definition excludes native plants, however, which can also be considered a threat to other species when growing in an invasive way (de la Cretaz & Kelty, 1999). For instance, the spread of bracken (*Pteridium aquilinum*) in the UK and elsewhere (Whitehead & Digby, 1997) and the saltmarsh grass *Elymus athericus* which, since the 1990s, has invaded large areas of the intertidal zone in Mont Saint- Michel Bay, France (Valéry, Bouchard & Lefeuvre, 2004). It also sets up the necessity for further definitions of natural or semi natural habitats, no easy task in an anthropogenically derived landscape like Britain (Hannah et al., 1994).

In order to reduce the considerable confusion that the competing terms generate, Richardson, Pysek et al. (2000) proposed the following definitions for plant species based on increasing plant frequency and ability to establish self-sustaining populations. (see Table 1.1).

Table 1.1. Definitions of alien, naturalised and invasive plant species (Richardson, Pysek et al. 2000).

Alien	A plant whose presence is due to intentional or accidental introductions as a result of human activity
Naturalised	An alien plant that reproduces consistently and sustains populations over many life cycles without direct intervention by humans often recruiting offspring freely, usually close to adult plants and does not necessarily invade natural, semi-natural or human-made ecosystems
Invasive	A naturalised plant that produces reproductive offspring, often in very large numbers at considerable distances (also defined) from parent plants and thus has the potential to spread over a considerable area

For the purposes of this study, the above definition is perfectly serviceable, although as Colautti and MacIsaac (2004) observe, a consensus on operational terminology is required if invasion ecology is to evolve into a more objective discipline.

### **1.3 Harmful effects of invasive alien species**

The detrimental effects of invasive alien species are now seen as one of the most pressing threats to the ecology and economy of the world (Pimentel, 2002). Article 8h of the Convention on Biodiversity proposes that participating signatories strive to “prevent the introduction of, control or eradicate those alien species that threaten ecosystems, habitats

or species” and that invasive aliens represent the second most serious threat to biodiversity after habitat destruction (Glowka, 1994; McGeoch et al., 2010). Cronk and Fuller (1996) list the following as negative results of the invasion of ecosystems by invasive alien plants:

1. Threatening native fauna
2. Smothering nest sites, food plants etc.
3. Alteration of soil chemistry
4. Alteration of geomorphological processes
5. Causal factor in plant extinctions
6. Alteration of fire regime
7. Alteration of hydrology

Among the other harmful effects attributed to alien plant invasion are the alteration of genetic diversity of the invaded community (Ellstrand & Schierenbeck, 2000) decrease in native species abundance and richness through competition, predation and indirect effects (Blackburn et al., 2004; Gaertner et al., 2009) and the alteration of soil ecosystem processes such as nutrient flux rates (Ehrenfeld, 2010; Raizada, Raghubanshi & Singh, 2008). These are just the effects for which data have been collected. As Simberloff (2011) makes clear, alien invasions are likely to cause other subtle effects on ecosystem processes long before the more obvious ones are manifested and noted by scientists.

Since the 17th century, invasive alien species have contributed to nearly 40% of all animal extinctions for which the cause is known (CBD, 2006). The cost of non-native species invasions in the USA, UK, South Africa, India and Brazil have been estimated at more than US\$ 314 billion per year for these six nations (Pimentel et al., 2001). The costs of controlling invasive plant species alone in the USA have been estimated to be in the region of \$125 billion per annum (Baker, 2001). In Britain, the costs of invasive plant eradication are said to be £2.7 billion per year (de Bruxelles, 2010). In May 2008, The Invasive Non-native Species Framework Strategy was launched. (DEFRA, 2008). It is intended to provide a strategic framework to facilitate the better co-ordination of control efforts between government departments and key stakeholders involved in invasive species control. One of their flagship campaigns “Be Plant Wise” was launched in 2010 to alert gardeners to the dangers of liberating invasive aquatic plants and provided information on their safe disposal (Anon, 2010).

Not all introduced species are considered to be harmful, however. Some alien species may be benign or beneficial in their effects on ecosystems (Davis, 2009; Manchester & Bullock, 2000). In some parts of the world, introduced plant species have a role as nurse crops early in the in the re-establishment of native vegetation (Cronk & Fuller, 1996; De Pietri, 1992; Williams, 1983).



## **1.4 The Role of Alien Plant Introductions in the United Kingdom**

Approximately 29% (558 species in total) of the British flora is composed of introduced species (Myers & Bazely, 2003; Williamson, 2002), which is the highest percentage in Europe. This figure is a result of Britain's isolation from continental Europe by rising sea levels at the end of the last ice age, leading to a low initial total number of indigenous plants - around 1400 species of angiosperms (Stace, 1997). By way of comparison, Germany has 2850 native plant species and France 4171 (Myers & Bazely, 2003). Williamson & Fitter (1996) proposed a simple "Tens Rule" to biological invasions: it is reasonable to expect 10% of introduced species to appear in the wild; of these introductions 10% become established and 10% of established aliens become invasive. This means that approximately 0.1% of the total introduced species are likely to present a threat due to their invasive capabilities. Although there are exceptions to this rule of thumb, it appears to hold true in the majority of cases involving angiosperms (Williamson & Fitter, 1996). Climate change and countrywide declines in the UK's biodiversity could create more opportunities for invasive organisms within existing ecosystems (Manchester & Bullock, 2000).

## **1.5 The genus *Impatiens* in the United Kingdom**

The genus *Impatiens* L, in the family Balsaminaceae, is composed of about 1000 species (Stevens, 2008) and is mainly found in the tropics and subtropics of the Old World. The majority of species are fleshy herbaceous perennials, with some annual species native to temperate Eurasia and North America.

*I. noli-tangere*, the touch me not balsam, is an uncommon native with a very limited distribution (Hatcher, 2003). It is currently found in a few sites in Cumbria and Wales, where it grows most frequently in damp woodlands, often by water (Hatcher, 2003). Several other *Impatiens* species have been introduced beyond their native ranges and are now considered to be invasive alien species (IAS),(G.I.S.P., 2002). In the UK, the three main IAS in the genus *Impatiens* are *I. glandulifera*, *I. parviflora* and *I. capensis*. *I. glandulifera* is by far the most common species of *Impatiens* in the UK and is considered to be one of the top three weeds in terms of its visual impact (Manchester & Bullock, 2000). Another species, *Impatiens balfourii*, from Kashmir, has recently been recorded as increasingly naturalised in both the UK and Europe (Adamowski, 2009; Schmitz & Dericks, 2010).

### 1.5.1 Relative invasiveness of *Impatiens* species in the United Kingdom

Perrins, Fitter & Williamson (1993) ranked the four longest established species in order of increasing invasiveness: *I. noli-tangere*, *I. capensis*, *I. parviflora*, *I. glandulifera*. They listed frost tolerance and seed production as important factors in the varying success of the species mentioned, although they also suggested that other as yet unidentified factors might be involved

The invasive abilities of these species do not appear to be linked to the size of their native ranges (Williamson, 1996) (Figure 1.1). Indeed *I. noli-tangere*, the rarest species in Britain has by far the widest range of the four species, whereas *I. glandulifera*, the most invasive, has much the smallest native range.



Figure 1.1. Native ranges of *Impatiens noli-tangere*, *I. capensis*, *I. parviflora* and *I. glandulifera* (From Dericks (2007) based on Williamson (1996) Used with permission.

## **1.6 *Impatiens glandulifera* in the United Kingdom**

### **1.6.1 History**

*Impatiens glandulifera* Royle was introduced to Britain in 1839 as an ornamental from the Kashmir region of the Himalayas (Coombe, 1956) and is now considered to be one of the three main terrestrial invasive alien weeds in the United Kingdom (Manchester & Bullock, 2000) and is, uniquely among the three, an annual. Within two decades of its introduction, it was recorded as commonly occurring in the Manchester area (Britten, 1900) and was thoroughly naturalised on the River Looe in Cornwall by the same time (Hume (1995) quoted in Grigson (1996)). It is commonly known in English as Himalayan balsam, or, less commonly, as Indian balsam. Other names in European languages include Drüsige Springkraut (German), Balsamine de l'Himalaya (French), Reuzenbalsemien (Dutch).

### **1.6.2 Native Habitat**

Data on the species in its native range are sparse (Polunin & Stainton, 1984); the literature does not suggest that it is considered to be weedy or invasive, although it shows a preference for similar habitats to those in its introduced range: roadside ditches and along watercourses (Gupta, 1989). It is found between 2000 – 2500 m a.s.l. from Kashmir to Gharwal,

although Polunin & Stainton (1984) record it as occurring at up to 4000m; Adamowski (2008) gives an altitudinal range of 1600-4300 m. Researchers from the Centre for Agricultural Bioscience International (CABI) report that in Himalayan Pakistan, *I. glandulifera* is a minor component of the flora and is mainly found as a weed in field crops (Tanner, 2006).

### **1.6.3 Distribution of *I. glandulifera* in Europe and the United Kingdom**

*I. glandulifera* is widespread and increasing in Europe from southern Sweden to the Mediterranean and in Central Europe (Beerling & Perrins, 1993). It is now recorded from 35 European countries (Lambdon et al., 2008). It is found in North America on both eastern and western sides (IPANE, 2009; Tabak & von Wettberg, 2008) and is also reported as invasive in New Zealand (Clarkson, 2006). It has increased its range in the United Kingdom dramatically in the last decade (see Figure 1.2).

It favours moist habitats such as watercourses and wet woodlands and is found in suitable habitats below 210 m above sea level. It is less common in Scotland and Ireland than in England and Wales, although it also occurs on isolated island groups such as Shetland, Orkney and the Scilly Isles.

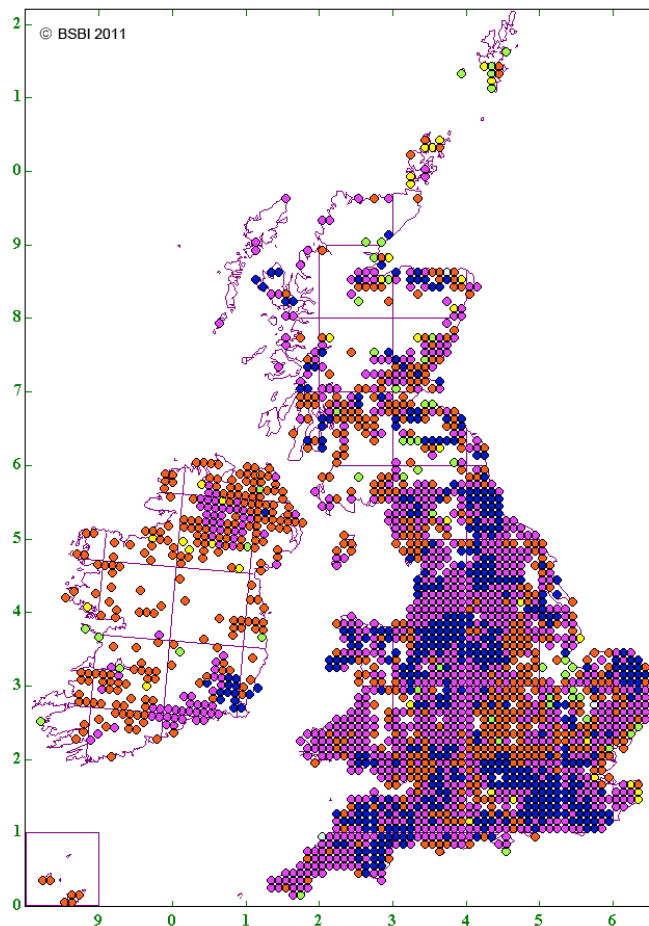


Figure 1.2 2011 hectad map of the distribution of *Impatiens glandulifera* in Great Britain and Ireland. Blue circles represent new records from 2010 onwards, pink 2000-2009, orange 1987-1999, green 1970-1986, yellow 1930-1969, pale blue up to 1930. This species is now recorded from 2100 hectads and has shown a very rapid expansion in range since 2000. ©2011 Botanical Society of the British Isles.

#### 1.6.4 Plant description

This brief account of *I. glandulifera* is based upon Beerling & Perrins (1993) with additional information by the author.

*I. glandulifera* is an annual herb up to 2.5 m tall.

Stems: hollow 5-50 mm in diameter, simple, sometimes branched.

Leaves: opposite or in whorls of three, 5–18 cm long, 2.5–7 cm in width.

Margins serrate, with 18–50 serrate teeth on each side.

Flowers: 3–12, born in axillary racemes, 2.5–4 cm long, hypogynous; stamens 5, ovary 5 celled with axile placentation, ovules anatropous; corolla drops from plant after flowering; colours range from white, through pink to dark purple.

Capsules: 1.5–3.5 cm containing 4–16 seeds approximately 4–7 mm in length and 2–4 mm wide. Seed mass 2–35 mg, released explosively when ripe capsule is disturbed.

Roots: reach a depth of 10–15 cm in soil, adventitious roots often appear at the base of the plant or further up the stem following lodging (see Plate 1.1).



Plate 1.1. Adventitious stilt roots appearing from the lower nodes of *I. glandulifera* stems, a common occurrence in large plants. Metre rule for scale. Dartington, Devon, July 2007.

## *Phenology*

Seeds require a period of cold stratification before germinating (Mumford, 1988). In the south west of the UK this usually occurs in February. Rapid plant growth then begins in April, with the onset of stem elongation and leaf expansion.

Flowering: usually occurs from July to October; often delayed by several weeks in shaded sites.

Senescence: plants begin to collapse by November, depending on weather conditions. Fragmented stems often persist as a layer of litter over the soil until the following year, through which *I. glandulifera* seedlings often emerge successfully (see Plate 1.2).





Plate 1.2. *I. glandulifera* seedlings emerging through litter of dead stems. Nettles (*Urtica dioica*) also present, shoots developing from existing rhizomes. Looe Valley, Cornwall, February 2009.

### ***Life History***

Details of its life history in Britain are based on the paper of Beerling & Perrins (1993) summarised briefly below:

- Frost and drought sensitive.
- Tolerates shading to 30% without suffering significant harm.
- Tolerates wide pH range: 3.5 – 7.7.
- Requires bare ground for successful establishment
- Occurs on exposed river sediments, river banks, waste ground, fens, grasslands, woodlands.

- Seed dispersal 5 m by dehiscence, further by water
- Seeds: require chilling, no persistent seed bank, negatively buoyant, viable up to 18 months. It is found in suitable habitats below 210 m above sea level and is less common in Scotland and Ireland than in England and Wales.

*Impatiens glandulifera* is the tallest representative of its life form (annual) in both Britain and Central Europe (Pysek & Prach, 1994) and has been described as a “mobile, opportunistic, rapidly growing annual” (Prowse, 2001). It is also showy, with large pink, purple or white flowers and excites comments by virtue of its explosive seed pods. In addition to its spread by natural means, humans have been implicated in sowing it in semi-natural and natural areas previously free of the plant and these populations may then serve as foci for new invasions (Rotherham, 2000). Seeds of *I. glandulifera* were still available commercially in UK seed catalogues in the mid-2000s (Bowden, 2007) and colour selections such as “Red Wine” were continuing to be sold in 2010 (Brown, 2010).

Himalayan balsam seeds are large for an annual, oily, and are palatable to humans (Facciola, 1990; Fowler, 2011) and presumably rodents and other seed feeding animals (Beerling & Perrins, 1993). Recent attempts have been made in Germany to market products made from

both *I. glandulifera* and *Fallopia japonica* as a means of offsetting the costs of control programmes (Becker, 2008).

### **1.7 Ecological Impact of *Impatiens glandulifera* on native plant communities**

Attitudes towards the plant and its perceived harmful effects vary. Prowse (2001) for example concluded that *I. glandulifera* did not have significant deleterious impacts on native plant species in the Manchester area: it often occurred in species-poor habitats, such as river banks and wet woodlands and a number of plants survived quite successfully with it due to temporal niche differentiation. It was suggested that *I. glandulifera* might have a negative effect on low frequency species, although it was concluded that this would presumably be the case with invasive native plants such as nettles (*Urtica dioica*) as well.

Hulme & Bremner (2006) reported that although the presence of *Impatiens glandulifera* may cause a reduction in species richness of up to 25%, the majority of excluded species consisted of widespread ruderals of low conservation value. The removal of *I. glandulifera* also caused a compensatory increase in abundance of other non-native weeds, thereby failing to achieve a reduction in non-native vegetation. Hejda & Pysek (2006) reported that *I. glandulifera* exerted negligible effects on invaded

riparian communities found along six rivers in the Czech Republic and did not represent a threat to the plant diversity of these areas.

Many conservation organisations, however, do not share these views and a considerable amount of time and effort is spent eradicating this species (Abbit, 2009). For example, the National Trust organises “balsam bashes”, where volunteers uproot or cut the plants to prevent them seeding on Trust-owned land (Anon, 2011).

### **1.8 Other deleterious effects of *I. glandulifera***

Effects of *I. glandulifera* on invertebrate populations have not been established, although it has been described as supporting an impoverished density of phytophagous insects (Beerling & Perrins, 1993). Its effects on the invertebrate fauna of exposed river sediments, often colonised, by *I. glandulifera*, are a potential cause for concern (Renals, 2005). There is also some evidence that balsam may attract pollinators away from native plants due to the higher sugar content of its nectar, leading to reduced seed set and possible genetic isolation in those species (Chittka & Schuerkens, 2001).

Other causes of concern are the displacement of perennial vegetation such as nettles by *I. glandulifera*, with potentially negative effects on erosion and sedimentation rates (Tickner et al., 2001b).

## **2. Allelopathy as a potential contributor to the invasive success of *Impatiens glandulifera***

“It is remarkable that this annual species is able to withstand competition by robust, competitively strong perennials.”

From Prach, K, (1994) Seasonal dynamics of *Impatiens glandulifera* in two riparian habitats in Central England in *Ecology and Management of Invasive Riverside Plants*, Chichester, UK. John Wiley & Sons 1994.

### **2.1 Introduction**

A general predictive theory of the invasiveness of introduced plants has remained elusive (Williamson, 1999) although it is known that successful and unsuccessful invaders often differ in performance related traits such as growth rate, size and fitness (Van Kleunen, Weber & Fischer, 2010). In their review of 150 studies of the literature on exotic plant invasions, Levine et al., (2003) found that less than 5% explored the mechanisms underlying the invasions such as competition, allelopathy, alteration of ecosystem variables or other processes. They suggested that further research be directed to redress this imbalance. Several life characteristics

have been linked to invasiveness in plants and some of these will be considered in relation to the spread of *I. glandulifera* in Table 2.1.

Table 2.1. The most common hypothetical predictors of a plant species' invasiveness based on Cronk & Fuller (1996) with supporting and conflicting evidence, if any in the case of *Impatiens glandulifera*.

Predictor	Supporting evidence	Conflicting evidence
Absence of native predators  Specialist predators/diseases control plant in native range	Limited fauna feeds on it (Beerling & Perrins, 1993)  Research on predators and pathogens in native range likely soon as part of biological control programme (Tanner, Djeddour & Shaw, 2008)	Heavily browsed by slugs (Prowse & Goodridge., 2003)  Viral pathogen discovered (Kollmann, Banuelos & Nielsen, 2007) – effects limited
Greater reproductive potential  Produces more seeds than natives; long lived seed bank		Seed production not markedly higher than native weed species (Perrins, Fitter & Williamson, 1993).  Seed bank very short lived (Beerling & Perrins, 1993)
Poorly adapted native species  Invader tolerates suboptimal resource levels better than native plants	Some evidence that <i>I. glandulifera</i> is able to tolerate lower nutrient levels than, for example, <i>Urtica dioica</i> (Beerling & Perrins, 1993)  Shade tolerance adaptations present (Andrews et al., 2005).  More frost tolerant than other <i>Impatiens</i> species (Perrins, Fitter & Williamson, 1993)	Colonises nutrient rich sites (Andrews et al., 2005; Güsewell, Zuberbühler & Clerc, 2005; Hejda & Pyšek, 2006)  Local adaptations to frost tolerance and other traits present in both <i>I. glandulifera</i> and other <i>Impatiens</i> species, making them unlikely to confer competitive advantages to <i>I. glandulifera</i> . (Skálová, Moravcová & Pyšek, 2011)
Chemical change  Eutrophication or pollution prior to invasion	Unknown, believed to tolerate polluted habitats, tolerates wide pH range (3.5–7.7) (Beerling & Perrins, 1993)  Some <i>Impatiens</i> species indicators/ bio accumulators of heavy metals (Tiagi & Aery, 1982)	
Balance of nature  Stability linked to diversity – more diverse habitats harder to invade	Riparian habitat of <i>I. glandulifera</i> inherently species poor, therefore unstable, facilitating invasion by <i>I. glandulifera</i> (Prowse, 2001)	
Empty niche/missing functional group  Life history traits not found in native plant species	<i>I. glandulifera</i> is a shade tolerant late season annual forb, with few equivalent functional group members.	<i>I. noli-tangere</i> occurs in similar habitats and shows similar life history traits to <i>I. glandulifera</i> . (Hatcher, 2003)
Niche vacant, no native organism adapted to make use of resources	Native species not maximising resource use, so <i>I. glandulifera</i> grows and spreads (Prowse, 2001)	
Disturbance	Seeds require bare soil in which to germinate (Beerling & Perrins, 1993)	

Although traits associated with plant reproduction and establishment, such as seed production, germination and seedling performance, are known to be different in successful and unsuccessful invaders (Moravcová et al., 2010; Rejmánek & Richardson, 1996), it is also known that invasive species can adapt rapidly to local conditions (Rice & Mack, 1991) and that contemporary evolution may play a more important role in invasion ecology than has been previously recognised (Maron et al., 2004). In the specific case of *I. glandulifera*, Kollman & Banuelos (2004) found that latitudinal trends in phenological traits such as time of flowering were present in populations from across the European range when they were grown in a common garden experiment. In a comparison of traits important in plant establishment, Skálová, Moravcová & Pyšek (2011) demonstrated that discrete populations of invasive *Impatiens* species and the native congener *I. noli-tangere* showed similar intraspecific differentiation in germination times, seedling emergence and seedling frost resistance, depending on the locations from which the seeds were gathered. Since local adaptations were indicated in both the native and invasive species studied, they concluded that the traits investigated were unlikely to provide the invasive *Impatiens* species with a competitive advantage against *I. noli-tangere*. The case for a general purpose genotype for invasive alien species as proposed by Baker (1965), was therefore not supported by these studies, nor were the observations of Perrins et al.



(1993) who believed that superior frost tolerance was one of the reasons for the spectacular success of *I. glandulifera* as an invader. Table 2.1 suggests that *I. glandulifera*'s suite of traits may not necessarily be sufficient to explain its characteristic invasiveness and that other factors may be involved.

## **2.2 Allelopathy - a missing component in the invasive success of *Impatiens glandulifera*?**

One area of research which until recently has received little attention is the potential for allelopathic inhibition of competitors to play a role in facilitating the spread of invasive alien plants. Allelopathic interactions between plant species have often been discounted in the past (Harper, 1975; Harper, 1977), but research over the last decade has suggested that they may be a factor in some plant invasions (Callaway & Aschehoug, 2000; Dorning & Cipollini, 2006; Hierro & Callaway, 2003).

The number of published studies specifically mentioning allelopathy as one of the competitive mechanisms used by *I. glandulifera* and other invasive *Impatiens* species are however, very limited (Krejčová et al., 2007; Scharfy et al., 2011). This thesis addresses this lack by providing a detailed study into the allelopathic potential of *I. glandulifera*. Before proceeding further, it is worth reviewing definitions of the word allelopathy and giving an overview of its history as a scientific discipline.

### 2.2.1 Allelopathy – definitions

The term allelopathy was first coined by Hans Molisch in his book *Der Einfluss einer Pflanze auf die andere-Allelopathie* (Molisch, 1937). He defined it (in translation) as an:

“inhibitory material or substance which is released from one plant and exerts an influence on another plant....It deals with an influence which is exhibited in a remarkable way in spatially separated plants” (Molisch, 2001).

Allelopathy was derived from the two Greek words “allelon” meaning “mutual” and “pathos”, meaning suffering. As Willis (2007) makes clear however, Molisch intended the term to cover both beneficial and detrimental effects of compounds on all classes of plants and also included micro-organisms. Other definitions are listed in Table 2.2.

What distinguishes allelopathy from other plant-plant interactions is that it is an additive mechanism of influence, rather than one involving the removal of necessary growth factors, such as light, water or nutrients; something is added rather than taken away. The effect can be either beneficial or inhibitory and the allelochemicals must be released from donor plants so that they are then available to acceptor plants for uptake and assimilation. In most cases, allelopathy is taken to be a form of interference, or negative influence of a plant on its neighbours (Harper, 1961); other forms of interference are resource competition and the

harbouring of inimical organisms such as herbivores or pathogens (Weidenhamer, 1996).

Table 2.2. Definitions of allelopathy

<b>Author</b>	<b>Definition</b>
Rice (1984)	Any direct or indirect harmful or beneficial effect by one plant (including microorganisms) on another through the production of chemical compounds that escape into the environment
Lovett & Ryuntyu (1992)	Best regarded as part of a complex network of chemical communication between organisms, in which groups of chemical compounds elicit similar, quantifiable responses from disparate organisms.
Pratley (1996)	Allelopathy is a process whereby plants provide themselves with a competitive advantage by putting phytotoxins into the near environment.
International Allelopathy Society (IAS) (1996)	Any process involving the secondary metabolites (allelochemicals) produced by plants, algae, bacteria, and fungi (excluding animals) that influence the growth and development of agricultural and biological systems, including positive or negative effects.
Rizvi et al. (1992)	Biomolecules, named allelochemicals, escape into the environment and subsequently influence the growth and development of neighbouring plants.

### 2.2.2 Historical references to allelopathy

Although the term allelopathy is less than one hundred years old, references to allelopathic interaction can be found in literature from several cultures, spanning more than two thousand years. A complete review of these sources can be found in Willis (2007). Some of the key references are summarised in Table 2.3.

Table 2.3. Historical references to allelopathy in world literature (from Willis 2007)

Source and date	Effects
Theophrastus ca. 350BC	Inhibition of weeds by chick pea ( <i>Cicer arietinum</i> )
Pliny (Plinius secundus) AD 1	Chick pea, barley and bitter vetch ( <i>Vicia ervilia</i> ) harmful to the development of wheat plants
Columella 1 <sup>st</sup> century AD	Oak root exudates harmful to olive trees, even when oak has been felled
Ibn al Awwamca. (1180)	Beans, rue, oregano and euphorbia harmful to Seville orange
Culpeper (1652)	Grapes and cabbages grow poorly together; basil and rue grow poorly together
Banzan Kumazawa - quoted by Lee and Monsi (1963)	Rain and dew dripping from needles of red pine ( <i>Pinus densiflora</i> ) inhibit the growth of plants growing beneath their canopy
De Candolle (1832)	Crop rotation necessary because plants “sicken the soil through root excretions “

### **2.2.3 Types of chemical compounds identified as allelochemicals**

Allelochemicals have been identified as plant secondary metabolites (PSM), i.e. compounds which are generally recognised as lacking any major role in plant metabolism, such as photosynthesis, solute transport, nutrient assimilation or differentiation (Fraenkel, 1959; Whittaker & Feeny, 1971). PSMs have long been recognised for their role in plant: animal interactions (Brattsten, 1979; Fraenkel, 1959; Harborne, 1987) and there is an increasing body of evidence to linking them to direct between-plant communication (Bertin, Yang & Weston, 2003; Chamberlain et al., 2001; Singh, Batish & Kohli, 1999; Weir, Park & Vivanco, 2004). Nearly all classes of PSMs have been implicated as allelochemicals (Putnam, 1988 ). They usually occur at concentrations of less than 10% of the total plant mass and vary in amount according to the species and tissue type which is under investigation (Van Beek, 1999).

PSMs are produced by two main biochemical pathways: the Shikimate Pathway and the Acetate Pathway (Rice, 1984). Under normal circumstances, approximately 20% of the carbon fixed by plants travels through the Shikimate Pathway (Haslam, 1974) and further reactions lead to the production of PSMs such as quinones, alkaloids and phenolic acids. Rice (1984) devoted a chapter to the wide range of compounds which have been shown to possess allelopathic activity. He classified

them into fourteen groups according to their origins. Fig 2.1 summarises this information:

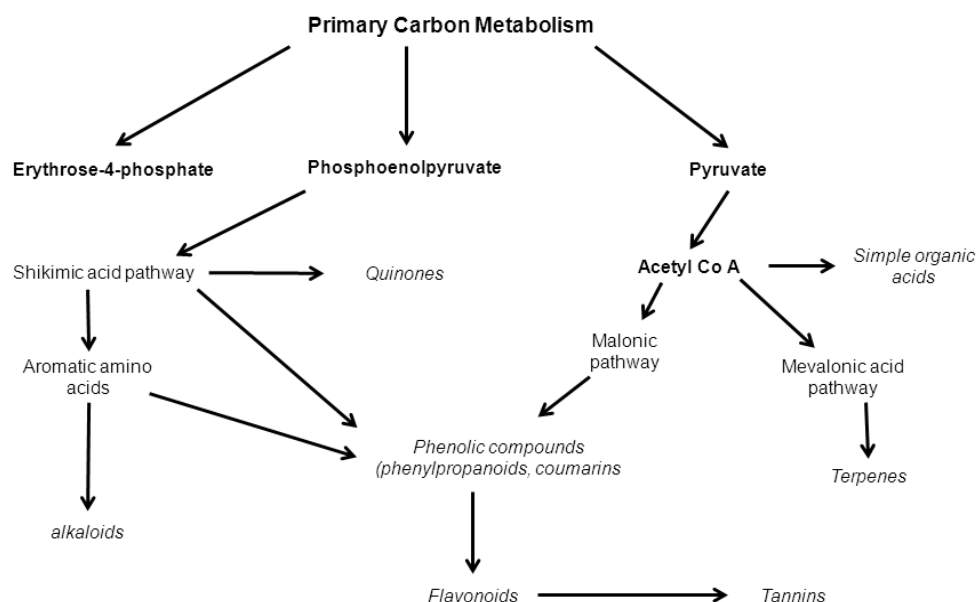


Figure 2.1 Plant secondary metabolites and their relationships with primary plant metabolism. Bold compounds are metabolites from primary plant metabolism; italic text denotes compounds with known allelopathic properties. Based on Rice (1984) and Taiz and Zeiger (1998).

Phenolics are considered the most important group of allelochemicals in temperate ecosystems (Inderjit, 1996; Mandava, 1985; Putnam & Duke, 1978), whereas terpenoids are believed to be more important in arid or semi-arid climates.(Horsley & Avery, 1991).

## 2.2.4 Mode of release of allelochemicals

PSMs with allelopathic potential are widely distributed in the majority of plant tissues, including flowers, stems, leaves, roots and seeds (Weir, Park & Vivanco, 2004). They are liberated by four processes, namely leaching, exudation, volatilization and the decomposition of plant residues (Einhellig, 1986; Kobayashi, 2004; Putnam & Weston, 1986; Rice, 1984; Weir, Park & Vivanco, 2004). This is represented diagrammatically in Figure 2.2.

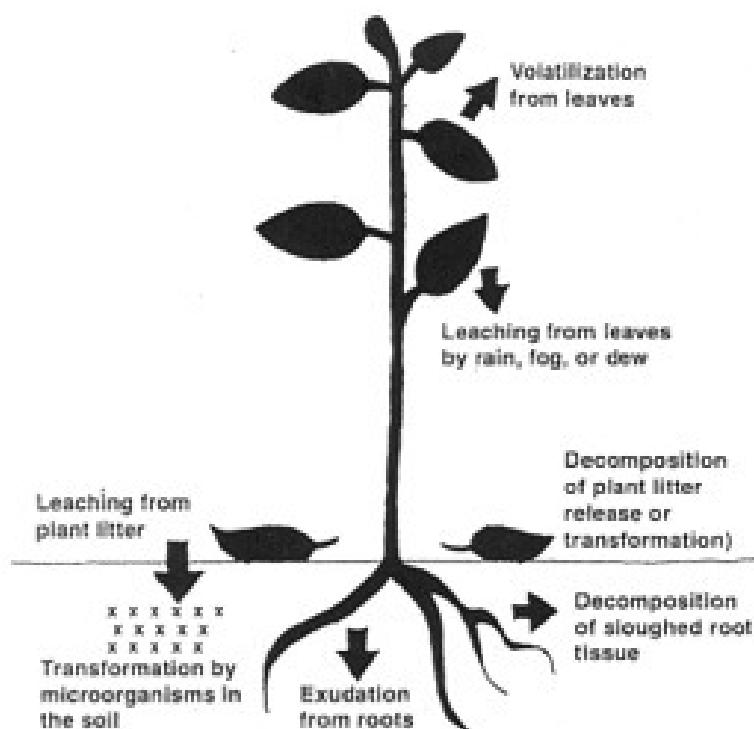


Figure 2.2 Modes of release of allelochemicals from plants, based on Chick and Kilbaso (1998) Used with permission of authors.

In the case of exudates, leachates and decay products, the amounts added to the soil are a function of source plant biomass and density and also the

concentration and solubility of the specific allelochemical (Weidenhamer, 1996).

### **2.2.5 Factors influencing the release of allelochemicals**

This subject is extensively covered in Rice (1984); for the purposes of this discussion, the most important of these factors will be mentioned below.

#### ***Resource limitation***

As previously mentioned, allelopathy is considered to be a process of interference (Harper, 1961; Rice, 1984) rather than resource competition; it is known, however, that plants undergoing stress caused by unfavourable nutrient, water or temperature regimes produce larger quantities of PSMs than those experiencing more favourable growth conditions. Tang et al. (1995) for example, reported increased concentrations of phenolics and terpenes in plants undergoing water deficit stress and Koeppe et al. (1976) found increased production of the allelochemical chlorogenic acid in sunflowers grown under varying levels of phosphate deficiency. Potassium and magnesium deficiencies produced similar increases in the concentration of scopolin in tobacco plants (Armstrong et al., 1971). Water stress can also increase allelochemical production (Fisher et al., 1990; Melkiana, 1992; Reigosa & Pedrol, 2002).



### *Temperature*

Unfavourable temperature regimes can affect both the rate of production of allelochemicals and their absorption by receiver plants. Glass (1976) found that barley seedlings subjected to a mix of phenolic acids showed reduced root growth at all temperatures, but the effect was most pronounced at high and low temperatures. Similarly, Einhellig & Eckrich (1984) reported that sorghum and soya bean growth showed the greatest reduction at temperatures close to the upper tolerance limits for these species, while Koeppel et al. (1970) found that chilling tobacco plants led to increases in chlorogenic acid concentrations of up to five times that found in unchilled plants.

### *Plant age*

The rate of production of allelochemicals may vary within an individual plant according to its life stage (Wardle, Nichol森 & Rahman, 1993). For example, Schumacher et al. (1983) found that wild oat (*Avena fatua*) became allelopathic to spring wheat crops at the four leaf stage with the release of vanillic and scopoletic acids. In the case of *Parthenium hysterophorus*, a highly invasive weed of warm climates, Kanchan & Jayavachandra (1979) found that allelochemical release was at its highest at

the rosette and flowering stages of the lifecycle. Walnut trees were reported by Ponder & Tandros (1985) to require an establishment period of over 15 years before any allelopathic effects were detectable. However, in the case of Sudex, an interspecies hybrid sorghum, Weston et al. (1989) found that allelopathic potential declined with age.

### *Pest and disease attack, herbivory*

It is now well established that plants respond to disease attack and herbivory by increasing their production of defence chemicals (Fraenkel, 1959; Harborne, 1987; Swain, 1977). Once released, some of these may also be able to act as allelochemicals against competitor plants (Arimura, Matsui & Takabayash, 2009 ; Arimura, Shiojiri & Karban, 2010; Thelen et al., 2005; Xu, Wang & Luo, 2005).

### *Light*

The quality and quantity of light received by plants is known to have an effect on production of allelochemicals. Rice (1984). For instance, Kato-Noguchi (1999) found that the release of the allelochemical 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) from the roots of germinating maize plants was stimulated by visible light and increased the plants' inhibitory effects on lettuce seedlings. Koeppe et al. (1969) reported an

increase in the allelopathic compounds scopolin and caffeoylquinic acids in tobacco and sunflower plants which had been exposed to increased levels of ultra violet radiation. Long photoperiods have also been found to increase the allelopathic potential of plants such as rock rose (*Cistus ladanifer*) by stimulating the production of the allelopathic compounds in its leaves (Chaves Lobón et al., 2002).

For a plant growing outdoors, facing a number of biotic and abiotic challenges, it is possible that many of the factors described above will act as multiple stressors on the plant in a way that may lead it to increase its production of potential allelochemicals (Kong, Hu & Xu, 2002). Laboratory or greenhouse culture, by contrast, may reduce allelochemical production due to the unusually favourable conditions usually provided by the researchers. For example, Woodhead (1981) reported that although the levels of phenolics in laboratory and field-grown sorghum plants followed similar patterns, the values for the field grown plants were consistently much higher than the corresponding ones grown in the laboratory.

### **2.3 Phytoalexins and Phytoanticipins**

In instances where defence compounds are synthesised and exuded following microbial infection of plant tissues, they are referred to as phytoalexins (Müller & Borger, 1940). Phytoanticipins by contrast, are

released prior to any elicited response to environmental challenges and may in fact help to neutralise such challenges (VanEtten et al., 1994 ).

Phytoalexins and phytoanticipins produced by plants are likely to be released from their roots into the surrounding soil (Dakora & Phillips, 1996) or sloughed from root border cells (Hawes et al., 1998; Hawes et al., 2000 ). This places them directly in the rooting zone of competitor plants and may facilitate their action as allelochemicals; the compounds associated with pest/ pathogen resistance and with allelopathy are not mutually exclusive and some compounds may fall into both categories (Field, Jordán & Osbourn, 2006). Among the compounds described as phytoanticipins are saponins, tannins, terpenes and flavonoids; phytoalexins include phenylpropanoids, alkaloids, terpenes, coumarin glycosides and glucosinolates. All of these groups have been implicated in allelopathic interactions (Rice, 1984).

The categorisation of compounds as either phytoanticipins or phytoalexins should be treated with some caution, however. There is evidence to suggest that some compounds can act in both ways, depending on circumstances - for example certain isoflavonoids found in legumes (Dakora & Phillips, 1996).

Over 350 phytoalexins have been characterised from over 100 plant species, including both monocots and dicots (Guest & Brown, 1997).

## **2.4 Mode of operation of allelochemicals**

It is likely that allelochemicals released from plants often consist of mixtures of different compounds, making the isolation and determination of each compound and its exact effects problematic (Einhellig, 1995). Nevertheless, some discussion of the mechanisms by which their effects are believed to be achieved is warranted. Most allelopathy studies concentrate on visible signs of changes to the receiver plant. These are, as noted by Winter (1961), outward manifestations of primary changes occurring at the molecular level.

There are two main modes of action – indirect and direct and (Rizvi et al., 1992).

### **2.4.1 Indirect**

Indirect modes of action include alteration of nutrient status of the soil and effects on resident populations of micro-organisms, insects and nematodes (Callaway & Howard, 2007; Klironomos, 2002; Stinson et al., 2006; Zhang et al., 2009).

### **2.4.2 Direct**

Direct modes of operation include effects on plant metabolism and growth.

A very wide range of sites and processes can be directly affected by allelochemicals (Rizvi et al., 1992; Wink, Schmeller & Latz-Bruning,

1998). Among those studied by allelopathy researchers to date are changes to transpiration rates, water utilisation, leaf expansion and the cell cycle (Blum & Gerig, 2005); reduction in Photosystem II (PSII) efficiency (Zhu et al., 2010); altered nutrient uptake (Bhowmilk & Doll, 1979) changes to dark respiration and adenosine triphosphate (ATP) synthesis (Inderjit & Duke, 2003; Weir, Park & Vivanco, 2004); impaired phytohormone metabolism (Bogatek & Gniazdowska, 2007); altered gene expression (Bais et al., 2003); changes to membrane permeability (Ding et al., 2007) and the production of reactive oxygen species (ROS) (Bais *et al.*, 2003; Bogatek & Gniazdowska, 2007; Weir, Park & Vivanco, 2004).

## **2.5 The challenges in establishing proof of allelopathy**

As a discipline, allelopathy has been the subject of considerable scepticism regarding methodological shortcomings and flawed conclusions based on laboratory tests, with little ecological relevance (Harper, 1975; Inderjit & Weston, 2000; Stowe, 1979). Perhaps the most notorious example of the misattribution of allelopathy to complex ecological phenomena was that of the work of C.H. Muller (Halsey, 2004). Muller had observed the spacing of desert annuals and shrubs in Colorado and concluded that their distributions were due to the effects of allelopathic interference on germination, which was confirmed by tests of leaf extracts on seedling germination (Muller, 1953). In a later study on

the ecology of the soft chaparral of southern California, he concluded that San Luis purple sage (*Salvia leucophylla*), coastal sagebrush (*Artemisia californica*) and chamise (*Adenostom afasciculatum*) produced volatile terpenes which affected the growth of competitors and was responsible for the characteristic mosaic-like pattern of plant stands with surrounding bare zones. Bioassays using these species showed that they produced volatile terpenes, which both inhibited plant growth and could also be adsorbed by the soil (Muller, 1966). Bartholomew (1970) found that, in contrast to Muller, the bare areas were successfully colonised by annuals, irrespective of the presence of terpenes, as long as the effects of seed predation by birds and small mammals and herbivory by rabbits was prevented by the construction of wire exclosures. Bartholomew also considered the concentrations of terpenes used in Muller's laboratory studies as excessively high and unrepresentative of those found in the field, a common criticism levelled at allelopathy studies.

As a result of these and other revelations, many plant ecologists concluded that allelopathy was not a significant factor in ecological interactions. Most notably, J L Harper (1977) in his highly influential book, *Population Biology of Plants*, devoted considerable space to a critique of allelopathy in the chapter on plant interactions. He systematically dismantled the claims of allelopathy researchers and questioned the relevance of laboratory studies to conditions pertaining in the field.

Harper's scepticism was encapsulated in the following sentence: "Almost any species can, by appropriate digestion, extraction and concentration, be persuaded to yield a product that is toxic to one species or another." He continued by providing additional arguments against the existence of allelopathic interactions between plants. Firstly, plants have been shown to evolve tolerance to environmental pollutants such as heavy metals and herbicides within measurable timescales, which suggests that they would be just as likely to evolve tolerance to allelochemicals produced by themselves or other competitors. Secondly, complex organic molecules, when liberated in the soil, undergo rapid degradation by microbes. As allelochemicals are themselves complex organic molecules, there is no reason to suppose that they would behave differently under similar conditions.

Finally, he stated that allelopathy research "has greatly widened our imagination but not contributed much to our understanding."

Two decades later, Romeo (2000) voiced his concerns about the standard of allelopathic research, citing the 40% rejection rate of allelopathy papers as significantly higher than those concerning other areas of chemical ecology. Among the issues he raised were the use of excessively high concentrations of allelochemicals, the limited range of test species used and the failure to identify the chemical structures of putative allelochemicals. He also criticised allelopathy researchers for



failing to distinguish between phytotoxicity – the negative effect of plant extracts, leachates or derivatives on the germination and growth of receiver plants with allelopathy – the demonstrable alteration of the growth of one plant by chemicals released by another into the soil.

In an attempt to avoid some of the inconsistencies outlined above, Fuerst & Puttnam (1983), building on suggestions made by Harper (1977), proposed that allelopathy studies should be confirmed using a modified version of Koch's Postulates, which are used in the identification of disease organisms. These require that:

- a) An allelopathic interaction should be demonstrated using suitable controls
- b) The causative chemicals should be isolated and tested against species that were previously affected
- c) When the isolated chemicals are added to the system, they should demonstrate similar toxic effects to the original experiments
- d) The quantity of putative allelochemical released into the environment should be measured and its presence should also be detected within the receiver plant.

Williamson (1990) questioned the validity of this approach, citing two objections to the adoption of Koch's Postulates when studying

allelopathic interactions: Firstly, micro-organisms are self-replicating and when introduced into a suitable host are capable of increasing in number and generating disease symptoms. Allelochemicals, by contrast, must be continually released by the donor plant in suitable quantities to generate an inhibitory effect. Secondly, the direct relationship between disease causing organisms and their hosts is more easily established than in allelopathic interactions. It is possible, for example, that most allelopathic effects are generated by chemical mixtures with complementary actions on the receiver plants (Einhellig, 1986).

Blum (2011) offered a comprehensive list of criteria which he believed were generally accepted by researchers engaged in allelopathy research:

- a) Patterns of stimulation or inhibition of plants on other plants must be shown
- b) The observed patterns cannot be solely explained by physicochemical modification of the environment (other than promoters and/or or inhibitors), utilization of substances as sources of nutrients, carbon and/or energy, transfer through mycorrhizae and/or root grafts and/or biotic factors such as resource competition, herbivory or disease

- c) The putative allelopathic plants or their residues must produce/ contain and release organic substances into the environment that will ultimately be capable of stimulating or inhibiting the function or growth of associated plants.
- d) The affected plants must come into contact with and interact with the organic promoters or inhibitors produced directly or indirectly (e.g. modified by microorganisms) by an allelopathic plant.
- e) The organic promoters and inhibitors must be at sufficient concentrations and be present for sufficient length of time to modify plant function and growth of receiving plants either directly (e.g., impact on root membranes and/or cell process) or indirectly (e.g., impact on nodule or mycorrhizae formation, development, and or function).

## **2.6 Types of experiments used to identify allelopathic effects**

Given the difficulties associated with providing reliable evidence of allelopathic effects outlined above, a number of different experimental approaches have been adopted by allelopathy researchers, ranging from simple bioassays, to more elaborate attempts to separate resource competition from allelopathic interference. Some of these are described below.

### **2.6.1 Bioassays**

The term bioassay as used in allelopathy studies has been described as body of procedures in which the amount or strength of an agent or stimulus is determined by the response of a subject (Hubert, 1992). The most commonly used bioassays in allelopathy research consist of seed germination rate, radicle elongation and greenhouse experiments involving the application of leachates or extracts from putative allelopathic plants to the soil of pot grown plants.

#### ***Germination bioassay***

Field studies on germination remain uncommon, while laboratory studies often do not involve any soil whatsoever and are generally used for broad scale screening of plants for their allelopathic potential. Nevertheless germination bioassays require small volumes of solution and are simple and fast (Leather & Einhellig, 1986).

#### ***Radicle elongation bioassay***

Radicle elongation is usually more sensitive than germination, because elongation depends on cell extension only - germination depends on cell

division and elongation. Compared to a germination bioassay, radicle elongation is more sensitive to certain groups of allelochemicals, for instance phenolics (Rasmussen & Einhellig, 1977).

Such bioassays are a quick, and relatively inexpensive screening method for potential allelopathic plants but do not provide definitive evidence of allelopathy in the field, because, as Inderjit & Callaway (2003) stated, “allelopathy is better demonstrated through experiments in which a toxic product is shown to be released from the putative aggressor, and arrives at the putative victim in functional concentrations under reasonably natural conditions”.

### *Soil bioassays*

Soil bioassays usually involve the addition of the putative allelochemical in the form of plant residues, leachates or extracts in order to simulate the release that would occur under natural conditions (Bhowmilk & Doll, 1984; Inderjit & Dakshini, 1992). The growing medium used should be similar to the soil in which the target plant is growing as naturally occurring microbes. In addition to liberating nitrates and other soil nutrients, these can also enhance allelopathic effects by the transformation of allelochemicals to more toxic forms, as has been shown in the case of actinomycetes (DeFrank & Putnam, 1985).

### *Stair-step pot designs*

More sophisticated experimental designs exist. For example, the effects of root competition and root exudation or leaching can be separated by the use of a stair-step apparatus in which donor plants are grown in pots above the receiver plants (Bell & Koeppe, 1972; Tang & Young, 1982; Viard-Cretat et al., 2009). Donor and receiver plants are linked by tubes along which leachates or exudates from the donor plants pass to the receiver plants. The root systems and above ground parts of the two types of plants are physically separated from each other by a suitable distance, thereby eliminating the confounding effects of nutrient, water and light availability.

### **2.7 Use of activated charcoal in allelopathy experiments**

There is an increasing recognition among allelopathy researchers that the direct action of allelochemicals on competitor plants is the exception, rather than the rule in allelopathic interactions and that most effects are indirect ones on the ecological milieu of the soil (Inderjit & Mallik, 2002; Inderjit & Weiner, 2001; Wardle et al., 1998). In order that comparisons can be made between living plants, a non-destructive means of neutralising the effects of allelochemicals while leaving the other properties of the soil unchanged is therefore desirable. This type of procedure would help to avoid the criticisms of ecological irrelevance that have been frequently levelled against standard bioassays (Inderjit &

Dakshini, 1995; Inderjit, Kaur & Dakshini, 1996; Inderjit & Weston, 2000). Activated charcoal (or carbon), (AC) is well known for its propensity to adsorb low molecular weight organic compounds such as those commonly held to be allelochemicals (Cookson Jr., 1978). It also has low affinity for inorganic electrolytes (Cheremisinoff & Ellerbusch, 1978) and has been used in a substantial number of allelopathy studies dating back two decades (Callaway & Aschehoug, 2000; Cipollini, McClain & Cipollini, 2009; Cipollini & Schradin, 2011; Ridenour & Callaway, 2001; Viard-Cretat et al., 2009; Zackrisson & Nilsson, 1992).

The use of activated charcoal has not gone unchallenged, however. Lau et al. (2008) found that the addition of AC to potting media affected both nutrient availability and plant growth. Viard-Cretat et al. (2009) reported that activated charcoal had unexpected effects on fertility and these effects were complex and dependent on the species involved.

## **2.8 Measurement of allelochemicals in situ**

Attempts have been made to measure the flux rates of allelochemicals in the undisturbed rhizosphere of allelopathic plants using polyurethane foam plugs (Weidenhamer, 1996) and polydimethylsiloxane (PDMS) tubing (Weidenhamer, 2005; Weidenhamer, 2007) with varying results. The chemicals sorbed by the PDMS were eluted with methanol and then analysed using High Performance Liquid Chromatography (HPLC)

techniques. Consistent success in this area would remove one of the last remaining experimental obstacles to the study of allelopathy (Weidenhamer, 1996).

## **2.9 Effects of plant density on allelochemical uptake**

Density-dependent responses to herbicide were shown by Thijs et al. (1994) in an elegant target-neighbour design where increasing densities of maize plants were grown around single soya bean plants. The herbicide atrazine was then applied. Neighbour density had a powerful influence on the response of the soya beans to the toxin. The dry mass of soya beans increased with increasing maize density, which ran counter to the predicted effects of resource competition. The authors suggested that density-dependent competitive uptake by maize roots, leading to soil detoxification, was responsible for this result. Weidenhamer *et al.* (1989) showed that tomatoes grown at varying densities in soil collected from beneath black walnut trees (*Juglans nigra*) suffered less phytotoxic inhibition at higher densities than at lower densities. They suggested that this was due to each tomato plant receiving a reduced share of the available phytotoxins, indicating a density-dependent relationship. Experiments using a variation of these methods could prove useful in ascertaining whether *Impatiens glandulifera* demonstrates any allelopathic effects on competitor species when grown in soil.



## **2.10 The role of allelopathy in plant invasions**

It has been suggested for some time that the exudation of by-products of primary metabolism (allelochemicals) may provide an alien plant species with a competitive advantage when it invades a new community (Rabotnov, 1982; Willis, 1999).

In a frequently cited breakthrough study, Callaway & Aschehoug (2000) reported that *Centaurea diffusa*, an introduced weed in North America had a more powerfully negative effect on the growth of native North American grass species than it did on closely related grasses from its home range. The success of *C. diffusa*, they postulated, was due to differences in the effects of its root exudates and how these exudates affected competition for resources.

In a later related paper, Callaway & Ridenour (2004) proposed the “Novel Weapons Hypothesis”. They argued that exotic invaders may produce chemicals which function as allelopathic agents, or as mediators of new plant-soil microbial interactions. Plants or soil microbes which have co-evolved are not so sensitive to these chemicals because of long-term adaptation, whereas they may inhibit the growth of neighbouring plants in invaded communities. They stressed that this theory required further investigation to establish its validity.

Other studies indicate that root exudates may be able to both initiate and manipulate the biological and physical interactions between roots and soil organisms, and therefore act as mediators between the roots of different species and their attendant microbes (Bais et al., 2004). Cappuccino & Arnason (2006) reported that highly invasive alien species in North America were more likely to have potent, previously unrecorded, secondary compounds than less invasive exotics.

The potential of allelopathic interactions to facilitate the spread of invasive alien plant species has now been investigated in a wide range of species across several continents: in Europe, Csiszár (2009); in North America, Cappuccino & Arnason (2006); Callaway & Vivanco (2007), Dorning & Cipollini (2006); in Australia, Ens, French & Bremner (2009); in Asia, Niu et al. (2007), Yang et al. (2007).; in Africa, Fatunbi et al. (2009).

However the topic has received very little attention in the UK thus far, perhaps due to the influence of J.L. Harper (Fitter, 2003).

### **2.11 Evidence for allelopathic effects in *Impatiens***

In a recent study of six invasive forbs in Switzerland, Scharfy et al. (2011) found that substrates which had previously grown *Impatiens glandulifera* showed the strongest allelopathic effects on shoot biomass production of the grass *Dactylis glomerata*. *I. parviflora* caused the second highest levels of inhibition. The authors suggested that the allelopathic behaviour of

these two alien *Impatiens* species may contribute to their success as invaders of perennial vegetation in Central Europe, an unusual ability among annual species in that area (Thompson, Hodgson & Rich, 1995). Allelopathic inhibition by *Impatiens glandulifera* exceeded that of *Phragmites australis*, the most allelopathic of twelve native species which were also studied and one which has been investigated for its allelopathic effects on several occasions (Bains et al., 2009; Qian, Fu-Geng & Pei, 2007; Rudrappa et al., 2007).

In a personal communication, Fujii (2007) reported strong inhibitory effects of root exudates of *I. balsamina* plants using the so-called “Plant Box Method” (Fujii et al., 2007). Intact root systems were encased in low temperature agar and a combined lettuce germination and radicle bioassay carried out. Radicle growth was progressively inhibited by proximity to the root system, due to the diffusion of allelochemicals from the plant roots. The causal compound, however, was not identified.

## **2.12 Naphthoquinones, a potential chemical basis for allelopathy in *I. glandulifera***

The genus *Impatiens* contains a range of naphthoquinones, a family of phenolic compounds with potent anti-microbial and allelopathic properties (Little, Sproston & Foote, 1948; Panjchayupakaranant et al., 1995; Sakunphueak & Panichayupakaranant, 2010). These are oxygen-

derivatives of naphthalene, often produced via the shikimic acid pathway (Babula et al., 2009b). In addition to the Balsaminaceae, their presence has been recorded in a number of other angiosperm families, such as Juglandaceae, Droseraceae and Plumbaginaceae as well as in fungi, algae and actinomycetes (Babula et al., 2009a). *Impatiens balsamina*, rose balsam, the most thoroughly investigated species, has been used for centuries in traditional Chinese medicine for its antimicrobial, anti-rheumatic and anti-tumour properties (Yang et al., 2001). *Impatiens capensis* sap has been used as a topical treatment for poison ivy rash (Long, Ballentine & Marks, 1997).

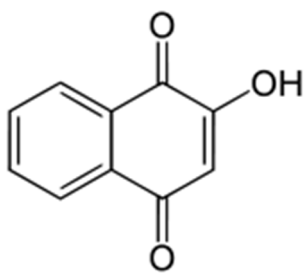
Seeds of *I. balsamina* have been found to contain novel antimicrobial peptides (Tailor et al., 1997), as well as baccharane glycosides (Shoji et al., 1994). They are also known to contain large quantities of linolenic and  $\alpha$ -parinaric acid, in addition to lesser quantities of other fatty acids (Gunstone, 1996). Data on the biochemistry of *I. glandulifera* seeds is currently lacking, as is the case for the other species found in the UK.

Little et al. (1948) isolated 2-methoxy-1, 4-naphthoquinone (2MNQ) from *I. balsamina* flowers, while Foote et al (1949) recorded that 2MNQ was a potent inhibitor of fungal spore germination and proposed that some kind of blocking action on the carboxylase system was responsible for its inhibitory effects. They also suggested that the substitution of

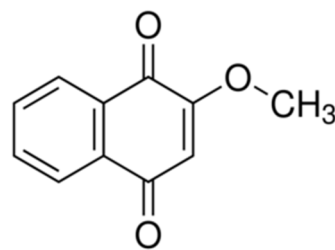
methoxy by hydroxyl groups would reduce the effectiveness of the naphthoquinone as a spore inhibitor.

Panjchayupakaranant et al. (1995) isolated two naphthoquinones, 2-hydroxy-1, 4-naphthoquinone (lawsone) and 2MNQ from root cultures of *Impatiens balsamina*, along with coumarin derivatives and a sterol. In addition, 2MNQ was found to have inhibitory effects on a range of bacteria and fungi in a bioassay carried out by Yang et al. (2001).

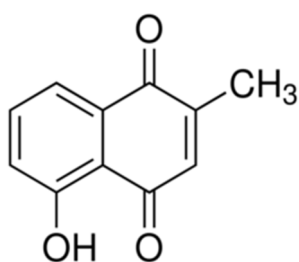
*Impatiens glandulifera* contains both lawsone and 2MNQ (Lobstein et al., 2001). Using reversed phase HPLC analysis of the aerial parts of *I. glandulifera*, the same authors noted that these naphthoquinones were found in much higher quantities in *I. glandulifera* than in the other native and naturalised species of balsam found in Europe, namely *I. noli-tangere*, *I. capensis*, and *I. parviflora*. In their analysis, *I. glandulifera* was the only species in which the levels of 2MNQ were higher than those of lawsone, with approximately four times as much 2MNQ as lawsone. Naphthoquinone production was shown to vary with plant age, declining markedly after flowering.



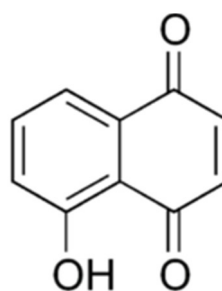
2-hydroxy-1,4-naphthoquinone (Lawsone)



2-methoxy-1,4-naphthoquinone (2MNQ)



5-hydroxy-2-methyl-1,4-naphthoquinone  
(Plumbagin)



5-hydroxynaphthoquinone  
(Juglone)

Figure 2.3. Naphthoquinone structures

Little research has been carried out to investigate the role of these naphthoquinones in *Impatiens* ecology (Krejčová *et al.*, 2007) although juglone, (5-hydroxy-1,4 naphthoquinone), an isomer of lawsone, has been studied for its potential as a powerful allelochemical for nearly ninety years (Chobot & Hadacek, 2009; Ercisli *et al.*, 2005; Massey, 1925; Rietveld, 1983). Produced by walnut (*Juglans*) leaves and roots, it is credited with credited with both phytotoxic (Rietveld, 1983) and antimicrobial properties (Clark, Jurgens & Hufford, 1990).

In experiments carried out by Vrchotová et al. (2011), various extracts from *Impatiens* species were found to be phytotoxic, with *I. glandulifera* having the greatest inhibitory effect. In their experiments, *Impatiens* extracts were much more phytotoxic than those obtained from Japanese knotweed (*Fallopia japonica*) and other closely related knotweed species and hybrids. Vrchotová et al. (2005) noted that naphthoquinones accumulated in parts of *Impatiens* plants other than the leaves; their results suggested that they were dominant substances in root extracts. They also stated that the high levels of naphthoquinones found in *I. glandulifera* were “interesting mainly from the perspective of invasive behaviour of this alien species”. Babula et al. (2006) reported the presence of another naphthoquinone, plumbagin, in both *I. glandulifera* and *I. parviflora*. The mode of action of the naphthoquinones juglone and plumbagin was elucidated by Babula et al. (2009b), who found that they generated reactive oxygen species which play an important role in programmed cell death and were also able to disrupt the mitochondrial respiratory chain. It is likely, therefore, that lawsone and 2MNQ have similar effects.

In addition to phytotoxic effects, naphthoquinones such as lawsone, juglone and plumbagin have been shown to have a suppressive effect on the growth of a wood decaying fungus, *Pleurotus sajor-caju* (Curreli et al., 2001). Lawsone and plumbagin, both found in *Impatiens*

*glandulifera*, were more potent inhibitors than juglone. Lawsone was found to be the most effective inhibitor of mycelial growth, with concentrations above 200 ppm killing the mycelium. Such findings suggest that root exudates from *I. glandulifera* might have effects on the growth and development of soil fungi, including saprophytes, parasites and mycorrhizae. *I. glandulifera* is described as rarely forming mycorrhizal associations (Harley & Harley, 1987).

Brigham et al. (1999) were able to show that the naphthoquinones produced by hairy root cultures of *Lithospermum erythrorhizon* had anti-fungal and bacterial inhibiting properties and functioned “as preformed and inducible microbial inhibitors regulated in a cell-specific manner to maximise the effect of highly toxic substances with minimal expense to the plant”. When the hairy root cultures were exposed to fungal elicitor factors or copper sulphate, coloured naphthoquinone pigments were exuded from epidermal and root hair cells. Babula *et al.* (2006) reported that a reduction in the pH of the agar growing medium led to the redirection of naphthoquinones from the tissues of Venus fly trap plants (*Dionaea muscipula*) into the agar. It is possible that low pH or other elicitors might lead to the increased release of naphthoquinones from *I. glandulifera* and other invasive *Impatiens* species and these might then act as allelochemicals in the rhizosphere of competing plants.



In a similar vein, von Kiparski et al. (2007) found that the roots of black walnut trees (*Juglans nigra*) released measurable quantities of juglone into the rhizosphere and that this could accumulate to inhibitory levels in the case of soils with low microbial activity. The fate of the naphthoquinones produced by *Impatiens* plants has not yet been studied in natural systems, but research using *Impatiens balsamina* root cultures suggests that the quantities released could be sufficient to affect microbial processes or the growth of plant competitors (Babula et al., 2009b).

### **2.13 Taxonomic basis for *I. glandulifera*'s invasiveness**

Examination of the cladogram of the genus *Impatiens* produced by analysis of Internal Transcribed Spacer (ITS) sequences indicates that *I. glandulifera* is phylogenetically quite distinct from the other balsam species found occurring wild in Britain and mainland Europe (Yuan et al., 2004). According to these authors, *I. noli-tangere* and *I. capensis* are both located in clade 10, *I. parviflora* is in clade 14 and finally *I. glandulifera* is placed in clade 15. These different phylogenies may provide the basis for the underlying differences in biochemistry between the species, in particular the quantity and variety of naphthoquinones present. See Figure 2.4.

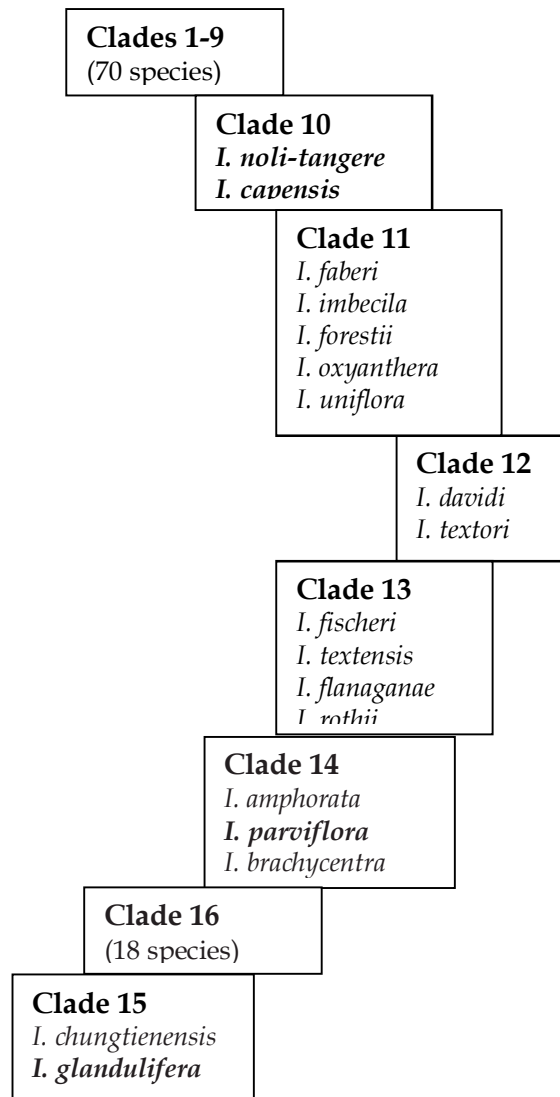


Figure 2.4. Simplified cladogram of Asian *Impatiens* species based on ITS sequences (Yuan et al. 2004) showing the relative locations and affinities of species. Those in adjacent clades have closer affinities than those further apart. Species highlighted in bold occur in the UK. *I. noli-tangere* and *I. capensis* are very closely related to each other, but only distantly related to *I. parviflora*. *I. glandulifera* is more distantly related still. For the purposes of clarity, tree branches have been omitted.

## **2.14 Rationale for the present study**

The synchronous, early germination and gregarious nature of *Impatiens glandulifera* often leads to the formation of dense stands of monospecific vegetation (Beerling & Perrins, 1993). It is conceivable, however, that in addition to the usual competitive mechanisms of light, water and nutrient

acquisition, *I. glandulifera* plants may exert an allelopathic effect on their competitors by the production of toxic leaf litter or root exudates. This aspect of *I. glandulifera*'s biology deserves further investigation as an additional mechanism by which it (and other *Impatiens* species) might be able to dominate sites where they grow.

There is certainly some evidence to support this as a possibility - notably the presence of large quantities of biologically active naphthoquinones in *Impatiens* tissues and also the generally supportive results of the limited number of studies carried out to date.

This study aims to assess *I. glandulifera*'s allelopathic potential throughout its lifecycle and compare this potential with other, less invasive species of *Impatiens* which grow wild in the UK. *I. glandulifera* is in several ways ideal for such a study. It is biochemically and taxonomically distinct from other native and alien *Impatiens* species as well as a much more aggressive invader. As an annual with a very limited seed bank, it needs to colonise or recolonise sites every year, so any allelopathic effects must become apparent during the lifecycle of the plant or shortly thereafter. It exhibits early and synchronous germination, making it a useful species for the study of density dependent phytotoxic effects as might be experienced by later germinating competitors.

As the number of studies exploring allelopathy in invasive *Impatiens* is so low, it makes sense to begin with pilot investigations into

the phytotoxicity of leachates and root exudates, as these are the two of the most important mechanisms by which allelochemicals are released; subsequent investigations into the effects of competition with other species will also be carried out.

Methodological development will be an important focus for these investigations so that appropriate experimental protocols can be devised to assist in the process of exploring *I. glandulifera*'s allelopathic potential. These will be described in the separate chapters devoted to the individual protocols used. Whereas most studies concentrate on the allelopathic effects of a plant at a specific stage in its lifecycle or use a specific organ as the source of the putative allelochemical, this study develops a more complete picture of the allelopathic potential of *Impatiens glandulifera* throughout its lifecycle. Comparisons of *I. glandulifera* with other invasive species will also be undertaken where possible in order to explore the possibility of differing allelopathic potential as a mechanism to explain the varying success of the species as invaders.

### **3. Materials and Methods**

#### **3.1 Introduction**

This chapter describes the general cultivation materials and methods that underpin this study. For brevity's sake they are mentioned here once rather than repeated in subsequent chapters. The conditions described were used for the production of the majority of plants used in these studies. Other experiment or chapter-specific methods are described in the appropriate chapters. Details are also provided of some of the problems encountered and subsequent steps taken to remedy problems.

#### **3.2 Location of study.**

All work was conducted within the laboratories and temperature-regulated growth rooms of the University of Plymouth, Drake Circus, Plymouth, Devon, UK, or in greenhouses and outside beds at the University's Skardon Gardens, located a short walk from the main campus at Plymouth.

### **3.3 Plant Production**

#### **3.3.1 Seed Collection**

At the time the study was conducted, there were no recorded colonies of *I. parviflora* or *I. noli-tangere* in the vicinity of the University of Plymouth. As both these species (as well as *I. glandulifera*) occur in the Lake District, Cumbria, seed was collected from there in autumn 2007. John Hooson, the National Trust's ecologist for the North Western region, provided information about suitable locations and granted permission to collect seeds of *I. noli-tangere* from National Trust owned land.

*I. parviflora* seeds were collected by Lake Windermere, Grid reference: SD3895 and *I. noli-tangere* from woodland adjacent to Coniston Water, Grid reference SD 3093.

No seeds of *I. capensis* were obtained during 2007 or 2008 due to ill health of the supplier, (Raymond. J. Morgan, holder of the National Impatiens Collection, West Glamorgan). When samples were obtained from two separate suppliers in 2009 and 2010 both failed to germinate.

Unlike the previous species, *I. glandulifera* was locally abundant in the region surrounding Plymouth and seed collection was successfully carried out at a number of sites over several years: Berryman's Marsh, Dartington, Totnes, Devon (map OL20, grid reference: 799617) September 2007, October 2008; Looe Valley, near Badham (map Explorer107, grid

reference 247587) 2009, 2010; Lostwithiel (map Explorer107, grid reference: 106025) 2009, 2010.

### **3.3.2 Seed storage and stratification**

As mentioned in Chapter One, seeds of *I. glandulifera* require a period of cold stratification before germination occurs (Mumford, 1988). This is also the case with the other species investigated, namely *I. parviflora*, *I. capensis* and *I. noli-tangere* (Coombe, 1956; Hatcher, 2003; Perglová et al., 2009). Rather than seed storage per se, the method used was one of holding the seeds until such time as their dormancy was broken and then using them in the studies described in subsequent chapters. Long term storage, involving fully imbibed seeds held at room temperature as described by Mumford (1988) for *I. glandulifera* was not attempted. The original intention was to produce seed crops from the study species grown indoors at regular intervals so that batches of seed could then be subjected to stratification at intervals, ensuring year round availability of seedlings and plants for the experiments described in the following chapters. Space restrictions, however, prevented this from being carried out.

Seeds of *I. glandulifera*, *I. parviflora* and *I. noli-tangere* gathered in September 2007 were initially stored on moistened blue laboratory paper in plastic boxes outdoors until November 2007. They were inspected on a

weekly basis and mouldy or otherwise damaged seeds were removed as they became obvious. In order to subject them to controlled stratification, they were then soaked in a 10% bleach solution for 10 minutes, rinsed 5 times with refrigerated, sterile distilled water and stored on moist filter paper in Petri dishes. The unsealed Petri dishes were placed within airtight plastic boxes and put in a refrigerator in the dark at a temperature of around 4° C. Seeds were inspected every two weeks and damaged and mouldy specimens were removed. In some cases the filter papers became discoloured and had a mouldy smell. In these cases they were discarded and the seeds were re-sterilised and placed on fresh filter papers.

In subsequent years, *I. glandulifera* seeds were gathered when ripe in the autumn, cleaned in the 10% bleach solution and then placed in a folded double ply sheet of moist laboratory roll so that both the top and bottom surfaces of the individual seeds were kept fully moistened. The folded sheets were placed in plastic boxes in the dark in a refrigerator at a temperature of 4°C. in order to break their dormancy requirements. They were checked every two weeks for signs of moulding, with affected seeds being removed.

### **3.3.3 Seed germination**

Germination of each species showed marked variation in timing: from the first batch of seeds collected in September 2007, *I. glandulifera* began



germinating in early February 2008. *I. parviflora* began germinating in March and *I. noli-tangere* in April. *I. capensis* seeds, when they were finally obtained, completely failed to germinate on several occasions, although they were freshly gathered prior to being stratified. Perrins, Fitter et al. (1993) reported the total failure of the germination of *I. capensis* and *I. noli-tangere* when the seeds were stored chilled at temperatures below 40°C prior to sowing. This figure presumably represents a typographical error; the more likely chilling temperature would be 4°C.

Problems with reliable germination of *I. noli-tangere* and *I. capensis* seeds have also been mentioned by others with experience of growing these species, including Hatcher (Hadacek, 2008; 2003; Morgan, 2009 ). In the case of *I. capensis*, however, Perglová et al. (2009) reported results which contradicted previous studies and communications. They found that *I. capensis* showed good germination in the laboratory and gave the highest percentage germination of the four species in a common garden experiment. Evans & Hughes (1961) report Coombe (1956) as stating that the seeds of *I. parviflora* require shallow immersion at 5°C for three months prior to germination. However, no such information is contained in the paper. For the purposes of this study, *I. parviflora* and *I. noli-tangere* seeds maintained in a moist condition on laboratory roll at 4°C were capable of germinating successfully without full immersion, albeit after a

longer stratification period than those of *I. glandulifera* (Perglová et al., 2009).

The initial delay in germination of *I. parviflora* and *I. noli-tangere* in 2008 prompted an investigation into their viability using tetrazolium chloride (TTC). Chilled, imbibed seeds were removed from the refrigerator and carefully bisected using a scalpel so that the embryos were themselves cut through. The bisected seeds were placed in a 0.1% TTC solution overnight at 20°C. Initially colourless, TTC accepts electrons from the mitochondrial electron transport chain of actively respiring tissues, reducing it to formazan, a pink compound which stains the tissues. The intensity of the staining is proportional to the respiration rate of the tissues. Examination of the embryos the following afternoon showed bright red formazan staining, indicating that the seeds were alive and actively respiring at the time of testing. The seeds were, therefore, assumed to be dormant.

Seeds of *I. glandulifera* collected and stored as described above at the beginning of October 2008 began germinating by the beginning of January 2009.

Seeds collected in November 2009 and stored at 4°C germinated in February 2010.

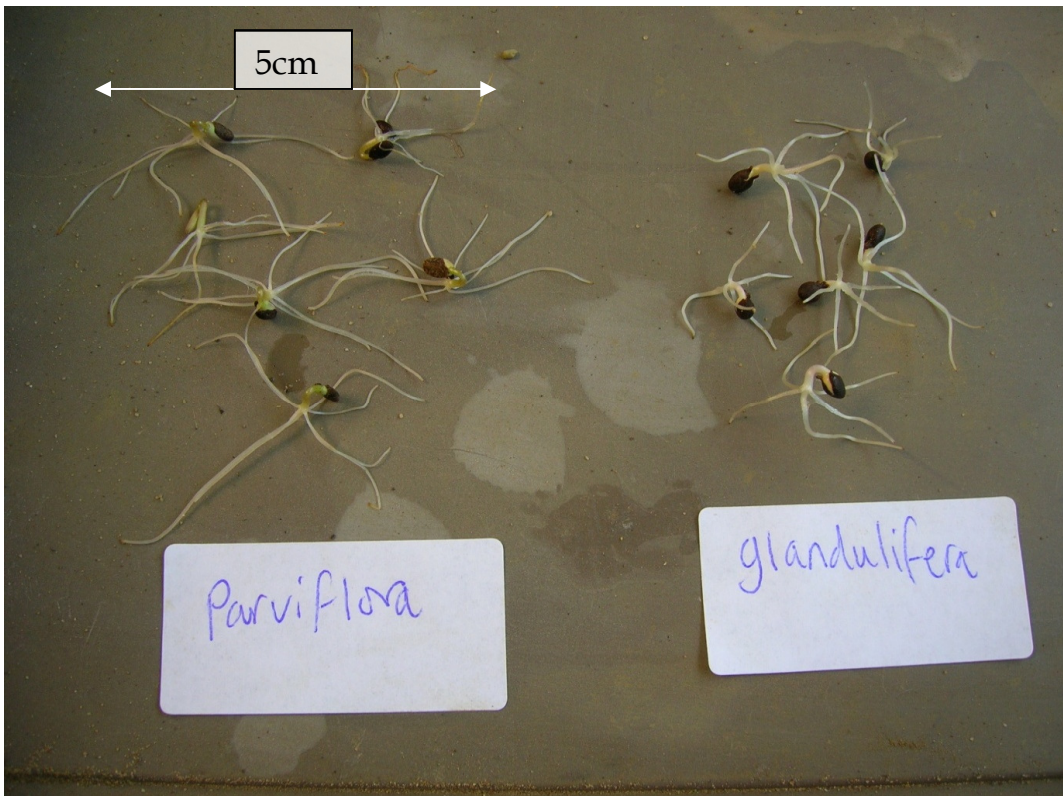


Plate 3.1. Seedlings of *I. parviflora* and *I. glandulifera*, showing characteristically branched root systems. Approximately 4 weeks after germinating at 4°C.



Plate 3.2. Germinating seeds of *I. noli-tangere*, showing numerous branching roots

### **3.3.4 Treatment of seedlings grown in sand**

#### ***Rationale***

For the purposes of the Plant Box Method described in Chapter 5, it was necessary to produce plants with intact, undamaged root systems, free of soil. In order to achieve this, plants were grown in a quartzite sand medium. Roots are unable to penetrate sand grains individually due to their hardness; they can however, grow successfully in the interstices between grains. With correct watering and fertilisation this leads to the production of a healthy root system resembling that of a plant grown in a soil based medium (Clark et al., 2011) More details of the cultivation methods can be found in Section 3.5. Following radicle emergence, germinating seeds were transferred from the Petri dishes to 9 cm square plastic pots, each with an approximate rooting volume of 280 ml. Pots were filled with slightly moist 3 mm graded quartzite horticultural sand (J Arthur Bowers, William Sinclair Holdings, Lincoln UK ) which was tested for pH prior to use and found to be neutral. The dry mass of the sand contained in each pot was approximately 400 g. To avoid excessive loss of sand during irrigation, the holes were sealed with a single layer of 'Enviromesh' (Agralan Ltd, Swindon, UK) a 1.35 mm aperture polythene mesh cut to fit the dimensions of the inside bases of the pots. This allowed for the free drainage of surface applied irrigation water through the drainage holes, whilst limiting the amount of sand washed out by the

same irrigation procedures. Four germinating seeds, with visible radicles, were planted approximately 1 cm from each corner of the pot, with their radicles facing down at a depth of about 5 mm below the surface of the sand (see Plate 3.3). Later growth, with fully expanded cotyledons and adult leaves can be seen in Plate 3.4.



Plate 3.3. Recently emerged seedlings of *I. glandulifera* growing in sand medium.





Plate 3.4. More advanced sand grown *I. glandulifera* seedlings at four leaf stage. 1 month after sowing.

In some instances the sand culture pots developed an algal and fungal crust. Microscopic examination revealed the presence of fungal hyphae and spores resembling those of *Rhizopus* sp. In subsequent experiments the sand was autoclaved before use, thereby eliminating pre-existing algal and fungal contaminants before the planting of *Impatiens* seeds.

### 3.3.5 Treatment of Seedlings grown in compost

Additional seedlings of *I. glandulifera*, *I. parviflora* and *I. noli-tangere* were planted individually in 40 cell module trays containing John Innes no 2 potting mix (J Arthur Bowers, William Sinclair Holdings, Lincoln UK), the medium suggested by Elias & Causton (1975) for the cultivation of *I.*

*parviflora*. These plants were then potted on individually into 90 mm plastic pots containing the same compost mix when the roots had completely filled the cells. However, this compost lacked structure and had a marked tendency to become waterlogged; some plants were lost through root rot as a result. In subsequent experiments a blend of equal volumes of John Innes no. 1 and a multipurpose compost (Westland, Sinclair Horticulture, Lincoln, UK) gave satisfactory results and allowed for adequate root development. Cuttings from the axillary shoots of *I. parviflora* and *I. glandulifera* plants which had succumbed to root rot were taken using a scalpel. These were placed in beakers of distilled water until roots had developed. They were then planted in the same compost mix and the majority rooted successfully.

After six weeks in the 90 mm pots, the plants were given a weekly feed of half strength dilute proprietary fertiliser (Miracle Gro (Scotts/ICL, Tel Aviv, Israel) as N:P:K 24:8:16) which was approximately the recommended rate for commercial bedding *Impatiens* of 150 mg L<sup>-1</sup> (Fischer, 2004). Plants receiving this fertilizer regime grew rapidly and their growth habit closely resembled that of plants occurring outside under natural light and temperature conditions.

### **3.4 Growing Conditions**

#### **3.4.1 Temperature**

Due to a lack of available greenhouse space during most of the study period, plants were grown under lights (see Section 3.4.2) in an air-conditioned room located in the Davy Building, University of Plymouth. The room's air temperature was set at 17°C. Plants were alternated between two light racks on a weekly basis. Moist sand temperatures were recorded as daily maxima and minima for a period of three weeks during March and April 2008 using two digital thermometers (Traceable, Novatech International Inc. Houston, Texas, USA) placed into same size pots containing moist sand located at either ends of the two growth light racks at a depth of 1 cm and results are shown in Figure 3.6. The left hand rack was referred to as Rack 1, the right hand one as Rack 2. The slight differences in temperature between the two racks may have been due to their positions relative to the door, which was opened frequently by the room's multiple users and the variable functioning of the fans in the air conditioning unit. There were, however, no significant differences between either the two mean maximum temperatures ( $p = 0.09$ ) nor the mean minimum temperatures ( $p = 0.28$ ) when they were compared using *t* tests.



Table 3.1. Sand temperatures ( $\pm$ standard deviations) recorded over a three week period in March/April 2008.

Mean Sand Temperature °C	Rack 1	Rack 2
Maximum	20.02 $\pm$ 1.13	19.83 $\pm$ 0.97
Minimum	16.05 $\pm$ 1.99	15.19 $\pm$ 2.00

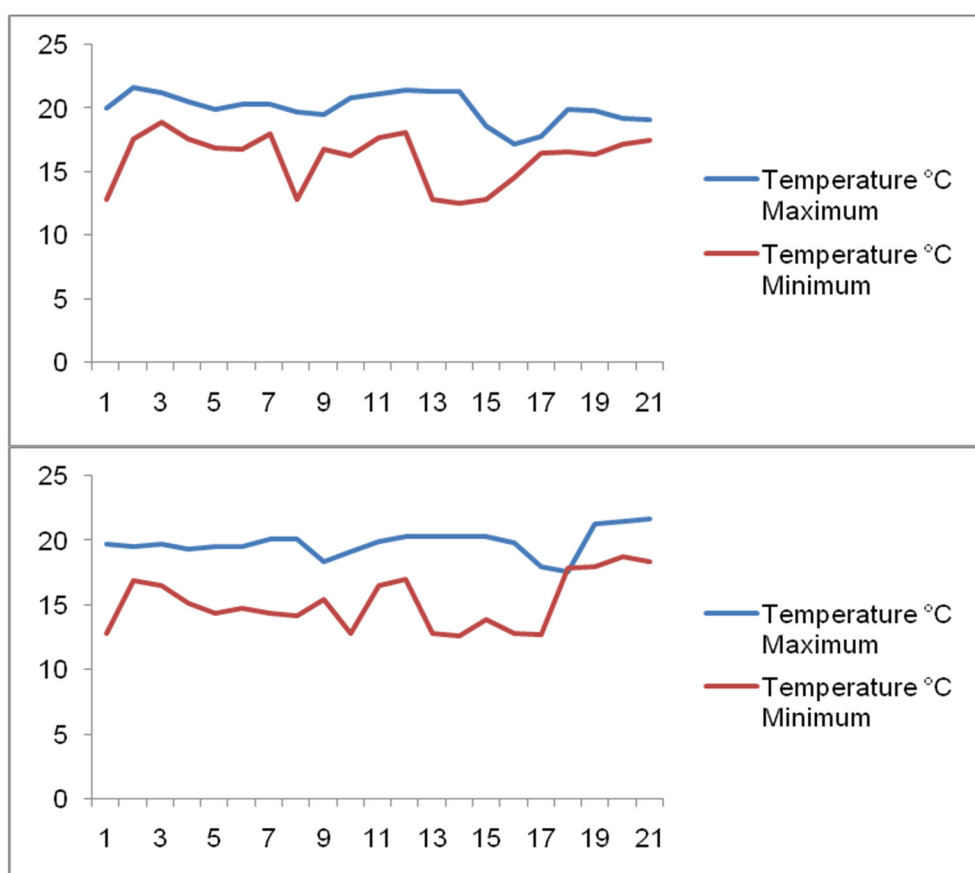


Figure 3.1 Maximum and minimum temperatures recorded in sand filled pots over a three week period in March/April 2008 Upper chart = Rack 1, Lower chart = Rack 2.

In February 2009, a limited amount of shared greenhouse space became available and some compost-grown plants were transferred there. A maximum-minimum thermometer located about 1.5 metres above the

floor in the greenhouse recorded maximum temperatures of 33°C and lows of 10°C during the cultivation period running from February to May 2009. The plants were, however, usually kept at a temperature of around 20-25°C during the day by means of opening the doors and vents on sunny days or keeping them closed when days were cloudy or cold. Night time temperatures around 10°C were maintained by means of a thermostatically controlled greenhouse heater. Photoperiod varied from 9.5 hours to 15.5 hours over the duration of the experiment. Details of the density dependent target-neighbour experiment carried out using these plants can be found in Chapter 7.

### **3.4.2 Illumination**

As mentioned briefly above, space restrictions meant that the majority of plants were grown under two adjacent light racks in a temperature controlled room. The racks consisted of 60 x 154 cm steel frames with light arrays set approximately 1 metre above the level of the pots. Each rack had the same light configuration, consisting of a mixture of 11 x 65W cool white, warm white and violet fluorescent tubes and 8 x 25W tungsten filament bulbs. The latter were prone to blow on occasion and were replaced as necessary. Photosynthetically active radiation levels (PAR) were measured at the level of seedling emergence using a Skye PAR meter (SKP 200, Skye Instruments Ltd, Llandrindod Wells, Wales,

UK). There was a slight variation between the two light racks, with the left rack giving a reading of  $60 \mu\text{molm}^{-2}\text{s}^{-1}$ , whereas the right rack gave a slightly higher reading of  $70 \mu\text{molm}^{-2}\text{s}^{-1}$ . This difference may have been a result of the latter's proximity to the window about 1m away. Mean PAR values ten times this can be obtained outdoors during the summer in Britain (Morecroft & Roberts, 1999), so these values are low; all the *Impatiens* species investigated during the course of this study are known for their ability to grow and flower successfully in shady environments, however (Andrews et al., 2005; Beerling & Perrins, 1993; Hatcher, 2003; Peace & Grubb, 1982).

### 3.4.3 Photoperiod

*I. glandulifera*, *I. noli-tangere* and *I. parviflora* are apparently day neutral plants which have not been reported as requiring specific day lengths to initiate key processes such as flowering (Beerling & Perrins, 1993; Hatcher, 2003; Hughes & Evans, 1963), although other species, such as *I. balsamina* are known to be short day plants (Poteau et al., 1997). Lights were set on 12/12 light/dark cycle using a timer switch. This illumination period was chosen to simulate the sort of ambient day lengths that plants might experience following germination and emergence in the spring. Plant habit, stem elongation and flowering appeared normal under this light regime.

#### **3.4.4 Relative humidity (RH)**

RH was measured with an RH85 Handheld thermohygrometer (Omega Engineering Ltd, Manchester, UK), with values falling between 45%–60% depending on the irrigation status of the plants. Highest values were obtained immediately following watering.

#### **3.4.5 Watering and fertilisation**

##### *Sand Culture*

Saturation capacity of the pots was determined by recording the volume of water required to saturate the sand and produce seepage through the drainage holes. This was found to be approximately 100 ml. Irrigations were carried out as required using distilled water to maintain the pots at about 75% of saturation capacity, around 75 ml per pot, as recommended by Smith et al (1983) as suitable for maintaining adequate moisture levels in sand cultures whilst avoiding anaerobic conditions in the rooting zones of the plants. The correct moisture content was estimated by feeling the weight of the pot and observing the moisture content of the sand just below the surface.

As plants grew larger, irrigation needed to be carried out on a daily or twice daily basis. When occasional unavoidable absences prevented

daily checking, the plants were irrigated to saturation capacity and the surface of the pots wrapped in aluminium foil to reduce water loss. Larger plants were wrapped in transparent plastic to reduce transpiration losses caused by the air conditioning unit.

A balanced commercial feed (Maxicrop Orchid Fertiliser, Maxicrop, Corby, UK with an N-P-K rating of 4.7:5.0:6.7 was diluted to half the recommended strength ( $2.5 \text{ ml l}^{-1}$ ) and added to the irrigation water once a week or every other irrigation according to the dryness of the pots. This provided a N supply of  $100 - 150 \text{ mg l}^{-1}$  which has been suggested as suitable for commercial production of bedding varieties of New Guinea *Impatiens* (Fischer, 2004). As the plants grew larger they were fed more frequently. Prior to application, the pH of the nutrient solution was adjusted to 6.2, as recommended for commercial *Impatiens* substrates (Fischer, 2004), by the addition of 1M HCl. pH readings were taken using a hand held pH meter (pHep, Hanna Instruments Ltd, Leighton Buzzard, UK), which was calibrated before use and at weekly intervals subsequently. Both *I. glandulifera* and *I. parviflora* appeared healthy using this fertilisation regime, with growth resembling that of plants growing outside.

In order to prevent loss of irrigation water, pots containing *Impatiens* plants were placed in shallow plastic trays. Excess water from

the pots drained into the trays, but usually did not form a continuous body of water around the bases of the plant pots.

### **3.5 Plant Development**

#### **3.5.1 Flowering and seed set**

Flowering of *I. glandulifera* and *I. parviflora* showed marked temporal differences. Some *I. parviflora* plants began flowering as little as 4 weeks after germination. By contrast, *I. glandulifera* plants showed no sign of flowering at 8 weeks, with the first flower buds present at 12 weeks after germination. The two species showed similar morphologies, with both undergoing a period of stem elongation and thickening, often with adventitious formation of stilt roots close to the base. This resulted in the development of unbranched, single stemmed plants. Flowering was initiated at the apex of the plants and was then followed by the development of axillary shoots bearing smaller racemes of flowers. Linked to the precocious flowering of *I. parviflora*, the resultant plants were shorter and had a bushier habit of growth, which led Evans & Hughes (1961) to comment that this species showed no apparent change in morphology when juvenile and reproductive phases were compared.

In the case of *I. parviflora*, seed pods developed, although there were no obvious pollinating insects. This ability to self-fertilise has been

reported previously for this species (EPPO, 2002) and both *I. noli-tangere* and *I. capensis* are known to produce cleistogamous flowers (Hatcher, 2003; Lu, 2002; Masuda & Yahara, 1994).

*I. parviflora* plants, which germinated in early March, began to produce ripe seeds by mid-April, approximately six weeks after germination. This was several weeks in advance of the flowering of the *I. glandulifera* plants, which seemed to invest more energy in the development of a tall stem during the pre-reproductive phase. The seeds produced by these *I. parviflora* plants were collected and stored at ambient humidity levels at 17°C in a dark cupboard. Seeds were tested once more with TTC and proved to be viable. Samples were stratified at regular intervals using the methods described above so that plants were available for successional sowing as germination commenced.



Plate 3.5. A comparison of growth habits of *I. parviflora* (left) and *I. glandulifera* (right). The relatively massive stem of *I. glandulifera* is clearly visible, while *I. parviflora* shows precocious flowering and pod development and the presence of axillary shoots.

### **3.6 Disease and cultivation problems**

#### **3.6.1 *I. noli-tangere***

During the cultivation period, the *I. noli-tangere* seedlings quickly developed severe chlorosis, which would suggest the possibility of iron or manganese deficiency induced by the waterlogged, anaerobic conditions in the substrate. This has been reported as a problem in commercial production of bedding plants including *Petunia* and *Impatiens*



(Smith & Fisher, 2004). Iron deficiency is considered to be one of the most difficult to understand and correct in plants (Wallace, 1982). The afflicted plants were repotted with the addition of horticultural grit at an approximate ratio of 1:1 v: v sand: grit. This however failed to remedy the situation, so the *I. noli-tangere* seedlings were fertilised using the same strength feed but with the pH adjusted to around 6.0 by the addition of hydrochloric acid; iron deficiency was reported as problematic in *Impatiens* when a substrate pH in excess of 6.2 was recorded and chlorosis was induced within 14 days by a pH of 7 (Smith & Fisher, 2004). Plants continued to make poor growth, however, and the chlorotic appearance remained. The *I. noli-tangere* plants grown in John Innes no. 2 compost also showed similar symptoms, with both mottling and necrosis on the leaves as well as the bleaching of young growth. Sequestered iron was added at a dilution of half strength to both sand and soil grown plants. Temporary greening of the leaves occurred in some instances, but once again, plants failed to make a permanent recovery, perhaps indicating some kind of systemic root infection. In some instances the bleached leaves took on a bright orange colour before wilting and desiccation occurred.



Plate 3.6. Chlorosis in *Impatiens noli-tangere*. Sand substrate is shown on the left, soil based compost on the right. All individuals show extreme chlorosis with defoliation and stunted growth also present.

Sunken black lesions were visible along the surface of some of the stems, raising a concern over possible infection by *Thielaviopsis basicola*, black root rot, a common pathogen of greenhouse grown bedding *Impatiens*. (Moorman, 2012). To check, 1 cm segments of unhealthy stem were excised from four plants showing the most severe symptoms and, placed in a saline solution, macerated with a sterile glass rod and the resultant liquid spread onto Petri dishes containing Potato Dextrose Agar (PDA). Dishes were incubated at 25° C for 7 days but no fungal growth was apparent at the end of the period.

The root system of *I. noli-tangere* is reported to be smaller than that of the invasive alien *Impatiens* species in Europe (Derricks, 2007), so this may have manifested itself as a greater susceptibility to unfavourable rooting conditions during the experiment. However, this species was observed by the author growing successfully in wet woodland flushes in the Lake District.

In order to secure a stock of healthy plants for use as had originally been intended, tissue culture was attempted with the surviving *I. noli-tangere* plants. Meristems from individual plants were removed with a sterile scalpel, dipped in 10% bleach solution for 10 minutes, then placed in sterile tubes on MS agar slants, with added 30 mg l<sup>-1</sup> sucrose and 2 mg l<sup>-1</sup> kinetin. Each plant was assigned a number and all meristems from each individual were given the same identifying number. Insertion into the growing medium was carried out under sterile conditions in a laminar flow cabinet. The tubes containing the *I. noli-tangere* meristems were initially placed in a growth cabinet at 20°C with a 12 h day, before space restrictions necessitated their removal to a laboratory windowsill, where they experienced ambient temperatures and day lengths. Unfortunately the plants did not thrive and it proved impossible to maintain and propagate the stocks, which were eventually discarded.

Possible reasons for failure may have been the presence of pathogens in the tissue selected, the poor nutritional status of the

meristems used, an inappropriate choice of growth medium (Martin, 2004) or an incorrect balance of plant growth regulators and nutritional imbalances within the growth medium (Basu & Chand, 1996). Different tissues from parts of the same plant may also have different nutritional requirements, often making correct medium selection a protracted process (Murashige & Skoog, 1962). Space and resources were not sufficient to allow such a programme of study to be undertaken.

This form of clonal propagation is advantageous for the experimenter however, as when carried out correctly, it allows for the production of batches of genetically uniform, disease-free propagules, within a short time period (Altman & Loberant, 2000). In the case of the bioassays undertaken in the following chapters, this would have enabled repeat testing and comparisons of lines of donor plants raised from specific individuals. Variation in plant size or maturity could also have been explored whilst removing the confounding factor of genetic variation. Among the disadvantages of plant tissue culture are the risks of media contamination, somaclonal variation (Pontaroli & Camadro, 2005) and the acclimatisation (“weaning”) of the propagules to conditions outside the culture vessels, where higher light levels and lower humidity levels are found (Fay, 1992). Cuttings of plants, taken as described below in Section 3.6.2. would also have been possible had healthy *I. noli-tangere* material been available. These would have been more robust than tissue

culture explants and could have been produced without the need to use sterile techniques. Physiologically mature *Impatiens* plants are likely to vary in their naphthoquinone content from those at a juvenile, pre-flowering stage, however (Lobstein et al., 2001).

### **3.6.2 Other species: *I. glandulifera*, *I. parviflora***

Although the majority of plants of these two species remained healthy and grew well, in some instances problems with inadequate pot drainage occurred. Affected pots became saturated and root rot ensued. Plants did not, however, develop the chlorosis present in *I. noli-tangere*. Sand as a substrate requires careful management due the ease with which all pore spaces become filled, driving out oxygen. Roots of affected plants became black and died back with the resultant rot spreading into the stems. A similar rot was observed by the author occurring in wild stands of *Impatiens glandulifera* where densely growing plants had lodged during wet weather. Of the two species, *I. parviflora* seemed to be more prone to root rot. The susceptibility of this species to waterlogged conditions has been previously noted (EPPO, 2002). However large numbers of adventitious root primordia developed along the hypocotyls of many plants (not just those with root rot). Softwood cuttings of the uninfected portions of shoots of both species were taken approximately 1 cm above the level of visible infection and the majority of them rooted very easily in

water. The rooted cuttings were potted into clean pots containing fresh sand; they grew and flowered satisfactorily. Plants produced from cuttings had a shorter, more branched habit and were not harvested for use in bioassays because of their atypical growth habit and the possible alteration in their naphthoquinone levels as described in section 3.6.2.

In autumn 2008, during a period of absence, plants of both *I. parviflora* and *I. glandulifera* numbering several hundred individuals suffered almost 100% mortality from an unknown cause, most likely linked to overwatering. This necessitated resowing and delayed some experiments, as replacement seeds had to be collected and stratified prior to recommencing experimental work.

A further problem was the appearance of large numbers of fungus gnats, *Bradysia difformis* which was thought to have arrived with the John Innes no. 2 compost. The maggot-like larvae appeared to be feeding initially on algal growths on the surface of some of the pots and additional colonies of maggots were found in the hollowed out bases of the stems of some plants. Affected plants usually died soon after.

A number of yellow sticky traps (Growing Success, William Sinclair, Lincoln, UK) were placed in the growing area in order to trap and control the adults. Large numbers were captured by this method and eventually populations were brought under control. Aphids (*Myzus persicae*) which appeared in August 2008 were initially controlled by

crushing, spraying with a rape seed oil based insecticide (Growing Success, William Sinclair, Lincoln, UK) or by the use of soap solutions prepared in the laboratory. None of these proved to be successful. Finally a garden insecticide containing 0.2 g<sup>l</sup><sup>-1</sup> pyrethrins (Wilkinson, Worksop, UK) was applied according to the manufacturer's instructions and this did prove effective. Spraying was carried out intermittently when aphid populations became visible and plants were not harvested for at least three weeks following an application so that the risk of sample contamination from pesticide residues were minimised. In 2009, *Aphidius* sp parasitic wasps were inadvertently introduced to the growth room via brassica plants raised as part of another study. These quickly colonised the *Impatiens* plants and resulted in the reduction of aphid populations to negligible levels where honeydew production and shoot tip distortion were no longer visible.

### **3.7 Harvesting and processing of samples**

Leaves and other plant material were harvested from the plants using scissors, or a scalpel, depending on the thickness or toughness of the particular plant organs. Individual items were then loosely wrapped in aluminium foil, labelled and placed in drying ovens at a temperature of 60° C for 24 hours. In the case of particularly fleshy material, such as stems, this period was extended for an extra day to ensure complete

moisture loss. When the specimens were completely dry, they were sealed in air tight plastic bags and placed in a dark cupboard at a temperature of approximately 15°C. Samples used for the bioassays described in later chapters were removed from the bags as required. In some cases dead material was collected. This too was dried in the oven before use.

In preparation for HPLC analysis, plant material was harvested, placed in a freezer at -20°C and subsequently freeze dried using an Edwards Modulyo Freeze Dryer (Edwards Vacuum, Crawley, W. Sussex, UK) Following freeze drying, the specimens were stored in airtight plastic bags until required.

### **3.8 Reflection**

Although notable for their ability to thrive outdoors in damp soil conditions, the *Impatiens* species cultivated in this study proved to be particularly susceptible to overwatering when cultivated indoors, with serious damage inflicted on their root systems within a few days of inundation. The sensitivity of *I. glandulifera* to excess water has been noted previously as a limiting factor in its ability to survive in competition with nettles (*Urtica dioica*) (Tickner et al., 2001b) and as mentioned previously, *I. parviflora* is even more sensitive to waterlogging (EPPO, 2002). Maintaining adequate, although not excessive water levels



was rendered more difficult due to the desiccating effect of the air conditioning unit and the low porosity of the sand, in which the water available to plant roots was limited to the capillary water present in the granular interstices and as films on individual sand grains. This meant that plants were susceptible to both anoxic root death through waterlogging when the medium was saturated and also to rapid wilting if water levels in the substrate were not carefully managed to maintain optimum levels. Plants grown in soil-based media were less susceptible to wilting, due to their higher organic matter content and the presence of finer particles, which provided a higher retentive capacity, although overwatering also produced root rots remarkably quickly. The presence of the plastic trays, although necessary to prevent flooding of the growth room, exacerbated the tendency to waterlogging as capillary action drew the standing water into the pots. This was particularly problematic in the case of sand grown plants. In order to reduce this problem in subsequent experiments, the trays were filled with horticultural grit to a depth of 2.5 cm, which lifted plant pots above the level of drainage water in most cases, but allowed the bases of the pots to remain in a humid environment. There was some evidence of increased root growth at the base of the pots as a result of this improvement.

Due to the heights of the plants, the proximity of the light bulbs and fluorescent tubes and the risk of overheating, it was not considered

practical to try and construct a plastic greenhouse within the frames, so that the drying effects of the air conditioning unit could be mitigated to some degree. Nevertheless, plants were able to grow successfully using this set-up, provided watering was attended to in a judicious manner. The constant air movement may have produced leaves with a thicker cuticle and a general morphology more closely resembling that of outdoor plants rather than the softer foliage commonly associated with high humidity environments with limited air movement. The low humidity experienced by the plants may also have led to a reduction in occurrences of fungal attack on the leaves of plants; these were noticeably absent during the experiments.

In some instances, plants of *I. glandulifera* grew so tall that they were close to making contact with the fluorescent tubes. These individuals suffered leaf scorch and were removed; no material from these individuals was used in any of the bioassays.

## **4. Investigations into the allelopathic potential of *Impatiens* leachates using the Sandwich Method**

### **4.1 Introduction**

The role of leachates in allelopathic interference has been the focus of sustained research in allelopathy. Early studies emphasised the role of leachates in the zoning of wild stands of vegetation (Halligan, 1973; Muller, 1966; Rasmussen & Rice, 1971).

Phytotoxic leachates have frequently been featured in studies on the allelopathic interference of weeds on crops (Inderjit & Dakshini, 1998; Kadioglu, Yanar & Asav, 2005; Rasmussen & Einhellig, 1975) and there has been an increasing emphasis recently on evaluating and developing crops which suppress weeds by the production of allelopathic leachates (Golisz et al., 2007; Lin et al., 2000). The development of ornamental ground cover plantings which produce weed suppressing allelochemical leachates is another area of current research interest (Shiraishi et al., 2002; Shiraishi et al., 2005 ). Finally, the effects of leachates from the leaves of invasive aliens have also been the subject of many studies (Dorning & Cipollini, 2006; Ghayal et al., 2011; Liu et al., 2010; Yang et al., 2007).

Leachates may be obtained using a range of techniques such as leaf washings (Lovett & Jackson, 1980), leaf extracts (Dorning & Cipollini,

2006), litter incorporation (Harun Rashid, Takashi Asaeda & Nazim Uddin, 2010) and solvent extraction (Krejčová et al., 2007).

The popularity of leachate studies probably reflects fundamental aspects of leaf and litter allelopathy. Leaf fall and subsequent accumulation as litter are large scale, obvious processes and it is easy to imagine that rainwater washing over fallen leaves might carry water soluble allelochemicals into the soil and affect the growth of other plants. Of the four principle pathways of allelochemical release, Tharayil (2009) ranked leaching as the second most important, exceeded only by volatilisation. When compared to the measurement of volatile allelochemicals however, leachates offer the researcher several advantages: firstly it is much easier to collect and store plant material for later analysis than it is to collect and measure the production of volatile chemicals from a living plant. Secondly, correctly dried and stored material can be kept for extended periods of time and thirdly, extracts can easily be prepared from samples using a range of solvents. These extracts can then be applied at a range of dilutions to receiver plants to assess their effects on their germination and growth.

#### **4.1.1 Leaf Deposition Rates**

Rates of leaf and litter deposition have been measured for temperate forbs at 200-600 gm<sup>-2</sup>year<sup>-1</sup>(Facelli & Pickett, 1991), with deciduous trees

producing between 300-400 gm<sup>2</sup>year<sup>-1</sup> (Jacob et al., 2010) and two and three times this quantity in the case of coniferous and tropical forests respectively (Fujii et al., 2004).

#### **4.1.2 Reports of allelopathic exudates from leaf material / leaf litter**

Reports of allelopathic leaf extracts and litter are common in the allelopathy literature and occur across many plant groups, including gymnosperms (Lee & Monsi, 1963); (Jameson, 1966). Among angiosperms, the species studied include monocarpic annual species such as *Kochia scoparia* (Wali & Iverson, 1978), sunflower (*Helianthus annuus*) (Wilson & Rice, 1968) fat hen (*Chenopodium album*) (Bhowmilk & Doll, 1979); herbaceous perennials such as catmint (*Nepeta meyeri*) (Mutlu & Atici, 2009); golden rod (*Solidago canadensis*) (Butcko & Jensen, 2002); nutsedge (*Cyperus rotundus*) (El-Rokiek et al., 2010); Siam weed (*Chromolaena odorata*) (Onwugbuta-Enyi, 2001) and shrubs such as boneseed *Chrysanthemoides monilifera* (Ens, French & Bremner, 2009), *Lonicera maackii* (Dorning & Cipollini, 2006) and trees such as *Eucalyptus* (Sasikumar, Vijayalakshmi & Parthiban, 2001), walnut (*Juglans regia*) (Ercisli & Turkkal, 2005) and camphor (*Cinnamomum camphora*) (Yumi, Yamaji & Katsuchiro, 2011).

*Impatiens glandulifera* produces whorls of leaves which tend to be dropped from the lower part of the stem throughout the growing season.

No published data are available for leaf litter production rates in *I. glandulifera* or any other invasive *Impatiens* species for that matter, but dense stands of this species must produce large quantities of leaf litter during their lives and the accumulations below mature plants could be substantial. Dicots growing in temperate climates have higher decomposability than monocots, (Cornelissen & Thompson, 1997). In studies of *I. glandulifera* sites undertaken by the author throughout the growing season, it appears that within a few weeks of falling, the majority of discarded leaves decay, irrespective of the date of shedding. Details of the study can be found in Appendix 2. The bulk of *I. glandulifera*'s biomass, is however, apparently contained within the stems. Following senescence, they collapse and come into close contact with the soil and presumably the seedlings of autumn-germinating plants. This stem litter often persists until the following season (personal observation) and *I. glandulifera* seedlings can frequently be observed emerging through it the following spring see Figure 1.4, Chapter 1. It is therefore possible that this might have a greater role in the suppression of competitor species than leaves alone.

As mentioned above, extracts prepared from leaves and other plant organs are one of the principle methods employed by allelopathy researchers investigating the effects of leachates. Extracts allow for standardised laboratory bioassays, but have been severely criticised for

their irrelevance to prevailing conditions outside the laboratory and the abundance of possible allelochemical candidates produced by the extraction processes used (Harper, 1977). Litter incorporation and mulch experiments attempt to mimic natural conditions, but leave the researcher open to complaints of confounding factors due to the many variables involved in such studies. A useful intermediate technique, which will be investigated further here, is to use agar as a medium into which relatively intact plant materials are incorporated. This allows putative allelochemicals to diffuse from the plant material in a way analogous to the adsorption of allelochemicals onto soil grains and their subsequent diffusion through the water film surrounding soil particles (Kobayashi, 2004). Its principle advantage is that, unlike a heterogeneous soil matrix, composed of different materials, particle sizes and pore spaces, it presents a uniform medium through which the allelochemicals can diffuse. Its transparent properties allow for the visual assessment of the rate of diffusion and quantity of leachates released and also enables differences in root development of receiver plants to be observed *in situ*.

Agar has a long history of use in plant experiments dating back to the 1920s when Went (1928) used it as a medium in his pioneering studies on plant growth regulators. The protocol adopted here is based on the Sandwich Method (Fujii et al., 2004) and is described below. This method is usually used as a preliminary screening method for multiple

plant species. For the purposes of this study, however, rather than focusing on one specific organ as is usually the case in screening studies, comparisons were made between different organs and plants of different ages so that a more complete picture of the comparative allelopathic potential of *I. glandulifera* tissues could be built up. The levels of naphthoquinones present in *I. glandulifera* and other *Impatiens* species are known to fluctuate through the growing season (Lobstein et al., 2001) and this may influence the allelopathic effects the plants produce. Although other studies have shown the allelopathic potential of extracts produced from invasive *Impatiens* species (Vrchotová, Šerá & Tríska, 2005), none to date have investigated the effects of whole plant material embedded in agar. This chapter aims to address this shortcoming by exploring the allelopathic potential of leachates from different organs and, where available, different species of invasive alien and native *Impatiens* at different stages in their lifecycles. This study is likely to be the first one to thoroughly investigate this aspect of *Impatiens* allelopathy.

## **4.2 Materials and Methods**

### **4.2.1 Overview of Sandwich Method**

The Sandwich Method involves the placing of pre-weighed samples of dried plant material into the wells of a six well plate. Each well has an



area of 10 cm<sup>2</sup>, so that when 10 or 50mg of leaf material is placed in each well, as specified in this protocol, this is equivalent to litter deposition rates of 10 gm<sup>-2</sup> and 50 gm<sup>-2</sup>; which lies at the low and high ends of natural litter deposition rates mentioned previously. Each well volume is close to 10 ml so the equivalent concentrations of 10 mg and 50 mg leaf material were therefore 1 mg ml<sup>-1</sup> and 5 mg ml<sup>-1</sup> that is 1 gl<sup>-1</sup> and 5 gl<sup>-1</sup> respectively. In cases where allelopathic effects are pronounced at the lower concentration of plant material, the plant can be said to be highly allelopathic. An equivalent effect at the higher concentrations would be less indicative of notable allelopathic effects.

#### **4.2.2 Sandwich Method Protocol**

Plant material from the Plant Box experiments and from other specimens grown as described in Chapter 3 were harvested and dried at 60°C for 24 hours in a drying oven. This temperature has been used for variety of leaf studies, including those investigating nutrient status (Zaiter et al., 1991) and PSM concentration in leaves in leaves (Balbaa, Hilal & Haggag, 1974) and was recommended by Fujii et al. (2004) as being sufficient to deactivate oxidative enzyme activity without impairing the content of allelopathic potential of the leaves. It also allowed a larger number of samples to be processed at any one time than was possible using the freeze-dryer. Following removal, they were placed in individual Ziploc

bags and stored at room temperature out of direct sunlight until used for experiments. Where necessary, leaves were cut into smaller pieces to enable accurate weighing prior to embedding in the agar.

Plant materials were carefully weighed on an accurate 4 figure decimal balance (Oxford Scientia 0.001g model, European Instruments, Oxford, UK) using a weighing boat. Plant material was manipulated using clean forceps to avoid contaminating the specimens with skin secretions. It was then gently tipped into the wells of a six well multiwell plate (Nunc Vita MD6, Nalge Nunc International, New York, USA). The top row of wells with designations 1, 2, 3 on the upper surface of the plate base were filled with 10 mg of plant sample per well. The bottom three wells, 4, 5, and 6 were filled with 50 mg plant sample per well. Three multi dishes were usually filled per sample, giving three replicates per sample, with three repeats. A control dish was set up for each experimental run, using agar without the addition of any dried plant samples.

#### **4.2.3 Type of agar and its preparation**

Following Fujii's protocol (Fujii et al., 2004), low temperature agar (Nacalai Tesque, Tokyo, Japan) with a gelling temperature of around 30–31°C was used. The agar was added to distilled water at a rate of 7.5 g l<sup>-1</sup>, giving a 0.75% concentration w/v. The mixture was boiled in a

microwave to ensure that the agar melted properly and it was then decanted into 500 ml borosilicate glass autoclave bottles (Schott Duran, Mainz, Germany). Bottles were autoclaved at 121°C for 15 minutes in a Boxer 4/500V autoclave. The bottles were placed in a water bath set at a temperature of 45°C until they had cooled to a temperature of 40–45°C. Five millilitres of the agar was then carefully pipetted into each of the sample and control wells using a pipettor (Gilson Co. Ltd, Villiers-le-Bel, France) and allowed to set. This took approximately 30 minutes. During this period, the open plates were carefully covered with laboratory roll to prevent dust entering. After the agar had set, a further 5 ml of agar was pipetted on top of the first layer. In some instances the application of fresh warm agar caused the plant material to rise up to the surface. In such instances a sterilised needle was used to push it below the surface so that a uniformly smooth upper surface was created when the agar set.

#### **4.2.4 Arrangement of seeds and incubation of multiwell plates**

After the agar had cooled and hardened, five lettuce seeds of the variety Great Lakes (Nicky's Nursery, Broadstairs, Kent, UK) were placed horizontally on the surface of the agar in each well using a pair of fine-tipped ceramic tweezers, which facilitated careful placement without the need to handle them. This variety was very similar to Great Lakes 366, the specific strain recommended by Fujii et al. (2004), which was not available from UK suppliers at the time of the experiments. The seeds

were arranged in a regularly spaced quincunx pattern. The lids of the plates were then closed and sealed with laboratory tape to prevent desiccation of the agar. The plates were wrapped in aluminium foil to eliminate light and then placed in a Sanyo incubator at a temperature of 20°C for three days (72h).

At the end of this time, the multiwell plates were opened for measurement. On some occasions measurement could not be completed promptly due to other commitments, so the plates were left wrapped and sealed and placed in a refrigerator at 4°C to prevent further development of the lettuce seedlings. Care was taken to note this when results were recorded.

The number of germinating seedlings was recorded and then the seedlings with the longest and shortest radicles in each well were discarded in order to remove outliers and maintain the central tendency and normality of the data so that parametric statistics could be used wherever possible for data analyses; the radicle and hypocotyl lengths of each of the remaining three seedlings were then measured. Experiments were repeated several times as sufficient plant material was available and the data pooled. Percentage elongation relative to control was calculated and converted to percentage inhibition, where 0% represents no inhibition and 100% complete inhibition. The experimental protocol is presented graphically in figure 4.1

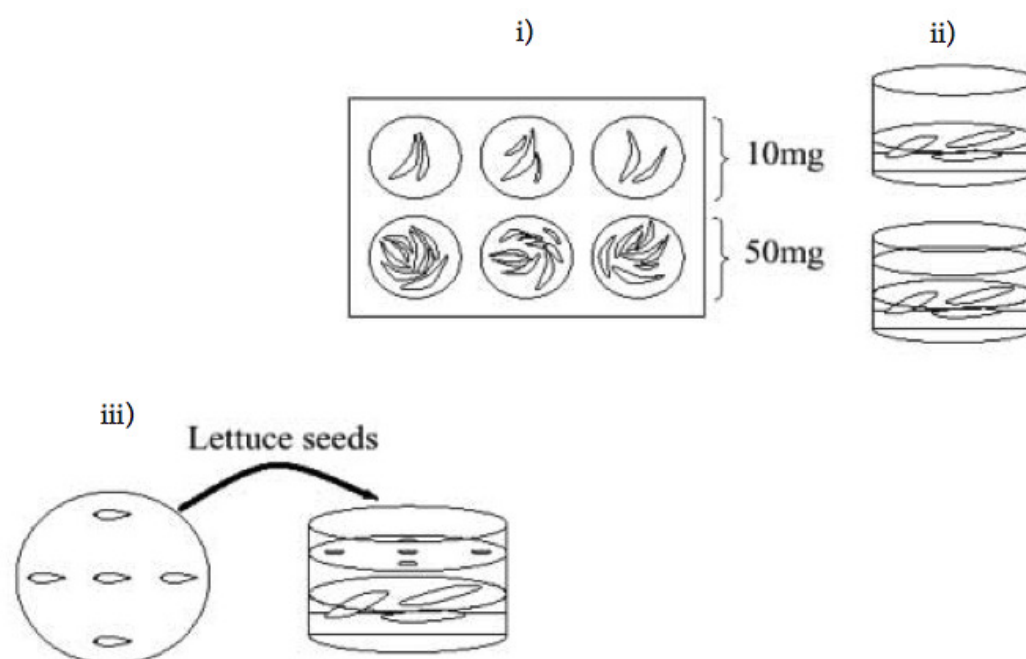


Figure 4.1. Diagrammatic representation of Sandwich Method (based on Fujii et al (2003) shows placement of 10 and 50 mg leaves in wells, the embedding of the samples between 2 x 5ml layers of agar, and the arrangement of lettuce seeds on agar surface. Used with permission of Dr Y. Fujii.

### **4.3 Data analysis**

Results were subject to one or two-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA, depending on the normality of the data, which was determined by the use of the Shapiro-Wilk test. Post-test comparisons also varied according to the initial analysis. Tests used were Dunn's Method, Tukey and Holm-Sidak as specified by SigmaPlot12 (Systat Software, San Jose, California, USA), the statistical package used for the analyses.

### 4.3.1 Overall Allelopathic Potential

In order to rank the data collected from separate experiments in terms of their allelopathic effects by plant organ and also by species, the concept of overall allelopathic potential (OAP) was developed for this study. Calculations were made using the formula:

$$\text{OAP} = \text{mean } (I_{10} + I_{50}) / 10$$

Where  $I_{10}$  = % inhibition of radicle growth compared to the control at 10 mg concentration and  $I_{50}$  = % inhibition of radicle compared to the control at 50 mg concentration. Using the mean of the sum of the radicle percentage inhibitions divided by 10, a score between 0.0 and 1.0 was obtained and the data were ranked according to this score. A maximum score of 1.0 would indicate that the test material had totally inhibited growth, while a score of 0.0 would indicate that no allelopathic inhibition had occurred. Hypocotyl data were not included as a previous study had established that there was a high degree of correlation between radicle and hypocotyl lengths (see Section 4.88, Figure 4.12).

The following classes were considered:

Table 4.1 Categories used to determine allelopathic potential using OAP score

OAP Score	Description
0-0.25	Non-allelopathic
0.26-0.5	Moderately allelopathic
0.51-0.75	Highly allelopathic
0.76-1.0	Extremely allelopathic

#### **4.4 Experiments into the allelopathic potential of *I. glandulifera* and *I. parviflora* leachates using the Sandwich Method**

##### **4.4.1 Comparison of healthy and senescent *I. glandulifera* leaves**

Leaves were gathered from the top three whorls of vigorous *I. glandulifera* plants grown under the light racks before flowering had occurred. Other studies have indicated that naphthoquinone content of leaves are at their highest at this time. (Lobstein et al., 2001). Leaves which were shed from plants as they grew were gathered and dried. Results are shown in Sections 4.8.1 and 4.8.2.

#### **4.4.2 Comparison of the senescent stems of *I. glandulifera* and *I. parviflora***

The stems from plants which were beginning to senesce or were close to doing so were gathered and dried at 60°C over a period of 24 or 48 hours, depending on their thickness. Results can be found in Section 4.8.3.

#### **4.4.3 Comparison of roots of *I. glandulifera* and *I. parviflora***

About 10 small plants each of *I. glandulifera* and *I. parviflora* were produced using the sand cultivation method described in Chapter 3, Materials and Methods. They were washed free of sand as described in chapter 3, the root systems removed and dried. Results can be found in section 4.8.4.

#### **4.4.4 Comparison of leaves of *I. glandulifera*, *I. parviflora* and *I. noli-tangere***

A small number of *I. noli-tangere* plants were eventually successfully germinated and these were grown outdoors in a common garden experiment with *I. glandulifera* and *I. parviflora* at the author's garden in Liskeard, Cornwall, in 2010. Leaves from the plants were collected in September 2010 and dried in the usual way. Results are located in Section 4.8.5.



#### **4.4.5 *I. glandulifera* flowers**

Flowers were gathered from plants as they opened and dried as described previously. Results are found in Section 4.8.6.

#### **4.4.6 *I. glandulifera* pods**

A small quantity of pods were produced by *I. glandulifera* in the growth room. These were collected following dehiscence and then dried as described previously. Results are found in section 4.8.7.

### **4.5 Ranking of different organs and species using their OAP (overall allelopathic potential)**

As the data gathered represented a series of discrete, separate experiments, a method was sought which would enable the various organs to be ranked according to their allelopathic potential – see Table 4.8, Section 4.8.9.

### **4.6 Determination of the sensitivity of the Sandwich method using increasing concentrations of powdered and flaked *I. glandulifera* leaf material**

An experiment was undertaken to determine the sensitivity of the Sandwich Method to concentrations of *I. glandulifera* leaf material ranging from 1 mg to 128 mg. In addition, the effect of grinding the leaf material

into a powder, thereby increasing its surface area was also investigated. Results are found in section 4.8.10.

#### **4.7 Effects of Pure Naphthoquinones**

A test into the allelopathic effects of pure naphthoquinones was carried out to ascertain whether their effects were measurable using the Sandwich Method protocol described here. Laboratory grade naphthoquinones lawsone (97%) and 2MNQ (98%) were obtained (Sigma Aldrich, St Louis, Missouri). They were dissolved in pure methanol to create stock solutions. When pipetted onto absorbent cellulosic material, the solvent rapidly evaporated, leaving a known quantity of naphthoquinone absorbed within the cellulose matrix. Initially 3 mm filter discs were used, but these required repeat pipetting and evaporation to reach the naphthoquinone concentrations described below. They were replaced by small cotton wool pellets. These showed much greater absorbency and were rolled by hand, whilst wearing laboratory gloves to avoid contamination by skin secretions. A size approximating 10mg in mass proved to be sufficient to absorb approximately 100  $\mu$ l of methanol. Stock solutions of lawsone and 2MNQ were prepared and dilutions from these stock solutions were pipetted onto the cotton pellets to produce a range of concentrations in the agar ranging from 2 to 10  $\mu$ gml<sup>-1</sup> of naphthoquinone. Lawsone concentrations

within this range have been shown to occur in *I. glandulifera* material (Lobstein et al., 2001). The dry pellets containing the naphthoquinones were then embedded in agar in the usual way. Radicle lengths were recorded as percentage elongation compared to the controls. Experiments consisted of three replicates per treatment and were repeated on three occasions, giving a sample size of 27. Results can be found in Section 4.9.

## **4.8 Results**

### **4.8.1 Healthy *I. glandulifera* leaves**

Healthy leaves were analysed using one way ANOVA. There were statistically significant differences between treatment means  $F_{5, 193} = 91.369$  ( $p < 0.001$ ). Holm-Sidak post hoc tests were carried out to compare treatment means. The mean values for the controls were 14.944 mm (radicle) and 8.529 mm (hypocotyl). At 10 mg dry weight, mean lettuce radicle elongation was 7.089 mm, showing highly statistically significant inhibition of 47% when compared to the control ( $t = 13.108$ ,  $p < 0.001$ ) whereas at 50 mg the inhibition was approximately 80% ( $t = 20.108$ ,  $p < 0.001$ ).

No significant difference was detected between the means of the hypocotyl control and the 10 mg concentration ( $t = 2.391$ ,  $p = 0.052$ ). The 50 mg concentration, however, gave a highly statistically significant

inhibitory percentage of 51% ( $t=6.437$ ,  $p < 0.001$ ). Data are presented graphically in Figure 4.2.

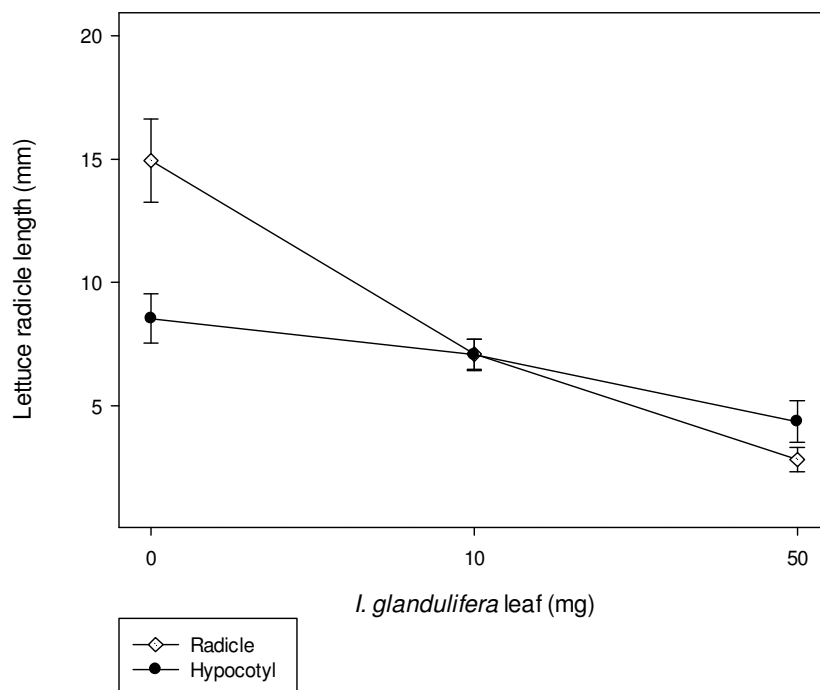


Figure 4.2. Effects of healthy *I. glandulifera* leaf material on lettuce radicle and hypocotyl elongation using 10 and 50mg of leaf material in the Sandwich Method protocol. Means are shown, with 95% confidence intervals.

#### 4.8.2 Senescent *I. glandulifera* leaves

Due to non-normality, test data were analysed using Kruskal-Wallis ANOVA on ranks. There were significant differences between treatments ( $p < 0.001$ ). Dunn's Method was used to compare medians of different treatments. The median values for the controls were 20 mm (radicle) and 10 mm (hypocotyl). At 10 mg dry weight, lettuce radicles showed an inhibition percentage of 50% compared with controls, whereas at 50 mg

the total was 80%. Both were statistically significant when compared to the control. R10 mg:  $Q = 4.598$ ,  $p < 0.05$ ; R 50 mg:  $Q = 8.613$ ,  $p < 0.05$ .

Hypocotyl length, however, was not affected at 10mg when compared to the control H10:  $Q = 4.189$ ,  $p > 0.05$ . The 50 mg concentration gave an inhibitory percentage of 20% when compared to the control, but this was not statistically significant H50:  $Q = 1.791$ ,  $p > 0.05$ ). Data are presented in Figure 4.3.

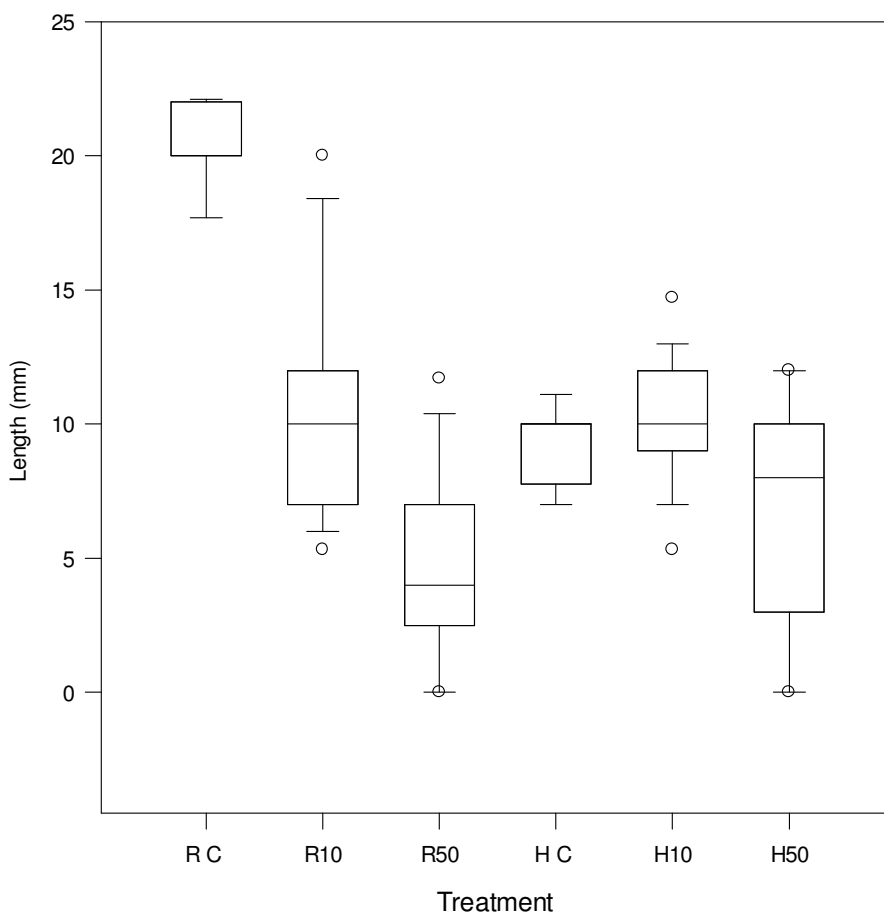


Figure 4.3. Box plots showing the effects of senescent *Impatiens glandulifera* leaves on lettuce radicle and hypocotyl elongation using the Sandwich Method. RC = radicle control, HC = hypocotyl control. 10 and 50 represent 10 mg and 50 mg treatments. Whiskers represent 5th and 95th percentiles, circles outliers.

### 4.8.3 Comparison of the senescent stems of *I. glandulifera* and *I. parviflora*

Due to non-normality, data from both species were analysed using Kruskal Wallis ANOVA.

In the case of *I. glandulifera*, significant differences were observed between treatments ( $H = 307.784$ ,  $p < 0.001$ ). Median values for the two controls were 20 mm (radicle) and 10 mm (hypocotyl). Lettuce radicles were inhibited by 50% at 10 mg and 80% at 50 mg, suggesting a powerfully allelopathic response. Hypocotyls, by contrast were less affected, with 0% inhibition at the 10 mg rate and 40% at the 50 mg rate. Data are shown in Figure 4.4

The measurement of *I. parviflora* was carried out separately but for convenience the results are discussed and plotted here. Kruskal-Wallis ANOVA, analysis was undertaken, followed by the Tukey Test for pairwise multiple comparisons.  $H = 163.770$ , with 5 degrees of freedom ( $p < 0.001$ ). Median values for the controls were 18 mm and 9 mm, slightly lower than those found in the previous experiment using *I. glandulifera*. Radicle inhibition at the 10 mg concentration produced 47% inhibition and 81% inhibition at the 50 mg concentration. There was no significant inhibition of either of the two hypocotyl values ( $p > 0.05$ ). Data are presented in graphically in Figure 4.5.

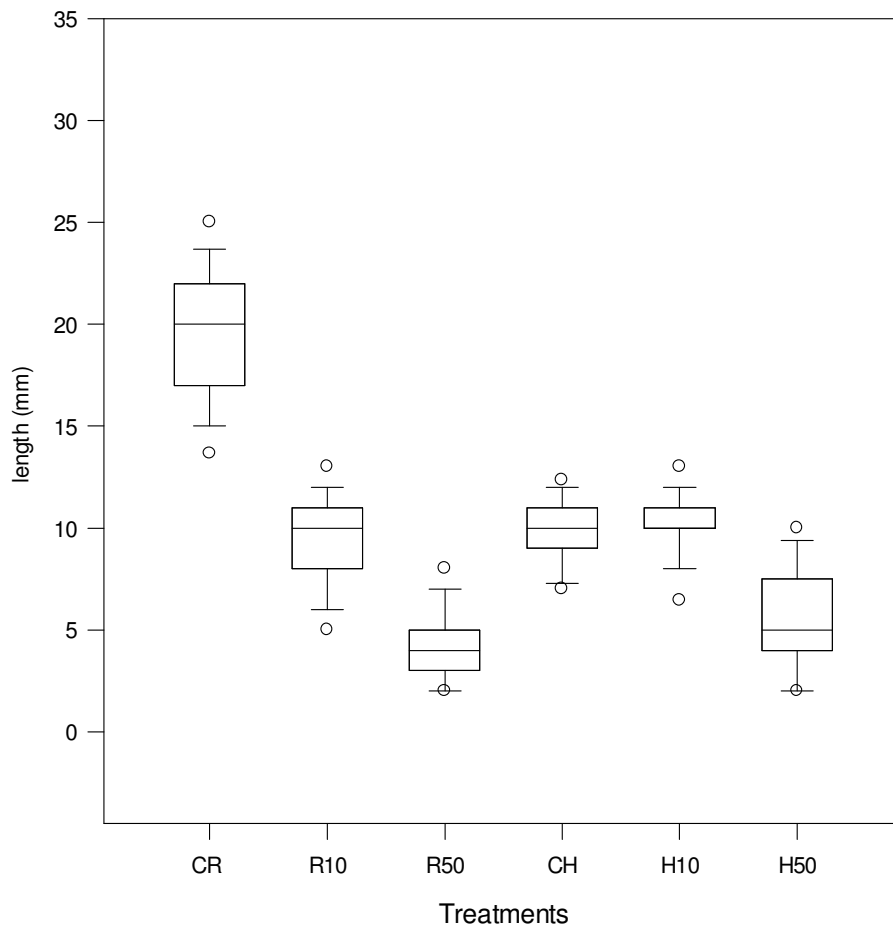


Figure 4.4. Box plots showing the effects of senescent *I. glandulifera* stem material on radicle and hypocotyl elongation in the Sandwich Method. CR = control radicle, CH = control hypocotyl 10, 50 = 10 and 50 mg treatments. Whiskers show 5th and 95th percentiles, circles are outliers.

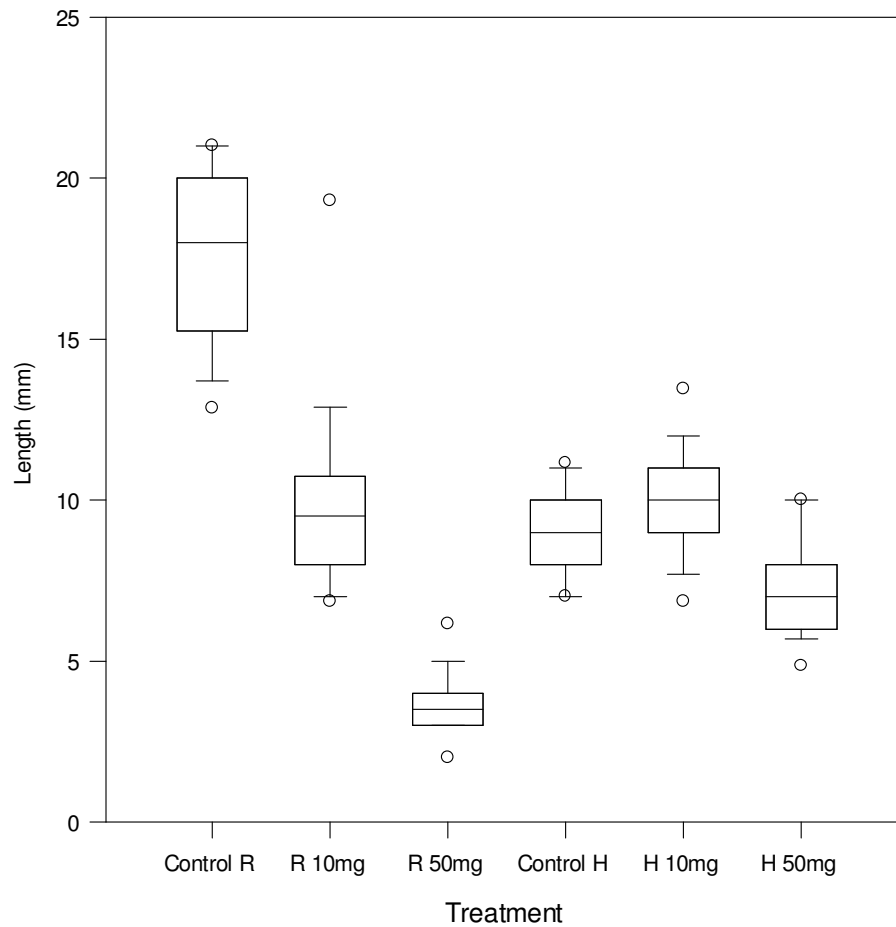


Figure 4.5. Box plots showing the effect of senescent *I. parviflora* stems on radicle and hypocotyl elongation of lettuce using the Sandwich Method. R and H = radicle and hypocotyl, 10 and 50 represent 10 mg and 50 mg concentrations. Whiskers show 5<sup>th</sup> and 95<sup>th</sup> percentiles, circles are outliers.

#### 4.8.4 Comparison of the roots of *I. glandulifera* and *I. parviflora*

These analyses were carried out on separate occasions when suitable root material became available. For the purposes of convenience, they are described together here. Both experiments were analysed using Kruskal-Wallis ANOVA. In the case of the *I. glandulifera* roots the analysis gave the following:  $H = 328.337$  with 5 degrees of freedom ( $p < 0.001$ ). Pairwise



comparisons were made using the Tukey test. Median values were 21 for the radicle and 11 for the hypocotyl. The effects on radicle inhibition were 31% inhibition at 10 mg and 67% at 50 mg, both being statistically significant ( $p < 0.05$ ). By contrast, there was a significant elongation of the hypocotyls by 18% compared to the control at the 10 mg concentration and a non-statistically significant inhibition of 9% at 50 mg, Figure 4.6.

In the case of *I. parviflora*, median values for the control radicles were 20.5 and 11 respectively. There was a 53% inhibition of radicles at 10 mg and 76% inhibition at 50 mg. The percentage inhibition values for the 10 and 50 mg treatments were both 9%, but neither was significantly different from the control ( $p > 0.05$ ), Figure 4.7.

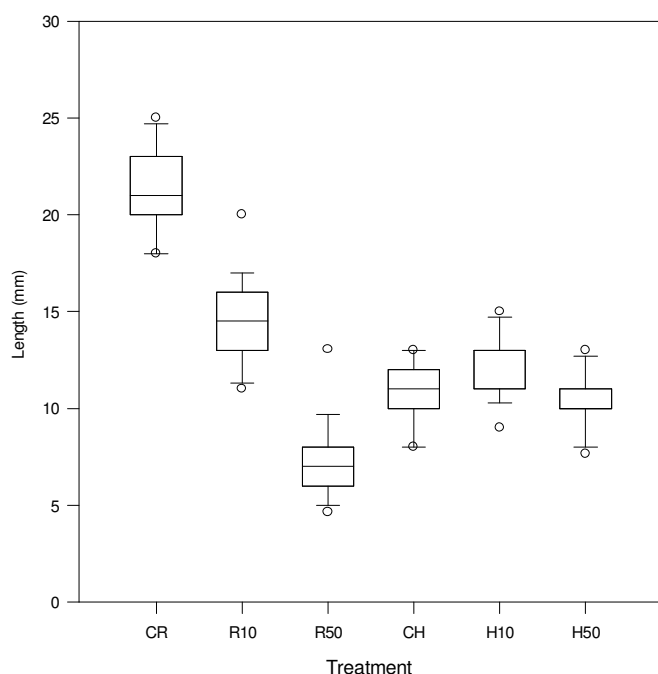


Figure 4.6. Box plots showing the effect of *I. glandulifera* root material on radicle inhibition at 10 mg and 50 mg treatment levels using the Sandwich Method. C = control radicle, R and H = radicle and hypocotyl, 10 and 50 represent 10 mg and 50 mg concentrations. Whiskers represent 5th and 95th percentiles, circles are outliers.

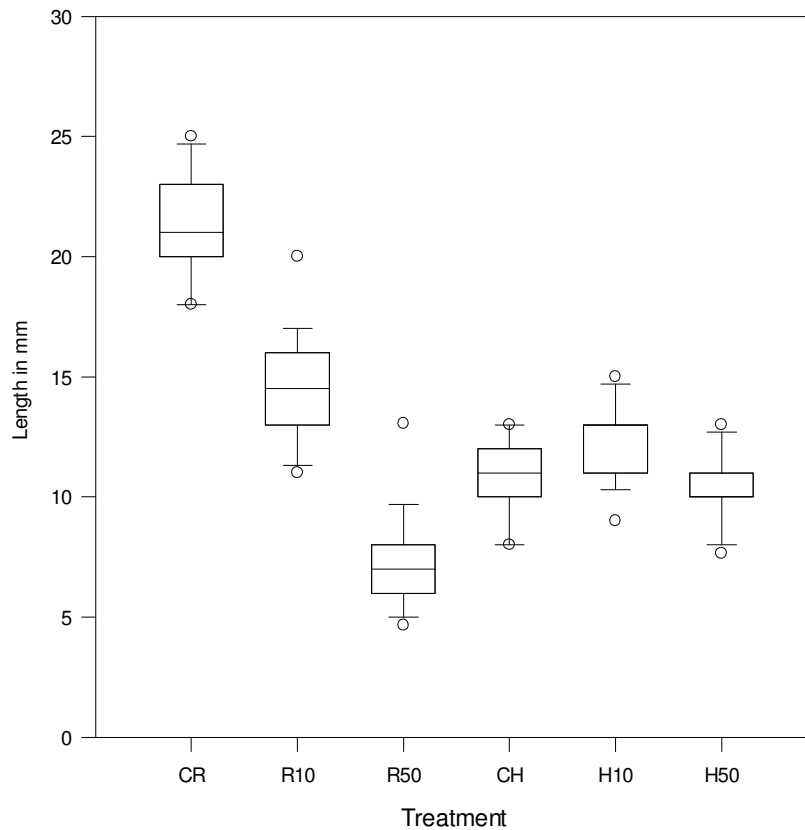


Figure 4.7.Box plots of the effect of *I. parviflora* roots on lettuce radicle and hypocotyl extension at 10 and 50 mg concentrations using the Sandwich Method. C = control, R = radicle, H = hypocotyl . R and H = radicle and hypocotyl, 10 and 50 represent 10 mg and 50 mg concentrations. Whiskers show 5th and 95th percentiles, circles are outliers.

#### 4.8.5 Comparison of the leaves of *I. glandulifera*, *I. parviflora* and *I. noli-tangere*

In all three species highly significant changes were seen in radicle elongation when compared with the control at both 10 mg and 50 mg treatments when analysed using two-way ANOVA,  $F_{3,154} = 5.553$ ,  $p < 0.001$ ). Of the three species, *I. glandulifera* showed the highest percentage inhibition of 42% at 10 mg, followed by *I. noli-tangere* with 29%

and *I. parviflora* with 23%. There were significant differences between *I. glandulifera* and *I. parviflora* at the 10mg concentration ( $p < 0.001$ ) and *I. glandulifera* and *I. noli-tangere* ( $p = 0.006$ ), but no significant difference between *I. parviflora* and *I. noli-tangere* ( $p = 0.226$ ). At the 50 mg concentration no significant differences were apparent between the species ( $p > 0.05$ ). Data are presented graphically in Figure 4.8.

In the case of hypocotyl lengths, for all three species 10 mg treatments produced significant elongation compared to the control ( $p < 0.05$ ). There were, however, no significant differences between the species at either the 10 mg and 50 mg concentration ( $p > 0.05$ ). There were significant differences between the 10 and 50 mg treatments in the case of *I. glandulifera* ( $q = 4.179$ ,  $p < 0.05$ ) and *I. noli-tangere* ( $q = 4.223$ ,  $p < 0.05$ ). There were no significant differences between the hypocotyls.

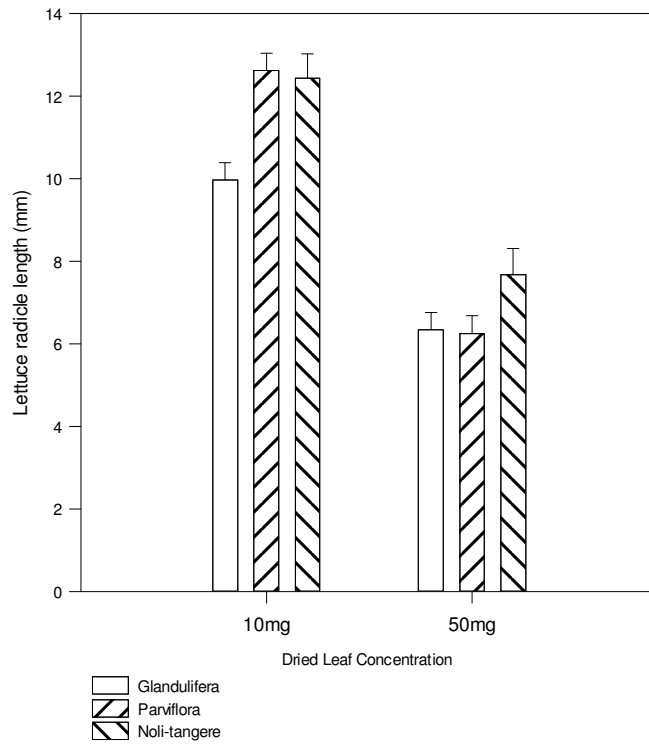


Figure 4.8. Effect of three species of *Impatiens* on lettuce radicle elongation using the Sandwich Method. N=27. Error bars represent 95% confidence intervals. All treatments were significantly different to the control.

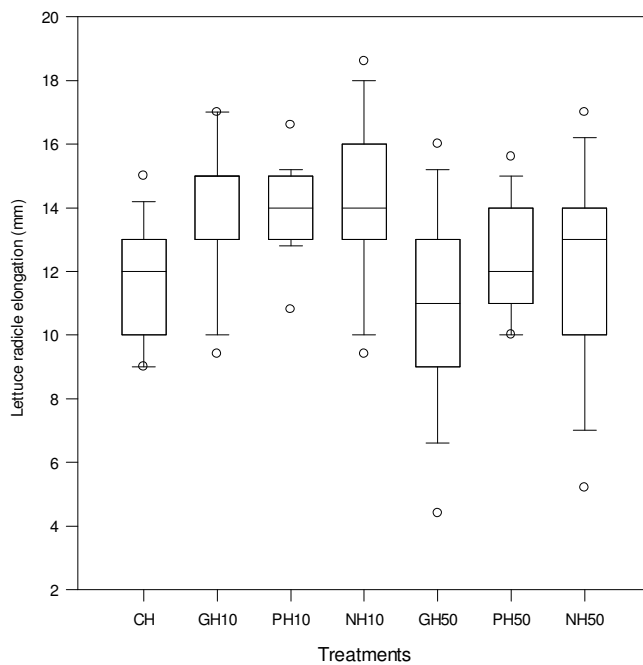


Figure 4.9. Box plots of the effects of three species of *Impatiens* on lettuce hypocotyl elongation using the Sandwich Method. CH = Control hypocotyl, GH = *I. glandulifera*, PH = *I. parviflora*, NH = *I. noli-tangere*. 10 and 50 = mg of leaf material used. N=27. Whiskers show 5<sup>th</sup> and 95<sup>th</sup> percentiles, circles are outliers.

#### 4.8.6 *I. glandulifera* flowers

Data were analysed using Kruskal-Wallis ANOVA followed by Dunn's Method for pairwise multiple comparisons. There was a significant difference between treatment groups ( $H = 344.058$ , with 5 degrees of freedom,  $p < 0.001$ ). Median values for the controls were 18 mm (radicle) and 9 mm (hypocotyl). Flowers produced a strongly and significantly inhibitory effect on lettuce radicle elongation ( $p < 0.05$ ) with 10 mg producing a 56% reduction in radicle length and a 73% reduction at 50 mg. Hypocotyl length was not inhibited significantly at the 10 mg concentration, showing a 12% reduction, but at the 50 mg concentration there was significant inhibition of 56 % ( $p < 0.05$ ).

Data are presented graphically in Figure 4.10:

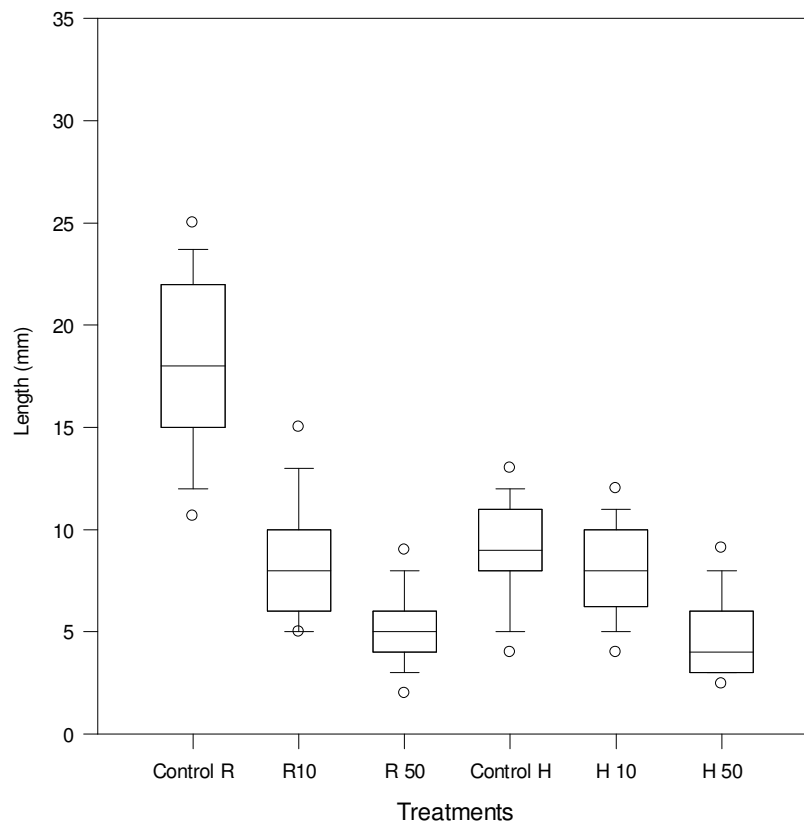


Figure 4.10. Box plots showing the effect of *I. glandulifera* flowers on radicle and hypocotyl elongation at 10 and 50 mg treatments, using the Sandwich Method. N = 108. R = radicle, H = hypocotyl. Whiskers show 5<sup>th</sup> and 95<sup>th</sup> percentiles, circles are outliers

#### 4.8.7 *I. glandulifera* pods

Data analysis was carried out using Kruskal-Wallis ANOVA, followed by Tukey test. Median values for the controls were 20 mm (radicle) and 11 mm (hypocotyl)  $H = 348.280$  with 5 degrees of freedom ( $p < 0.001$ ). Pods showed a powerfully inhibitory effect on lettuce radicle elongation with 10 mg producing a 40% reduction in radicle length and 85% inhibition at 50 mg. Hypocotyl length was not inhibited at the 10 mg concentration,

but at the 50 mg concentration, it was inhibited by 64 %. Data are presented in Figure 4.11.

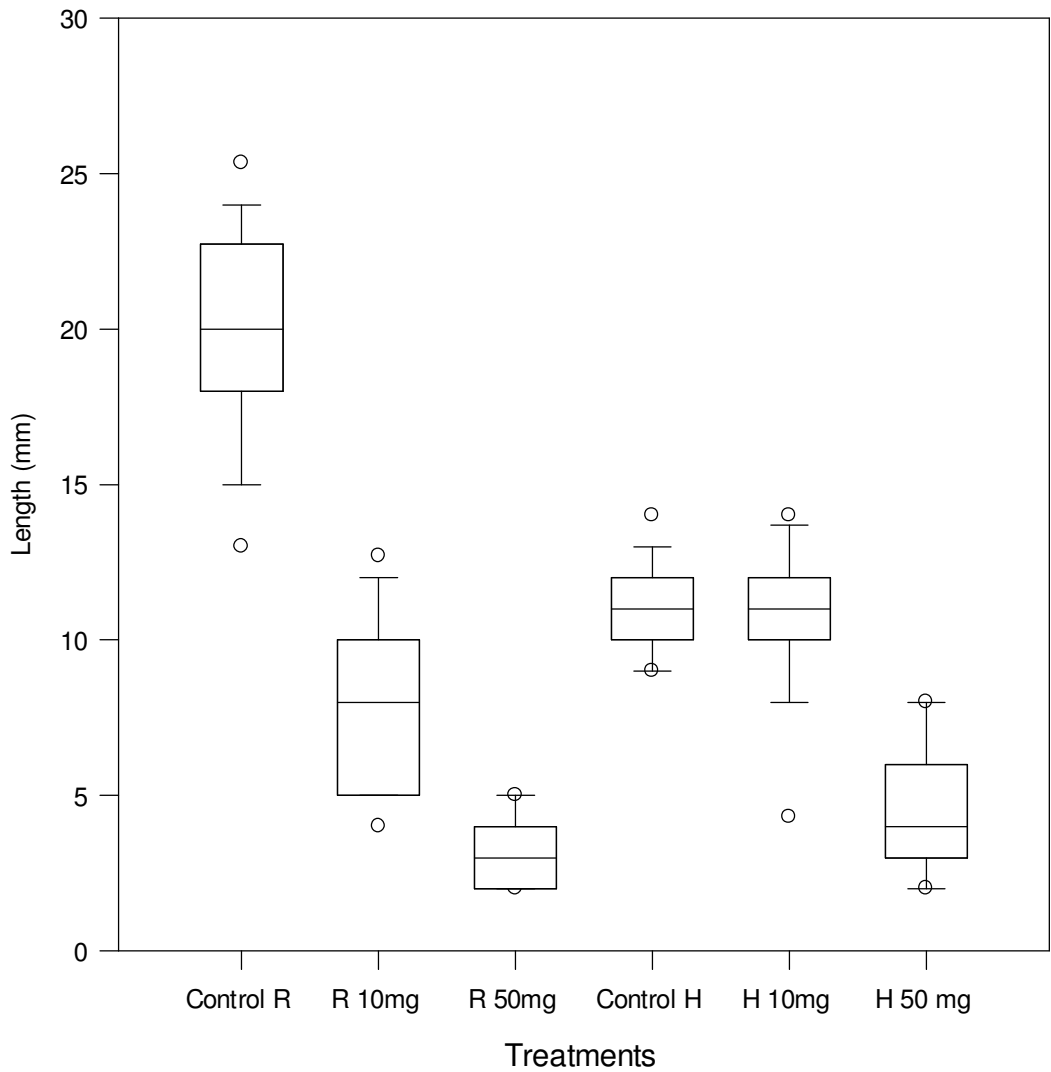


Figure 4.11. Box plots showing the effect of *I. glandulifera* pods on radicle and hypocotyl elongation at 10 and 50 mg treatments, using the Sandwich Method. N = 27. R = radicle, H = hypocotyl. Whiskers show 5<sup>th</sup> and 95<sup>th</sup> percentiles, circles are outliers.

#### 4.8.8 Correlation between allelopathic inhibition of radicles and hypocotyls

When the percentages of inhibition experienced by radicle and hypocotyls in separate Sandwich Method experiments were plotted as a linear regression, there was a highly significant correlation between the two variables  $r = 0.87945$ ,  $r^2 = 0.773$ ,  $p < 0.001$ , indicating a strong association between radicle and hypocotyl lengths.

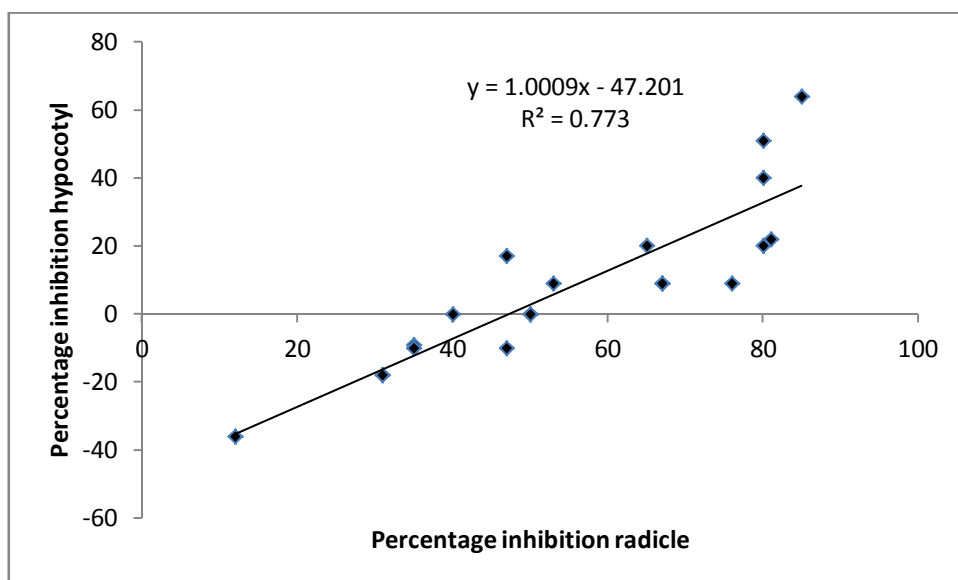


Figure 4.12. Correlation analysis of percentage inhibition radicle length and percentage inhibition hypocotyl length, using data from 16 separate Sandwich Method experiments. Axes represent percentage elongation compared to controls. Minus values indicate a stimulatory effect. A strong association was found.

#### 4.8.9 Overall Allelopathic Potential (OAP)

Using the data gathered in the previous experiments, the OAP values for the different organs and species were calculated. As Experiment 4.8.7 had



shown that there was a good correlation between radicle and hypocotyl inhibition when using the Sandwich method, data from the radicles alone was used. They are placed in ascending order of allelopathic potential in Table 4.2.

Table 4.2. Overall Allelopathic Potential of the plant materials tested

Species	OAP score	Allelopathic potential
<i>I. noli-tangere</i> leaves	0.425	Moderate
<i>I. parviflora</i> leaves	0.430	Moderate
<i>I. glandulifera</i> roots	0.505	High
<i>I. parviflora</i> roots	0.570	High
<i>I. glandulifera</i> flowers	0.585	High
<i>I. glandulifera</i> leaves	0.600	High
<i>I. glandulifera</i> senescent stems	0.600	High
<i>I. parviflora</i> senescent stems	0.605	High
<i>I. glandulifera</i> pods	0.635	High

Using the aggregated data from stems, roots and leaves for *I. glandulifera* and *I. parviflora*, a grand mean OAP was calculated for the two species and they were compared using an equal variance t test. There were no significant differences between the means of the two species: mean *I. parviflora* = 53.5, mean *I. glandulifera* = 56.833 ( $t = -0.536$ , 4 degrees of

freedom;  $p = 0.620$ ). Both species showed high OAP according to the criteria previously established.

#### **4.8.10 Effects of increasing concentrations of two types of *I. glandulifera* leaf material (flaked, powdered) on allelopathic inhibition of lettuce**

##### *Effects on radicles*

Both the flaked and powdered treatments caused a broadly linear decline in radicle elongation with doubling of the concentration of leaf material. There were significant differences between the two treatments when one way ANOVA was carried out ( $F_{17, 2251} = 752.068$ ,  $p < 0.001$ ). At lower concentrations, the powdered leaf material produced significantly less inhibitory effects on lettuce radicle elongation than the flaked material (see Figure 4.12). At high concentrations, this situation was reversed. In the mid-range (4, 8, 16, and 32 mg concentrations) there was no significant difference between the treatments. Concentrations above 64 mg produced some germination failures in both treatments, with higher germination failures in the powdered material. In the case of the powdered leaf material at 128 mg concentration, germination failures reached approximately 23%.

In the case of the powdered leaves, all pairwise comparisons were significantly different from one another, with the exception of the control and 1 mg concentration ( $p = 0.968$ ) and the 64 and 128 mg ( $p = 0.123$ ) concentrations. Values obtained for flaked leaves were all significantly different from each other apart from 2 and 4mg concentrations ( $p = 0.131$ ) and 2 and 1mg concentrations ( $p = 0.122$ ). When the two treatments (flaked and powdered) were compared, the following treatments were not significantly different from each other: 4 mg ( $p = 0.118$ ), 8 ( $p = 0.809$ ), 16 ( $p = 0.438$ ) and 32 ( $p = 0.969$ ).

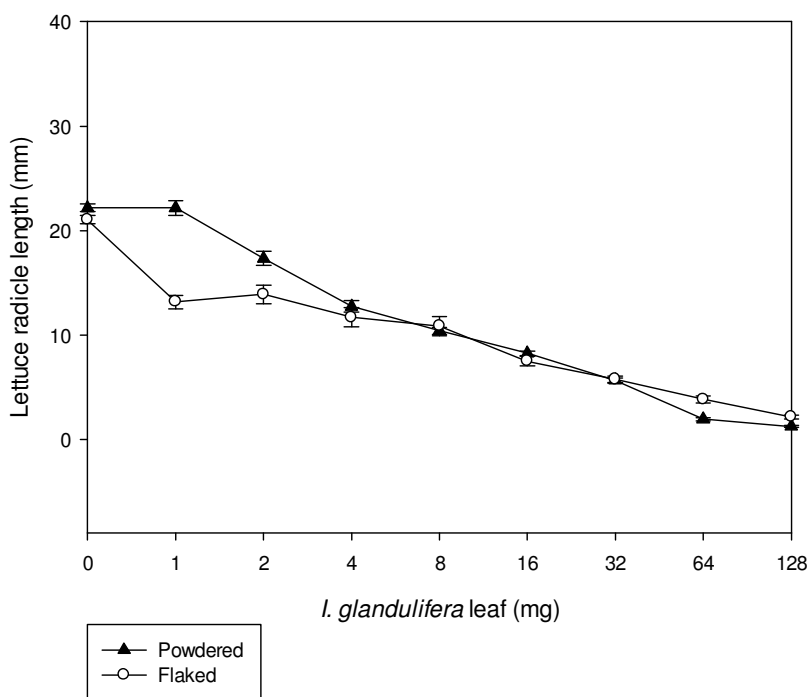


Figure 4.13. Effects of doubling quantities of powdered and flaked *I. glandulifera* leaf material on lettuce radicle elongation. Error bars represent 95% confidence intervals. N=108

### *Effects on hypocotyls*

There were significant differences between treatments ( $F_{17, 2241} = 254.585$ ,  $p < 0.001$ ), and once again the powdered material caused less inhibition than the flaked at the lowest concentrations. In this instance, the differences were maintained throughout the range of concentrations, apart from at the 32 mg concentration, where there were no significant differences ( $p = 0.926$ ). At low concentrations, the powdered material caused a marked elongation of hypocotyl length when compared to the control, which was maintained until the 8mg concentration and thereafter declined. At the highest concentrations (64 and 128 mg), the powdered material once again showed a greater degree of inhibition than the flaked material (see Figure 4.14).

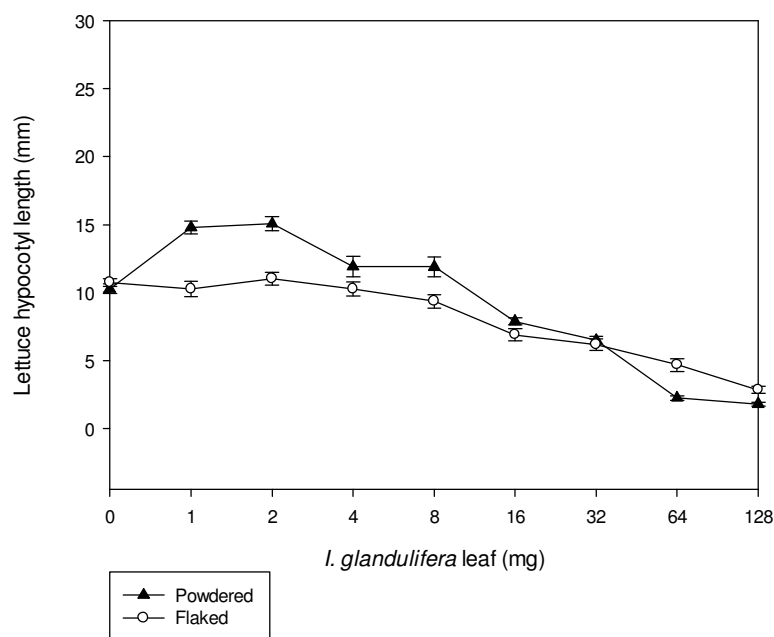


Figure 4.14. Effect of doubling quantities of powdered and flaked *I. glandulifera* leaf material on lettuce hypocotyl elongation. Error bars represent 95% confidence intervals. N=108

#### **4.8.11 Effects of AC on allelopathic inhibition of *I. glandulifera***

##### ***Determination of no effect concentration (NEC) of AC using the Sandwich Method***

0.125, 0.25, 0.5 and 1.5% AC were added to the agar used to prepare the Sandwich Method. The effect was compared with an AC free control. Data analysis was carried out using ANOVA and pairwise comparisons using the Holm-Sidak Method. It was found that the highest concentration of AC not affecting radicle elongation to a significant degree was 0.25% ( $p = 0.929$ ). This concentration was then used in a second experiment to investigate the effects of AC on the inhibition caused by 10 and 50 mg concentrations of *I. glandulifera* leaf material.

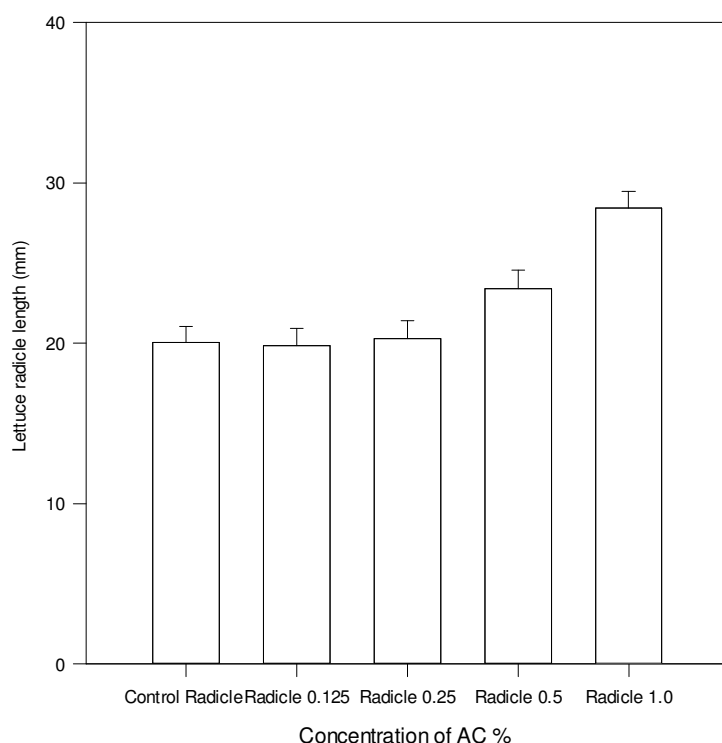


Figure 4.15. Effect of the addition of varying quantities of activated charcoal on lettuce radicle elongation used in the Sandwich Method.

#### ***Effect of 0.25% AC on lettuce radicle and hypocotyl elongation***

The addition of 0.25% AC caused an increase in radicle elongation of 48% at the 10 mg concentration and 70% at the 50 mg concentration. These increases were significantly different when compared with the control ( $F_{9,20} = 164.187$ ,  $p < 0.001$ ). Hypocotyl lengths were also affected, with a 5% increase at 10 mg and 39% increase at 50 mg. Data are presented in Figure 4.16.

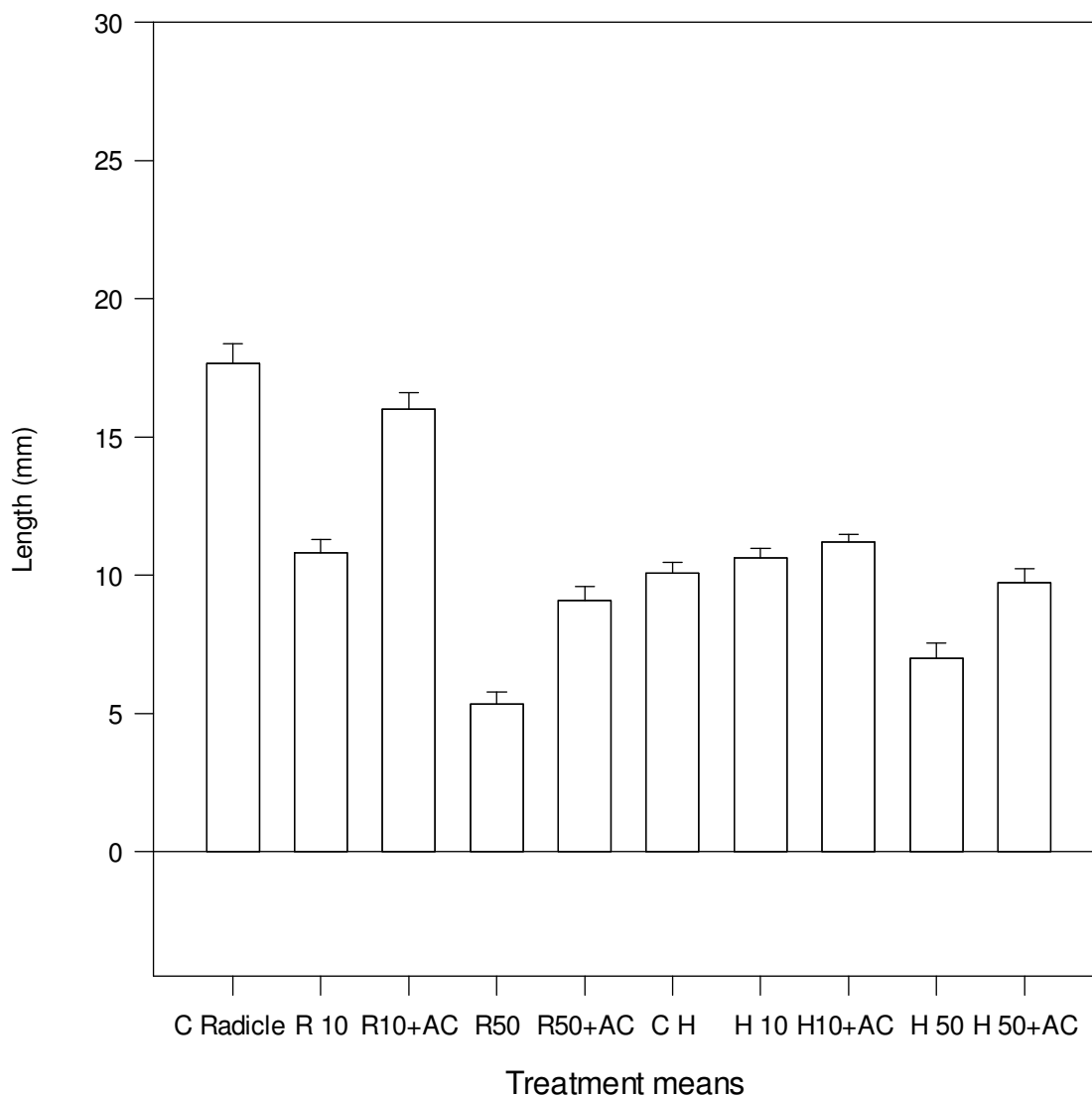


Figure 4.16. Effects of the addition of 0.25% AC to the agar containing *I. glandulifera* leaf material on lettuce radicle elongation using the Sandwich Method. R = radicle, H = hypocotyl; 10 and 50 mg are concentrations of leaf material +AC indicates the presence of activated charcoal.

#### **4.9 Effect of pure naphthoquinones on lettuce radicle elongation**

The two naphthoquinones showed visibly different effects on lettuce radicle elongation, with 2MNQ showing a greater inhibitory effect than lawsone at all the concentrations measured. When data were analysed

using ANOVA, there were significant differences between the two chemicals at identical concentrations  $F_{9, 29} = 164.7$  ( $p < 0.05$ ).

Neither chemical showed significant differences between the 8 and 10 mg ml<sup>-1</sup>, treatments: lawsone ( $p = 0.076$ ) and 2MNQ ( $p = 0.802$ ).

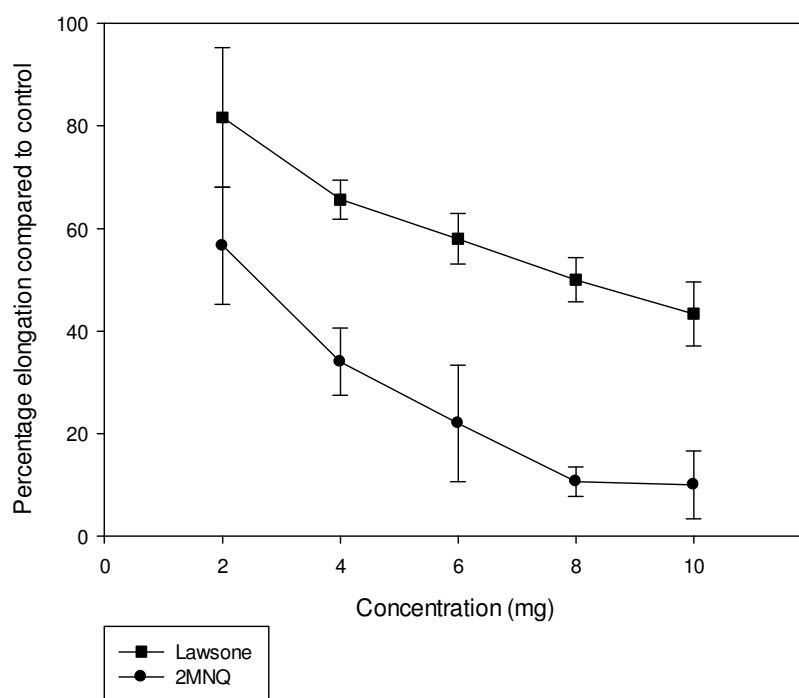


Figure 4.17. Effect of the naphthoquinones lawsone and 2MNQ on lettuce radicle elongation using the modified Sandwich Method. Error bars show standard error of the mean. N=27.

## 4.10 Discussion

The experiments conducted in this chapter demonstrated that both *I. glandulifera* and *I. parviflora* tissues were capable of producing allelopathic effects and that these effects varied according to the type of material used and the age of the plant from which it was collected. The small quantity of *I. noli-tangere* leaf material gathered also produced statistically



significant inhibition of lettuce radicles, although the degree of inhibition caused was less than *I. glandulifera*. This was, in fact, the only appreciable difference in allelopathic effects between the two species and merits more thorough investigation.

Limited space and cultivation problems meant that plants were often grown sequentially rather than contemporaneously as had originally been intended. As a result, data from separate comparative experiments were combined. The OAP calculations were useful in gaining a sense of the general effects of disparate organs and species. Perhaps the most interesting result was the small variation in allelopathic potential shown. Using the classes as described, the majority of plant material sits squarely in the highly allelopathic category, with only the root material falling below the class threshold into the moderately allelopathic grouping. It is interesting to note that *I. noli-tangere* showed the lowest OAP and *I. glandulifera* had the highest, although this was not a statistically significant difference. Pods showed the highest OAP, perhaps indicating that they are a competitive sink for naphthoquinone production as the season progresses and their concentration in the pods may offer some protection against herbivory or seed predation while the seeds are still developing.

Although lettuce hypocotyl lengths were generally less responsive to changes in concentrations of allelopathic materials than radicles when

using this protocol, they did show a respectable degree of correlation with radicle length inhibition, with an  $r^2$  value of 0.773,  $p < 0.001$ . This difference in degree of response could be due to increased distance of the developing hypocotyl from direct contact with the agar or may represent a lower sensitivity of hypocotyl tissue to the allelochemicals present in the agar. They were not as consistently significant when compared to the controls however and were probably not therefore as reliable an indicator of allelopathic potential as the radicle values were. This suggests that concentrating on radicle elongation as the main indicator of allelopathic activity makes sense when using the Sandwich Method.

One of the more interesting results was that the stems of both *I. glandulifera* and *I. parviflora* produced a large and significant inhibition in radicle extension of around 50% at 10 mg concentration and 80% at 50 mg concentration. This was equivalent to the degree of inhibition produced by *I. glandulifera* foliage, which is usually considered to contain a high level of naphthoquinones (Lobstein et al., 2001) and also to show elevated allelopathic activity when compared to other organs (Vrchotová, Šerá & Tríska, 2005). Usually stems are not considered to be an important source of allelopathic activity in plants, but the results presented here show distinct and statistically significant allelopathic potential in the stems of *I. glandulifera* and *I. parviflora*. They also appear to make up the bulk of the biomass in mature specimens of both these species and therefore their

ability to suppress plant growth under controlled conditions may indicate that they play an ecologically important role in the development and maintenance of natural *Impatiens* stands. In the case of *I. glandulifera*, stems can collapse, forming a thick mulch over the otherwise bare soil in winter (see Fig 1.4). This makes it feasible that leaching of allelochemicals from the collapsed stems might inhibit the growth of competitor seedlings emerging in the autumn. It is also possible that this effect might extend to the following spring, if weather conditions are suitable and microbial breakdown is not excessive. This is an area of research that warrants further investigation and could be carried out by collecting stem material at various times over the autumn and winter and measuring its effects on radicle elongation.

The comparison of the leaves of the three species *I. glandulifera*, *I. parviflora* and *I. noli-tangere* produced significant differences between the species. The differences between effects on the radicles and hypocotyls were marked. In the case of the radicle experiment, there was a clear pattern at the 10 mg concentration, where *I. glandulifera* showed the highest level of inhibition of 42% followed by *I. noli-tangere* 23% and *I. parviflora* 29%. There were no statistically significant differences between species at the 50 mg treatment level, however. Also of interest were the differences between the allelopathic potential of the dried root material of

both species. In this instance, *I. parviflora* appeared to be more allelopathic than *I. glandulifera* at the lower concentration of 10 mg.

The results of experiments carried out using powdered and flaked *I. glandulifera* material (Section 4.8.10) were contrary to the original expectation that powdered leaf material, having a greater surface area, would lead to a greater degree of radicle and hypocotyl inhibition when compared to leaf fragments. It is possible that grinding the leaves to powder allowed for the more rapid release of nutrients when compared with the flaked leaves, which stimulated growth of both radicles and hypocotyls at low concentrations. Another possibility is that the powder was distributed more diffusely and evenly through the agar medium and that localised concentrations of allelochemicals in the vicinity of the germinating lettuce seeds were reduced when compared to the much larger pieces of leaf material lying in close proximity to the germinating seeds.

The use of AC is well established in allelopathy research and it has been used to good effect in allelopathy studies involving invasive species (Kulmatiski & Beard, 2006) Callaway (Callaway & Aschehoug, 2000). Its mode of action is believed to be through the adsorption of low molecular weight organic compounds, such as allelochemicals (Cookson Jr., 1978) whilst it displays little affinity for inorganic electrolytes.(Cheremisinoff & Ellerbusch, 1978). It is commonly used in plant tissue culture media,

where its ability to irreversibly adsorb toxic metabolites and phenolic exudates has long been recognized and valued (Thomas, 2008). When AC was incorporated into the agar medium at a level previously determined to have no effect on lettuce radicle elongation (0.25%), it reduced the inhibition seen in the lettuce radicles significantly when compared to treatments without the addition of AC. This provides good evidence that the inhibitory effects seen in treatments lacking AC were caused by phytotoxic leachates which diffused from the plant material encased in the agar. Where AC was present, they were adsorbed and their allelopathic effects reduced as a result. Although the identity of these putative allelochemicals was not established, the presence of naphthoquinones in *Impatiens* tissues (Lobstein et al., 2001) and the inhibitory effects of pure naphthoquinones described briefly below strongly suggest there might be a link.

Although the principle advantages of the Sandwich Method protocol are its simplicity and cheapness, the results of the modified protocol described in this chapter showed that it is sensitive enough to be used over a much wider range of concentrations than was originally intended. This may be an incentive to other allelopathy researchers to adopt the Sandwich Method as a basic bioassay. The results obtained here suggest that statistically significant effects could be obtained from concentrations adjusted to levels based on real rather than notional litter

accumulation rates and varied appropriately according to sites and species. Further aspects of the Sandwich Method's versatility were demonstrated when a novel modification was made to the basic protocol so that the allelopathic effects of the two naphthoquinones, lawsone and 2MNQ could be determined. It proved successful in distinguishing the allelopathic effects produced by the two naphthoquinones and this modified protocol could be further developed so that blends of pure chemicals could be tested at a wider variety of concentrations. Another version of the basic Sandwich Method protocol involving a combination of agar and soil shaken from plant roots and known as the Rhizosphere Method will be described in Chapter 7.

The main limitation of the Sandwich Method is its reliance on one test species, lettuce. As might be expected, plants are variously susceptible or resistant to the effects of allelochemicals and a broader, more ecologically relevant assessment of a plant's allelopathic potential would be obtained by using a range of test species. Even though the longest and shortest radicles obtained per test well were discarded in order to reduce the presence of outliers and maintain the central tendency and normality of the data, they often showed distinct non-normality and unequal variance, necessitating the use of the less powerful non-parametric Kruskal-Wallis ANOVA. This could be considered a function of the large sample sizes sometimes used and perhaps an effect caused by

the inherent genetic variability of the lettuce seeds and their response to the allelochemicals present in *Impatiens* material. The variety of lettuce selected, Great Lakes, was chosen for its uniform germination and growth response, however and in the main proved to be very reliable in terms of its germination behaviour.

In order to address some of the concerns raised by the use of lettuce as the sole test species, nettle (*Urtica dioica*) was investigated as a potential second test species. *U. dioica* is a common competitor of *I. glandulifera* in riparian habitats (Beerling & Perrins, 1993; Tickner et al., 2001b). Seeds were collected from wild nettle stands occurring in the Looe Valley in the autumn of 2008 and stored for two months before sowing, in order to overcome after-ripening requirements (Thompson, Grime & Mason, 1977). They were then subjected to moist stratification at 4°C for two months (Luna, 2001) before sowing on filter papers in Petri dishes at a temperature of 20°C to determine their germination rate and suitability for inclusion in the Sandwich Method experimental runs. Germination was hampered by the development of mould and was erratic, with the majority of stratified seeds failing to germinate within 72 hours, the experimental duration time used for the Sandwich Method. In order to eliminate poor quality seeds as an explanation, commercially produced *U. dioica* seed was purchased (Herbiseed, Twyford, UK), but once again, the reliable production of ready-to-germinate seeds in sufficient

quantities was not achieved. The small size of *U. dioica* seeds and the difficulties implicit in handling them both pre and post germination, along with their dormancy requirements and irregular germination led to their rejection as a second test species. Similar problems with nettle germination were experienced in the target-neighbour experiment carried out in Chapter Seven (Section 7.4.1) and once again, its use was abandoned.

#### **4.11 Conclusion**

The experiments conducted provide strong support for the assertion that allelopathy could play a role in the ecology of *Impatiens* species through the chemical suppression of competitors. The effects appear to be limited, in the main, to inhibition of germinated seeds rather than a prevention of germination itself. Root material showed slightly lower allelopathic potential than leaves and stems. However, its location in the soil means it is particularly well placed to impact on the roots of other plants through the release of exudates into the soil in which they grow. This aspect of *Impatiens* allelopathy, using another agar-based bioassay known as the Plant Box Method, will be investigated further in Chapter 5.



## **5. Investigations into Root Exudate Allelopathy in *Impatiens glandulifera* and *I. parviflora* using The Plant Box Method**

### **5.1 Introduction - Root system overview**

The plant root system has been described as the “hidden half of plant” (Bohm, 1979 ; Feldman, 1984). The root architecture of any individual plant depends upon a number of factors such as the species to which it belongs, the soil structure and the numerous interactions between the two (Uren, 2000). Although little studied, the root zone of plants is known to involve complex interactions between micro-organisms, plant species and has been described as the “Underground superhighway”, in reference to the complexity of interactions occurring (Bais et al., 2004).

Roots are not merely passive bystanders in soil processes, but are actively involved in the interaction dynamics of the soil: “roots affect soil structure, aeration and biological activity as they are the major source of organic inputs into the rhizosphere and are also responsible for the depletion of inorganic compounds” (Bertin, Yang & Weston, 2003). Roots rarely interfere with one another directly, however and as a result of the heterogeneity of the soil structure, there tends to be a spatial separation of roots from one another (Young, 1998).

### 5.1.1 Rhizosphere – definition and importance

The zone of greatest activity within the soil matrix usually lies within 0–2 mm of the root surface and is significantly influenced by their presence (Bertin, Yang & Weston, 2003). “It consists of longitudinal and radial gradients occurring with expanding root growth, nutrient and water uptake and subsequent microbial growth” (Uren, 2000). Rhizosphere soil differs drastically in its properties from bulk soil (Bertin, Yang & Weston, 2003). Roots are the key source of carbon in the soil and biological activity is therefore concentrated in this zone, with marked difference between the properties of rhizosphere and bulk soil (Bertin, Yang & Weston, 2003). As an example, acidity levels 10 times greater have been recorded in this zone when compared to bulk soil (Darrah, 1993; Hubel & Beck, 1993). This is manifested as a one point drop on the pH scale, which is an inverse logarithm scale of hydrogen ion concentration.

The study of interactions between living roots have been dogged by methodological difficulties due to the highly complex nature of the medium and the problems implicit in sampling in a subterranean environment whilst maintaining the integrity of the substrate and its many interactions (Weidenhamer, 1996). However, some promising methodological advances have recently allowed the collection and analysis of putative allelochemicals *in situ* (Weidenhamer, 2007).

### **5.1.2 Root exudate composition**

Living root hairs as well as actively growing roots release exudates in large quantities (Bertin, Yang & Weston, 2003). Root exudates include a range of compounds, including high molecular weight polysaccharides, which can combine with soil particles and microorganisms to form a protective lubricating coat known as mucigel (Bertin, Yang & Weston, 2003). Of more relevance to the phenomenon of root exudate allelopathy are the terpenes, steroids, tannins and alkaloids exuded by plant roots. Low molecular weight compounds involved are sugars and simple polysaccharides, amino acids and of particular relevance to *Impatiens* allelopathy, phenolic compounds, which are known modifiers of the root environment (Bertin, Yang & Weston, 2003).

### **5.1.3 Mechanisms of root exudation**

Three mechanisms of root exudation are described by Bertin, Yang & Weston (2003). These are outlined below.

#### ***Diffusion***

Diffusion occurs when low molecular weight compounds such as sugars and phenolics are released via a passive process involving net movement across concentration gradients between the cytoplasm of intact root cells

to the lower concentrations found in the surrounding soil. Membrane integrity and therefore the rate of release, can be influenced by nutrient and mineral concentrations, (Cakmak & Marschner, 1988) temperature extremes or oxidative stress (Rovira, 1969).

### *Ion Channels*

Ion Channels which mediate the transport of compounds unable to diffuse through the roots as described in 1) above, due to stresses such as metal toxicity or suboptimal nutrient status present in the roots (Zheng, Ma & Matsumoto, 1998).

### *Vesicle Transport*

High molecular weight compounds such as mucilage polysaccharides are moved via Golgi vesicles, whereas proteins are transported via the endoplasmic reticulum. (Neumann & Romheld, 2002)

## **5.2 Examples of root exudate allelopathy**

Many phytotoxic compounds are released by plants directly into the soil (Inderjit & Duke, 2003). Among those that have been characterised and studied are sorgelone produced by sorghum (Duke et al., 2001; Einhellig & Souza, 1992 ) and (-)catechin exuded into the soil by *Centaurea*

*diffusa*, an invasive alien knapweed found in western USA (Bais et al., 2004). Other studies linking root exudates with invasiveness include those by Flores et al. (1999) and Bais et al. (2006).

Of particular interest in relation to this study, is the case of juglone, a naphthoquinone exuded by the roots of black walnut tree (*Juglans nigra*) and long associated with allelopathic effects (Funk et al., 1979 ; Ponder & Tandros., 1985; von Kiparski, Lee & Gillespie, 2007).

### **5.2.1 Supporting evidence in the case of *Impatiens* species**

The roots of *Impatiens* species are known to contain naphthoquinones, including in the case of rose balsam (*I. balsamina*), lawsone and 2MNQ (Panichayupakaranant et al., 1995). Under sterile conditions and with the correct light, nutrient and temperature regimes, *I. balsamina* roots can be cultured indefinitely and their naphthoquinones harvested. Production can be increased by the use of elicitation factors, such as methyl jasmonate (Sakunphueak & Panichayupakaranant, 2010) which suggests that environmental factors and challenges can affect the rates of release and may do so in wild populations of *Impatiens* plants.

The occurrence of naphthoquinones in the roots of invasive alien species of *Impatiens* found in Europe has not been the subject of any published studies to date. They are, however, described as being the dominant components of root extracts in HPLC analyses carried out on *I.*

*noli-tangere*, *I. capensis*, *I. glandulifera* and *I. parviflora* by Šerá, Vrchotová & Tríska (2005). The authors did not, however, indicate which naphthoquinones were found in each species, nor the relative quantities present.

### **5.2.2 Methodological difficulties in measuring root exudates**

Although root exudates have been mentioned in a number of allelopathy studies such as those by Callaway & Ashehoug (2000), Stinson et al. (2005) and Abhilasha et al. (2008), their identification and measurement *in situ* present methodological challenges to researchers. (Weidenhamer, 1996). As the wounding, defoliation or cutting of plants has been shown to affect the production of allelochemicals, (Rice, 1984; Thelen et al., 2005) it is not desirable to use methods involving the removal of sections of roots for the production of leachates.

The use of intact plants in a heterogeneous growing medium presents its own problems due to the multiplicity of confounding factors and the technical problems associated with collecting root exudates *in situ*. Two of the most successful approaches to date are those developed by Weidenhamer (2007) and Fujii et al. (2007) whose Plant Box Method will be explored here.

## **5.3 Plant Box Method**

### **5.3.1 Overview of Plant Box Method**

The advantage of this method is that it enables the allelopathic potential of root exudates to be determined using relatively undisturbed root systems placed into a standardised, homogeneous agar medium as was used successfully to determine the allelopathic potential of leachates using the Sandwich Method described in Chapter 4. The increasing proximity of the lettuce seedlings to the enclosed root system of the donor plant enables any differences in radicle elongation to be easily recorded. This reduces the problems inherent in a heterogeneous and opaque medium such as soil.

## **5.4 Materials and methods**

Donor plants were grown in sand according to the description given in Chapter 2. When plants were considered a suitable size for the Plant Box Method, they were removed from the growth room and prepared as described below in the section on sample preparation

### *Root zone separation cylinders*

In order to obtain reliable results, it was necessary that the root system of the donor plant was kept at a fixed distance from the lettuce seeds. This was achieved by inserting the whole root system into specially constructed root zone-separation cylinders which were obtained courtesy of Dr Yoshiharu Fujii of the National Institute of Agro-environmental Sciences, Tsukuba, Japan (Plate 5.1). They were constructed from 65 mm sections vinyl acetate water pipe, with an outer diameter of 32 mm and an inner diameter of 25 mm, giving a wall thickness of 7 mm. A section was cut out from the pipe to create an open window, which was covered with polyester gauze (Toray Teron, Japan #C-119 Skylark). The adhesive used to affix the gauze (Ethron) contained cyclohexane, methyl ethyl ketone and acetone. A 2 mm circular base plate was fitted to the tube using the same glue and the tubes were heated to 60°C for several hours to facilitate the evaporation of all solvents. Following this, the cylinders were washed in distilled water and allowed to dry before being dispatched to the UK.



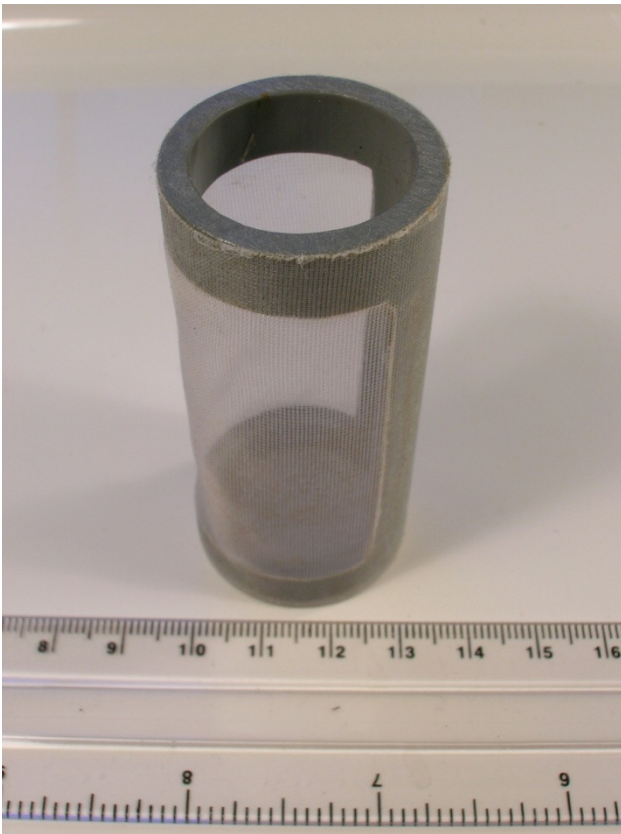


Plate 5.1. Root zone-separating cylinder into which donor plant root systems were inserted

### *Agar Preparation*

Low temperature agar (Nacalai Tesque, Tokyo, Japan, with a gelling temperature of 30-31°C) was used as in the Sandwich method (Chapter 4) at the same concentration of 0.75% w/v. It was autoclaved and cooled to a temperature of around 40°C in a water bath before use.

### *Preparation of plants*

Pots containing sand-grown plants of a suitable size were submerged in a bowl of distilled water and as a result, the sand medium was quickly and

easily washed away, leaving the exposed root system. Plants prepared in this manner were then removed and wrapped in moistened laboratory roll which was readily available in the laboratory. To prevent the plants from wilting before being placed in a shallow water-filled container while the plant boxes were being prepared. Each plant was given a label recording its species, number and date. Just prior to placing the tubes into the plant boxes, the moist laboratory roll was removed and the plants were placed in the root zone-separating tubes, with the plant's stem positioned so that the original planting depth was maintained when the agar was poured. The root zone-separating tube and the plant it contained was then placed into the Magenta vessel and fixed into one corner using cellophane tape attached to the stem of the plant. Individual boxes were labelled with the plant name, date of sowing and date of experiment.

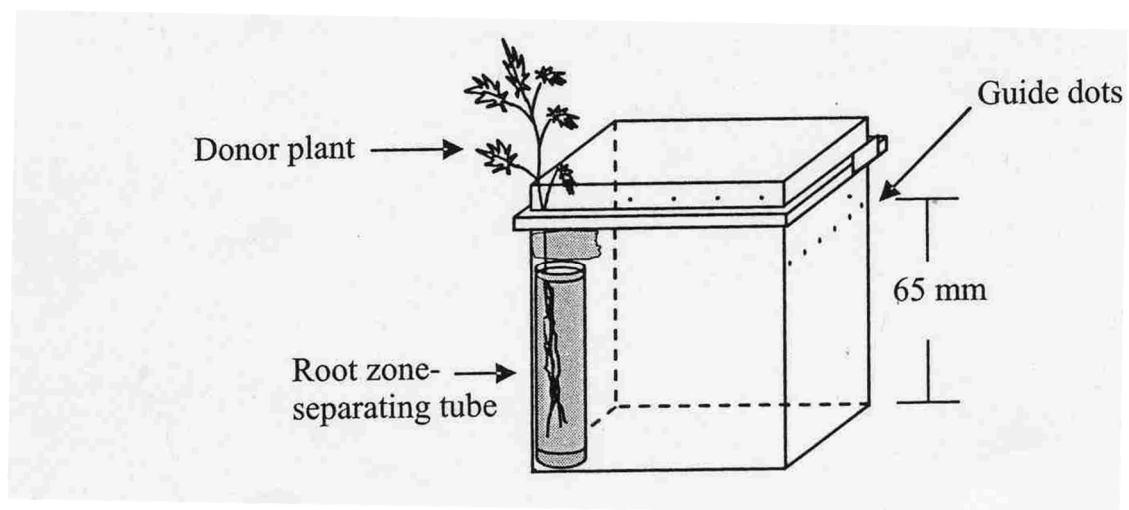


Figure 5.1. Positioning of donor plant in one corner of Magenta GA7 plant culture vessel prior to pouring agar. From Fujii (2004) used with permission of Dr Y. Fujii.

### *Preparation of Magenta GA7 Plant Culture vessels*

Magenta GA7 plant culture vessels (Magenta Corporation, Chicago, USA) measuring 60 x 60mm and 100mm height were marked with permanent ink dots at a height of 65mm from the vessel base (see Figure 5.1). This was to aid the correct placement of seeds as shown in Figure 5.2.

The agar was removed from the water bath and allowed to cool a little further before it was carefully poured into the Magenta, minimising the production of bubbles. The vessels were filled until the agar surface was in line with the line of guide dots. Immediately after pouring, the vessel was transferred to an ice water bath which ensured the temperature of the agar was reduced rapidly and thermal shock to the roots was minimised. Once the agar had solidified, the vessel was removed from the water bath and allowed to equilibrate to room temperature for an hour before lettuce seeding was carried out.

### *Seeding Process*

Lettuce seeds were inserted into the agar using ceramic tweezers, which gave the necessary fine control for accurate positioning. They were inserted with the narrower end (from which the radicle emerges) downwards to a depth of approximately half the seed's length. Thirty

three seeds in total were placed in each vessel in the arrangement shown in Figure 5.2.

Each vessel prepared as above was then inserted into an opaque black plastic sleeve reaching to the height of the agar surface so that the effects of light on the root system could be reduced to a minimum. A control vessel, with the same number of seeds arranged in a grid was also prepared using the seeding pattern illustrated.

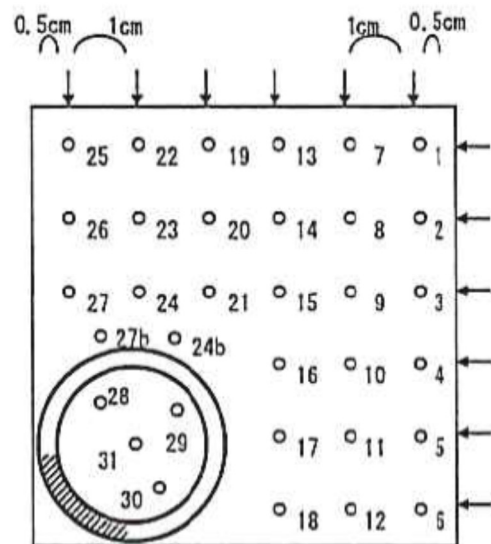


Figure 5.2. Seeding arrangement in Plant Box Method (from Fujii 2004). Numbers represent the order in which the seedlings are removed and measured rather than the order of seeding. Root-zone separating cylinder is shown in bottom left corner. Used with permission of Dr Y. Fujii.

### *Incubation*

In order to reduce evaporation from the surface of the agar and limit bacterial contamination, the tops of the vessels were sealed with polythene cling film.

The vessels were then placed in a Sanyo incubator with 25/20°C 12h/12h light/dark setting. Light intensity was measured using a Skye PAR meter (SKP 200, Skye Instruments Ltd, Llandrindod Wells, Wales, UK) and gave a reading of  $120 \mu\text{mol m}^{-2}\text{s}^{-1}$ . The plants were checked daily. To prevent transpiration losses from the plants which would have caused agar shrinkage and alteration to its density, the vessels were topped up with distilled water as required so that the agar surface remained level with the guide dots.

### *Measurement*

At the end of the five day incubation period, vessels were removed from the incubator and the lettuce radicles and hypocotyls measured using a sheet of laminated graph paper. Measurement of the radicles and hypocotyls were recorded on worksheets and then the data for each experiment were transferred to a customised Excel spread sheet, which produced a scatter plot of lettuce radicle elongation against distance from the donor plant's root system and calculated the coefficient of

determination  $r^2$  for the interaction between the two factors. The spreadsheet also calculated the percentage growth shown by the total of lettuce roots per treatment when compared to the control. As the lettuce hypocotyl measurements showed no correlation with distance from the donor plant's root system, they were not included in subsequent analyses.

Following the measurement of the lettuce seedlings, each donor plant was removed from the agar and its height recorded. The roots were separated from the rest of the plant using either scissors or a scalpel and both portions weighed before drying at 60°C until constant weight was reached, usually one or two days depending on the moisture content and the size of the samples. They were then reweighed and dry mass recorded.

## **5.5 Experiments carried out**

### **5.5.1 *I. glandulifera***

A total of 25 repeats of the Plant Box Method using *I. glandulifera* plants were carried out, consisting of four separate runs with varying numbers of plants according to their availability, correct size and suitability for inclusion in the Plant Box experimental system. Results are located in section 5.6.1.

### **5.5.2 *I. parviflora***

A total of 38 repeats of the Plant Box Method were carried out using *I. parviflora*, which consisted of four separate runs, with varying numbers per run, depending on the numbers of plants available and their suitability for inclusion in the Plant Box experimental system. Results can be found in section 5.6.2.

### **5.5.3 Effects of defoliation and damage of *I. glandulifera* plants on their allelopathic inhibition of lettuce radicles.**

As there was no conclusive support for the role of root mass in determining the allelopathic effects recorded in the two previous experiments, 5.5.1. and 5.5.2., a further experiment involving damaged *I. glandulifera* plants was undertaken. As noted previously, there is evidence to suggest that browsing or other physical damage to plants can affect their production and release of allelochemicals (Thelen et al., 2005; Xu, Wang & Luo, 2005) and many plants experience some degree of damage during the course of their lives; in the case of *I. glandulifera* slug herbivory has been identified as a major cause of plant damage and mortality (Prowse & Goodridge., 2003). Plants selected for this

experiment consisted of five visible nodes (1<sup>st</sup> node at cotyledon level) with four whorls of leaves.

Plants were randomly assigned into three treatment groups as follows:

- i. Intact – plants were not subject to defoliation or other damage.
- ii. Partially Defoliated. The upper two whorls of the plants were removed with a scalpel immediately prior to insertion into the Plant Box Method. Approximately 30% of the total leaf area was removed by this process.
- iii. Heavily defoliated, stems crushed. All foliage above the second node (approximately 75%) was removed and the stem was crushed firmly between the fingers above the first node before inserting into the root zone-separating tubes. Crushed stems had been noted previously to undergo rapid dieback at room temperature, leading to plant death within a few days. (See Figure 5.3.)



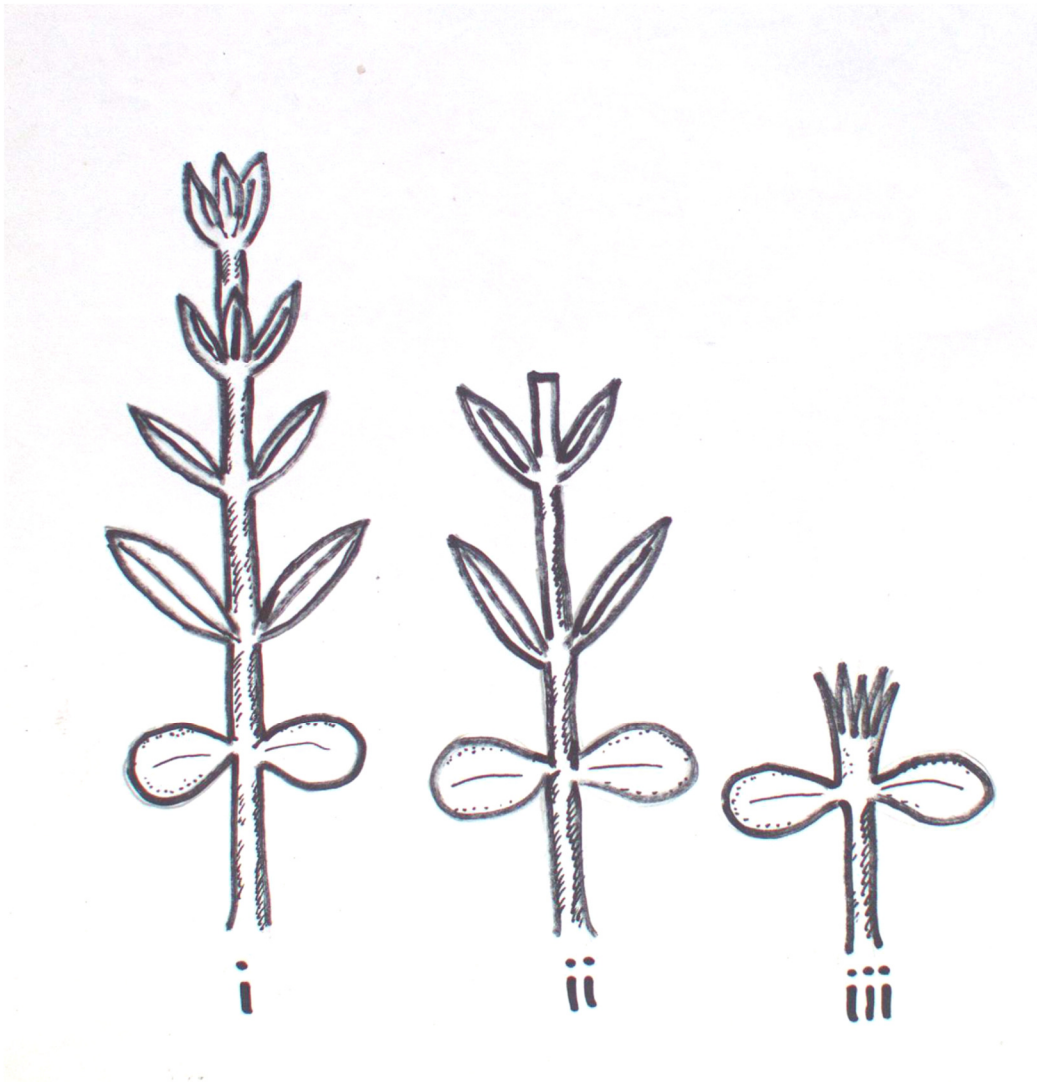


Figure 5.3. Treatments used in defoliation experiment, 5.5.3. i) intact, ii) approximately 30% defoliated, iii) approximately 75% defoliated, stem crushed above cotyledons.

Each treatment consisted of five replicates with a control vessel set up without the addition of an *I. glandulifera* plant as described in the general method in Section 5.4. The experiments were repeated twice. Results are described in Section 5.6.3.

## **5.6 Results**

### **5.6.1 *I. glandulifera***

The summary data for the four experimental runs are shown in Table 5.1. Data proved to be normally distributed when the Shapiro Wilk test was applied. W statistic = 0.943, p = 0.077. Mean value was 22.176, with standard deviation of 12.571, indicative of the wide dispersion of the data. They indicate a very highly statistically significant correlation between the distance from the donor plant's root system and the degree of lettuce radicle inhibition evident, with p values mostly below 0.001. Of the 25 plants tested, only one had a p value greater than 0.05. Figure 5.5. shows the individual plants plotted as percentage elongation compared to control. There was a wide range of values, with the maximum inhibition percentages of 3% of control and the minimum 47% of control. In all cases lettuce radicle elongation was inhibited by more than 50%.

Table 5.1. Summary data collected for 25 repeats of the Plant Box Method using *I. glandulifera*

<b>Nº</b>	<b>Root mass mg</b>	<b>Slope</b>	<b>Intercept</b>	<b>% growth</b>	<b>r</b>	<b>n N=33</b>	<b>r<sup>2</sup></b>	<b>p</b>
1	121	0.793	7.06	19	0.7614	31	0.5798	<0.0001
2	936	0.633	1.915	5	0.7084	25	0.5019	<0.0001
3	292	0.937	7.456	20	0.8605	31	0.7405	<0.0001
4	246	0.678	17.753	47	0.5831	30	0.34	0.0007
5	307	0.729	3.479	9	0.8749	32	0.7654	<0.0001
6	71	0.65	12.707	34	0.5341	33	0.2852	0.0011
7	147	1.306	12.448	33	0.7804	33	0.6091	<0.0001
8	258	1.153	12.488	36	0.8408	32	0.7069	<0.0001
9	126	1.146	8.705	23	0.8139	33	0.6625	<0.0001
10	540	0.304	2.881	17	0.5965	23	0.3559	0.0046
11	417	0.142	8.038	47	0.373	22	0.1392	0.0873
12	1290	0.394	6.288	37	0.6155	33	0.3789	0.0001
13	1429	0.416	5.247	30	0.8456	31	0.715	<0.0001
14	719	0.344	3.889	23	0.6125	23	0.3751	0.0001
15	331	0.651	3.762	22	0.7838	29	0.6144	<0.0001
16	802	0.63	5.754	15	0.3905	26	0.1525	0.0486
17	650	0.616	4.097	11	0.6023	32	0.8627	0.0002
18	164	0.789	9.119	24	0.7063	31	0.4989	<0.0001
19	249	0.63	3.966	11	0.7818	29	0.6113	<0.0001
20	550	0.621	2.863	8	0.7496	30	0.5619	<0.0001
21	582	1.001	1.05	3	0.8307	29	0.6901	<0.0001
22	354	0.321	6.144	16	0.5787	27	0.3349	0.0016
23	490	0.713	3.647	10	0.8795	30	0.7735	<0.0001
24	217	0.357	7.409	20	0.7433	33	0.5526	<0.0001
25	222	0.89	5.321	14	0.8534	33	0.7284	<0.0001

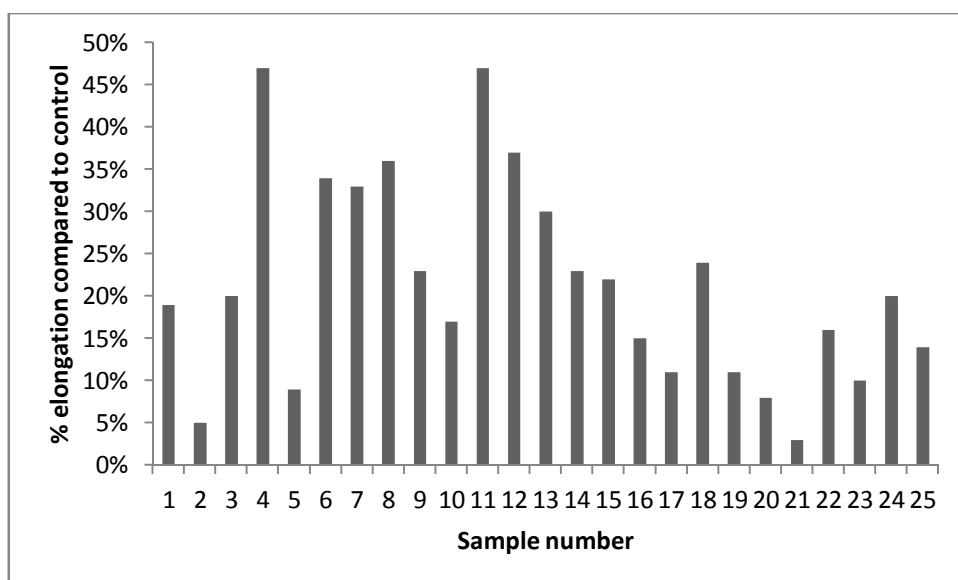


Figure 5.4. Percentage lettuce radicle elongation compared to the control in 25 repeats of the Plant Box Method using *I. glandulifera* as donor plant. In all cases inhibition exceeded 50%.

The scatterplots and regression lines were produced for all individual experiments. Included here are three representative examples showing very high inhibition, high inhibition and moderate inhibition, namely 9, 20 and 36% elongation compared to the control (figure 5.6). The results offer strong evidence in support of the assertion that root exudates are responsible for the inhibition seen. When increasing root mass was compared with degree of lettuce inhibition, no significant correlation was observed ( $r^2 = 0.0348$ ) (see Figure 5.6). This was demonstrated by Sample 4 and Sample 11 in Table 5.1, which show the same percentage elongation compared to the control (47%), yet the root mass of Sample 11 is nearly twice that of Sample 4. Seed germination numbers were not affected by increases in root mass either ( $r^2 = 0.01529$ ), see Figure 5.7. In fact two of the largest root masses recorded (Sample 13 = 1290 mg and Sample 14 =

1429 mg) gave seed germination numbers of 33 and 31 respectively. This supports the finding of the experiments carried out in the previous chapter where there was little evidence of germination inhibition using the Sandwich Method.

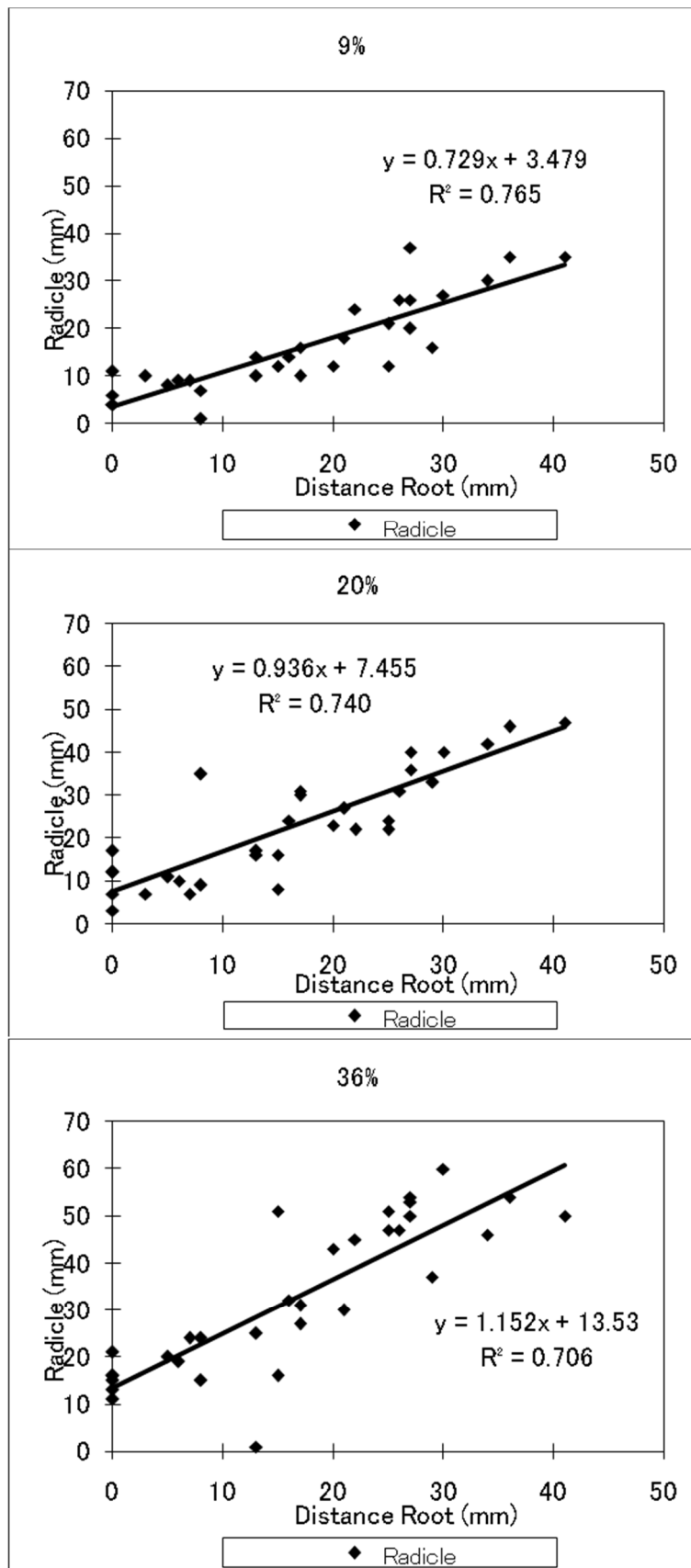


Figure 5.5. Regression analysis for three Plant Box experiments using *I. glandulifera*, with percentage elongations compared to the control of 9% (very high inhibition), 20% (high inhibition) and 36% (moderate inhibition).

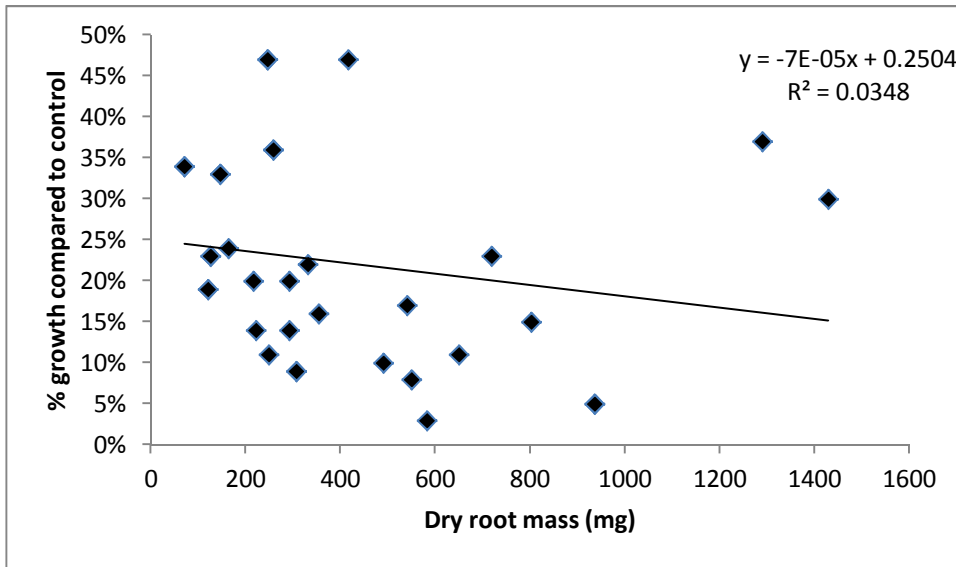


Figure 5.6. Effect of increasing *I. glandulifera* root mass on the elongation of lettuce radicles in the Plant Box Method experiments. There was no significant interaction between factors.

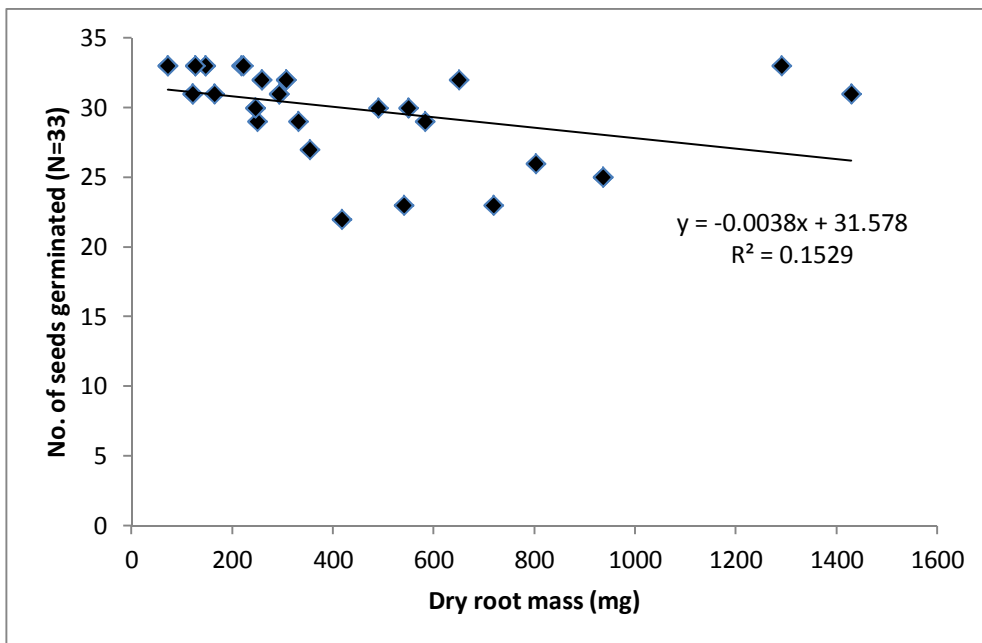


Figure 5.7. Effect of increasing *I. glandulifera* root mass on successful lettuce seed germination in Plant Box Method experiments. There was no significant interaction between factors.

One of the more notable effects produced by the *I. glandulifera* plants in the Plant Box Method experiments was the staining of the agar by a bright orange pigment which clearly originated from the roots. This can be seen in Plates 5.2., 5.3. and 5.4. These suggest that the pigment produced might have been involved in the allelopathic inhibition observed. Some of the most heavily stained agar was removed and frozen, with the intention of extracting the pigment and identifying it by means of HPLC. Unfortunately this was not possible during the duration of this study. An outline of the work originally envisaged can be found in Appendix 1.

Also visible was the reduction in lettuce seedling size with increasing proximity to the root zone-separating cylinder, although there was no evidence of reduction in the numbers successfully germinating in this experiment. In fact, seedlings can clearly be seen successfully emerging from the top of the root zone-separating cylinder itself. The variability in staining which developed can be seen when Plates 5.2 and 5.4 are compared.



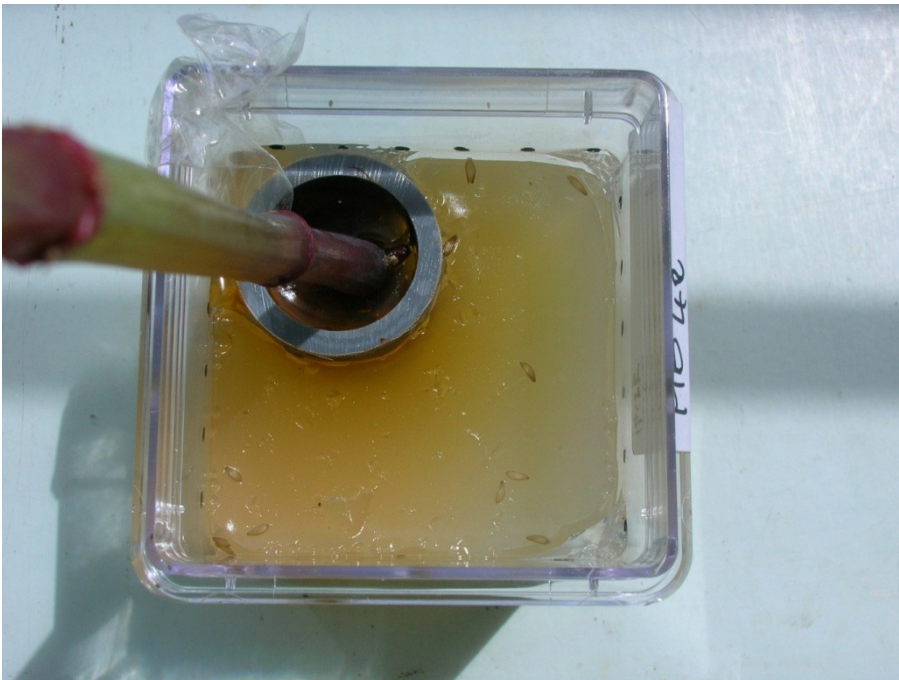


Plate 5.2. Aerial view of *I. glandulifera* in Plant Box Method, showing clear zone of staining around the root zone-separating cylinder (lettuce seedlings removed for measurement)

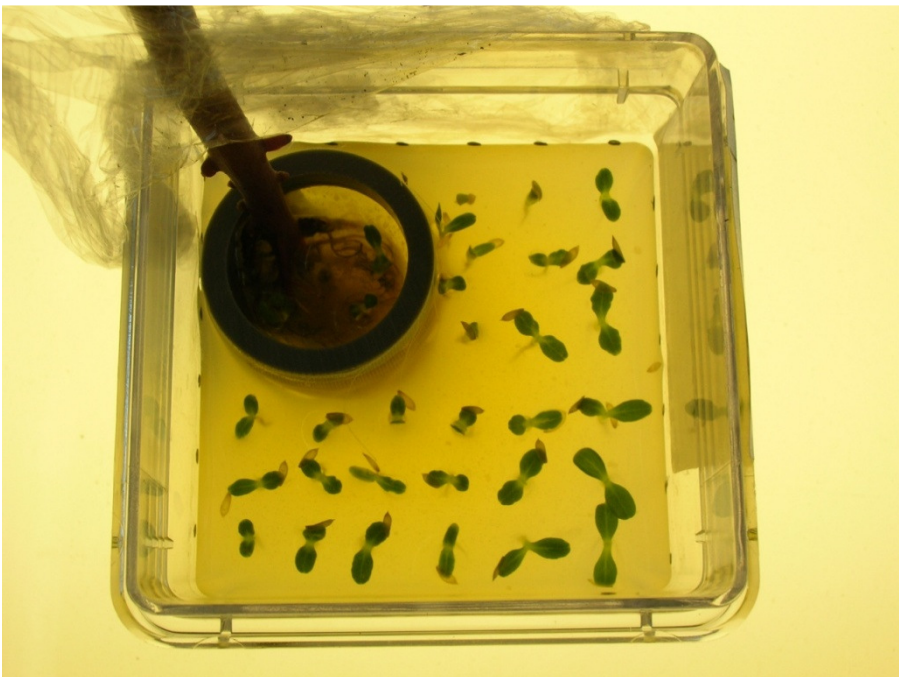


Plate 5.3. Aerial view of *I. glandulifera* in Plant Box Method, showing clearly visible reduction in the size of lettuce seedlings as they approach the root zone-separating cylinder, although germination does not appear to be affected.



Plate 5.4. Side elevation showing the effects of increasing *I. glandulifera* root proximity on lettuce radicle inhibition. Orange staining of the agar is clearly visible

### 5.6.2 *I. parviflora*

A total of 38 plants were used in the experiments, which consisted of 4 separate runs. The summary data is shown in Table.5.2. As in the case of *I. glandulifera*, a highly significant correlation between the distance from *I. parviflora* root system and the amount of inhibition experienced by the lettuce radicles was evident, with the majority of samples having p values of less than 0.001. Only two samples, 22 and 38, did not show statistically significant correlations between distance from roots and the degree of inhibition experienced by the lettuce radicles.

Figure 5.12 shows the heights of the individual plants plotted as percentage elongation compared to the controls. Results varied widely, with the maximum inhibition of lettuce radicles reaching as low as 2% of control and a minimum inhibition of 55% of control.

Typical scatterplots taken from experiments producing very highly inhibitory, highly inhibitory and moderately inhibitory results are shown in Figure 5.13. As in the case of *I. glandulifera*, there was little variation in the number of seeds germinating, with the mean value being close to 30  $\pm 3$ .

Table 5.2. Summary data collected for 38 repeats of the Plant Box Method using *I. parviflora*

Nº	Root mass mg	Slope	Intercept	% growth	r	n N=33	r <sup>2</sup>	P
1	295	0.78	9.131	25	0.6264	31	0.3924	0.0002
2	346	0.316	3.208	9	0.6868	32	0.4718	<0.0001
3	234	0.715	3.439	10	0.7838	26	0.6134	<.0001
4	263	0.589	5.948	17	0.8288	30	0.6869	<0.0001
5	40	0.664	5.325	15	0.6064	31	0.3678	0.0003
6	430	0.513	4.526	13	0.7377	29	0.5442	<0.0001
7	251	0.388	5.674	16	0.5685	29	0.3232	0.0013
8	172	0.312	7.967	22	0.5856	30	0.343	0.0007
9	346	0.924	8.859	25	0.843	31	0.7106	<0.0001
10	412	0.394	4.44	12	0.7134	28	0.509	<.0001
11	81	1.195	16.563	32	0.8378	31	0.702	<0.0001
12	192	1.268	11.74	23	0.8974	31	0.8053	<0.0001
13	143	0.804	27.103	53	0.609	31	0.3708	0.0003
14	394	0.849	19.102	37	0.6213	33	0.386	<0.0001
15	136	0.986	3.583	7	0.8425	23	0.7098	<0.0001
16	125	1.086	11.265	22	0.8176	30	0.8716	<0.0001
17	67	1.473	7.498	15	0.897	32	0.8047	<0.0001
18	87	1.556	14.396	28	0.8479	29	0.719	<0.0001
19	324	1.017	10.55	21	0.6996	28	0.4895	<0.0001
20	109	1.22	3.164	6	0.9065	32	0.8218	<0.0001
21	88	1.188	11.364	22	0.8789	31	0.7725	<0.0001
22	128	0.308	21.135	41	0.2578	15	0.2578	0.3536
23	179	1.272	14.023	27	0.9132	33	0.8839	<0.0001
24	656	0.903	0.533	2	0.8243	30	0.6794	<0.0001
25	342	0.893	4.042	13	0.8408	33	0.707	<0.0001
26	687	1.134	9.999	33	0.8992	30	0.8085	<0.0001
27	215	0.932	3.546	12	0.7729	32	0.5973	<0.0001
28	183	1.249	13.36	44	0.8678	29	0.7531	<0.0001
29	186	0.718	10.858	35	0.8082	33	0.6532	<0.0001
30	467	0.774	7.464	24	0.9268	33	0.859	<0.0001
31	498	0.812	10.517	34	0.8502	33	0.77228	<0.0001
32	106	0.996	16.713	55	0.9039	32	0.8171	<0.0001
33	533	0.574	3.638	12	0.7724	33	0.5967	<0.0001
34	224	1.291	5.511	18	0.9445	33	0.8921	<0.0001
35	419	0.334	8.018	26	0.5959	30	0.3551	0.0005
36	223	1.218	6.54	21	0.8885	33	0.7895	<0.0001
37	307	0.932	5.755	19	0.9432	30	0.8896	<0.0001
38	403	0.11	15.922	52	0.1942	33	0.0377	0.2788

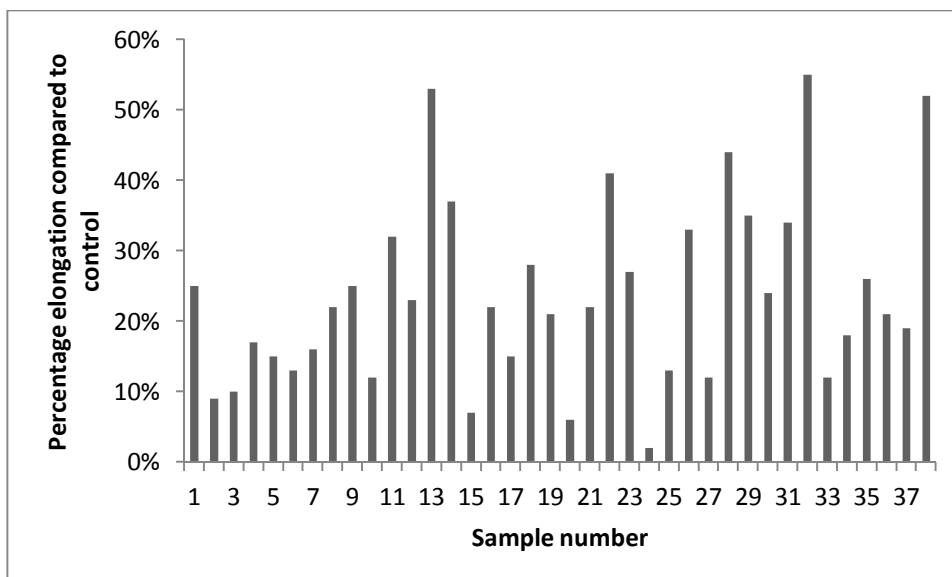


Figure 5.8. Percentage lettuce radicle elongation compared to the control (100%) in 38 repeats of the Plant Box Method using *I. parviflora* as donor plant

As with *I. glandulifera*, the data proved to be normally distributed when the Shapiro-Wilk test was applied. W statistic = 0.942,  $p=0.061$ . Mean value was 24, with standard deviation of 13.282, indicative of the wide dispersion of the data as might be expected from the graphical presentation of the data in Figure 5.12 above.

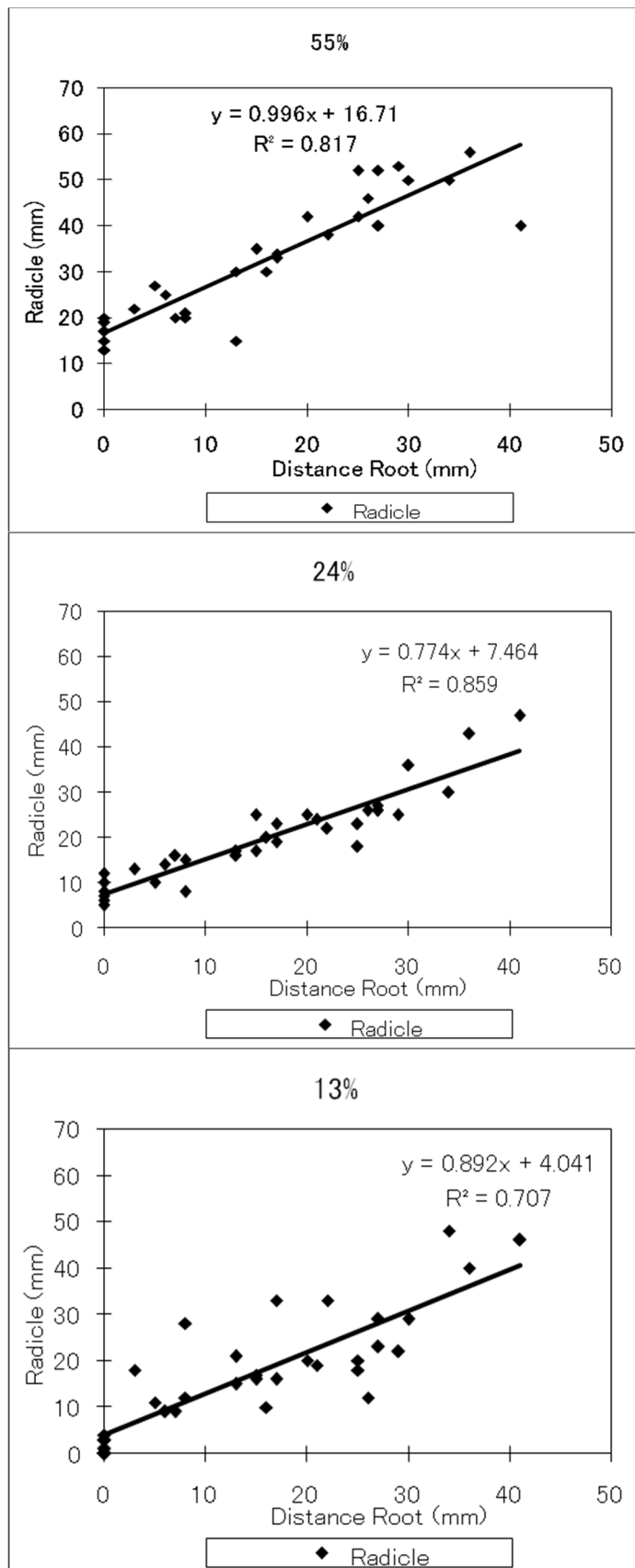


Figure 5.9. Regression analysis for three Plant Box experiments using *I. parviflora*, with percentage elongations of 13% (very highly inhibitory), 24% (highly inhibitory) and 55% (moderately inhibitory) when compared to the control.

When dry root mass versus lettuce radicle elongation were plotted, there did not appear to be significant interaction between the two factors, as evidenced by the low coefficient of determination ( $r^2 = 0.0166$ ). Similarly, the number of seeds which successfully germinated were not affected by dry root mass ( $r^2 = 0.0296$ ). Data are presented graphically in Figures 5.14 and 5.15.

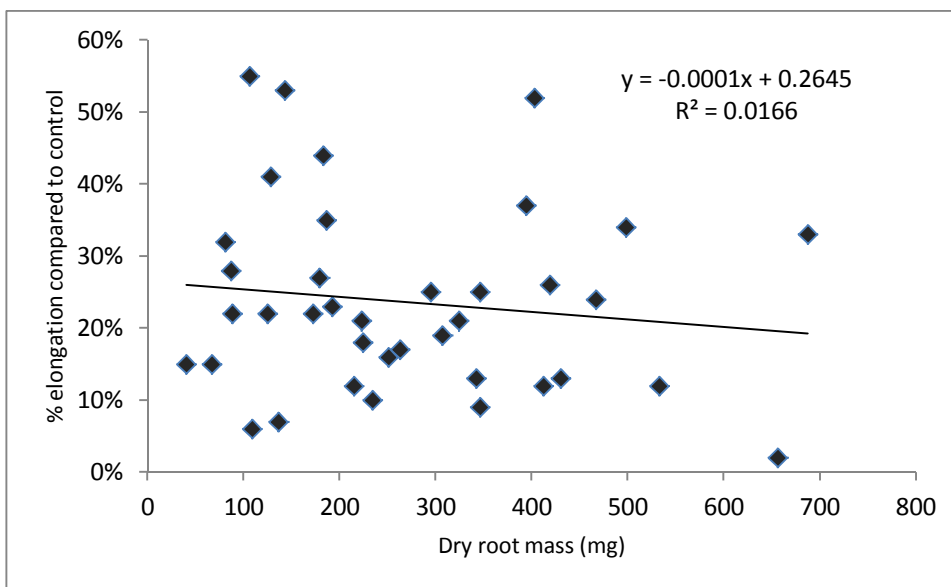


Figure 5.10 The effects of increasing *I. parviflora* root mass on the elongation of lettuce radicles in the Plant Box Method. There was no statistically significant interaction between the variables.

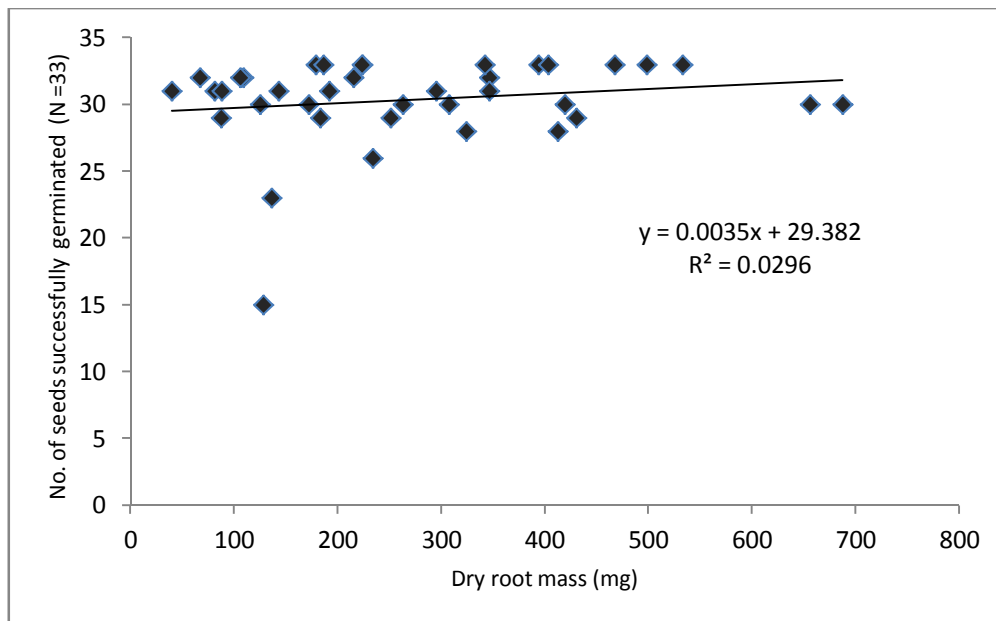


Figure 5.11. The effects of increasing *I. parviflora* root mass on successful lettuce seed germination in the Plant Box Method. There was no statistically significant interaction between the variables.

### 5.6.3 Comparison of the allelopathic potential of *I. glandulifera* and *I. parviflora*

When the two species were compared using the Mann-Whitney Rank Sum Test, no statistically significant difference was evident, indicating that their allelopathic potentials, although individually highly significant, were not distinguishable from each other. (Mann-Whitney U statistic = 536.500,  $T = 1158.55$   $n(\text{small}) = 34$ ,  $n(\text{big}) = 36$ . ( $p = 0.573$ ).



#### 5.6.4 Effects of defoliation and wounding damage on allelopathic potential of *I. glandulifera*

To normalise the data collected, results were  $\log_{10}$  transformed before being subjected to one-way ANOVA. There were no significant differences between the treatments,  $F_{2, 26} = 1.663$ ,  $p = 0.209$ , although when presented graphically, a slight variation in mean height was visible between the treatments (Fig 5.16).

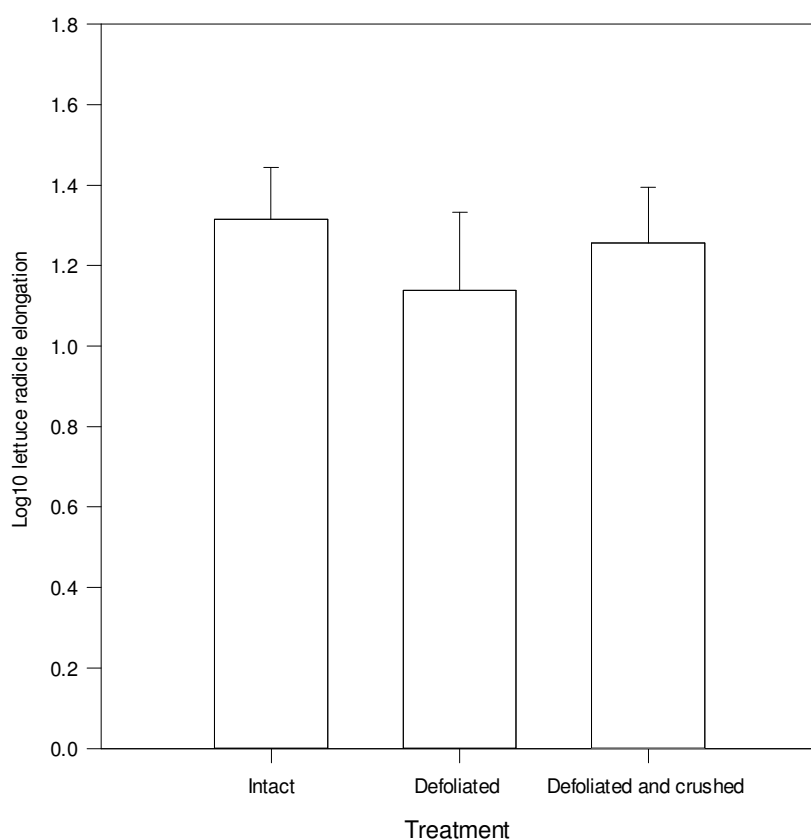


Figure 5.12. Comparison of defoliation with defoliation and stem crushing on the allelopathic inhibition of *I. glandulifera* on lettuce radicles using the Plant Box Method. Data were  $\log_{10}$  transformed. Error bars represent 95% confidence intervals. There was no significant difference between treatments ( $p = 0.209$ )

## **5.7 Discussion**

Both *Impatiens* species tested caused a large and statistically significant inhibition to lettuce radicle elongation and there was a strong correlation between the proximity of the lettuce seedlings to the roots of the donor plants and the amount of inhibition they experienced. There was, however, no significant difference between the two species in terms of their effects on lettuce radicle elongation. This suggests that differences in root exudate allelopathy are not likely to be the single most important factor in determining the relative invasiveness of the two *Impatiens* species studied. Other factors may act synergistically or antagonistically to amplify or reduce these effects.

Neither *I. glandulifera* nor *I. parviflora* showed any significant interaction between increasing root mass and the degree of inhibition caused to the lettuce radicles when compared to the controls. This suggests that the effects observed are not density dependent as might have been expected from the results of other experiments involving allelopathic effects. For example, samples 4 and 11 of *I. glandulifera* (Figure 5.1) show the same reduction of lettuce radicle length to 47% of the control, yet the root mass of sample 11 is close to twice that of sample 4. Similarly, the number of lettuce seedlings which successfully germinated was not affected significantly by the root mass of the donor plants. This suggests that the allelopathic effects which can apparently be

attributed to *Impatiens* root exudates do not inhibit germination at the levels produced in the Plant Box experimental system. Inhibitory effects are, in fact, limited to seedlings which have already begun radicle extension.

When plants were deliberately defoliated and wounded, a process which has been linked to the increased production of allelochemicals (Rice, 1984; Thelen et al., 2005), effects were not distinguishable from plants which were left undamaged. This suggests that the effects observed were located in the response of the roots to conditions within the Plant Box system rather than induced responses to the damage inflicted on aerial parts of the plants.

Rice (1984) reported that allelopathy researchers were frequently faced with the kind of variability shown in these experiments and suggested that the differences might lie in a genetically heterogeneous response to environmental variables. In the case of the Plant Box Method, differing responses of genetically unique individuals to temperature, oxygen levels, or some other unidentified factor might have accounted for the variation seen. In most published studies using the Plant Box Method the degree of replication has been limited to 3 to 5 replicates. This study has considered a much larger data set and therefore the results are likely to be more representative of the degree of variation seen within populations of plants which show allelopathic effects on other species.

One way to test this possibility would be to produce cuttings from a single plant. As mentioned in Chapter 3, it was easy to root stems and axillary shoots of both *I. glandulifera* and *I. parviflora*. A number of different individual plants could be used as stock plants from which cuttings are taken; these could then be grown in the sand medium under controlled conditions as described previously in Chapter 3. The resultant plants, with their identical genetic make-up could then be tested using the Plant Box Method to ascertain the influence of genetic factors on the degree of inhibition induced.

The production of large quantities of an orange pigment, resembling lawsone, suggests that this and other naphthoquinones could be responsible for some of the inhibition observed. Before any definite conclusions can be drawn, however, identification of the compound or compounds responsible for colouring the agar should be carried out. In addition to the naphthoquinones present in *Impatiens*, other similarly pigmented phenolic compounds such as quinones, flavonoids and tannins, could also be responsible for the effects seen (Dayan , Howell & Weidenhamer, 2009; Li et al., 2010; Netzly & Butler, 1986).

In some instances some of the roots of the plants appeared to have died during their time in the agar. Their sensitivity to waterlogging has been mentioned previously.

The effects shown by the Plant Box experiments were pronounced; it is highly likely that these effects would be much less obvious in the case of plants growing in a heterogeneous soil matrix containing a healthy population of microorganisms. Under such circumstances, accurate root measurement *in situ* would be impossible to achieve and some proxy measurement would need to be taken instead. Soil based experiments which investigate an alternative approach to root exudate allelopathy using a target-neighbour design in 1 litre plant pots are described in Chapter 7.

Whether or not the data are an artefact of the method, the evidence supporting the possibility that *Impatiens* species may affect the growth of competitors by means of allelopathic root exudates is strengthened by this study.

As mentioned previously, it was intended to use HPLC to identify the naphthoquinones present in the *Impatiens* species, but this was not completed. An outline of the experimental aims, equipment and methods used can be found in Appendix 1.

## 6 An investigation into seed and seedling allelopathy in *Impatiens glandulifera* and *I. parviflora* using the Modified Equal Compartment Agar Method (MECAM)

### 6.1 Introduction

The Plant Box Method (Fujii et al., 2007) described in Chapter 5 demonstrated that the root systems of well developed (although not mature) plants of *I. glandulifera* and *I. parviflora* were capable, under controlled experimental conditions, of inhibiting the development of the test species, *Lactuca sativa*; these allelopathic effects intensified with increasing proximity to the donor plant's root system. This suggested that the diffusion of water soluble allelochemicals from the donor's roots were likely to be responsible for the inhibitory effect demonstrated.

The *Impatiens* plants used for the Plant Box studies would be expected to take several months to reach an equivalent size or life stage when growing outside, an event which would normally occur sometime in late spring or early summer. By this date, however, it is likely that any late germinating competitors would have already been suppressed or killed by the combination of soil moisture deficit, shading and nutrient acquisition caused by the dense and precocious growth of the *Impatiens* plants (Beerling & Perrins, 1993; Winsor, 1983). Thus any allelopathic or phytotoxic effects, if present, might be obscured. The true ecological

relevance of this allelopathic potential could therefore be open to question, at least at this relatively advanced stage in the plant's lifecycle.

A previously unexplored possibility is that in addition to a strategy of synchronous, early germination, *Impatiens* seeds and seedlings may also release allelochemicals into the soil as part of the germination process. This would provide them with a further competitive advantage over the emerging seedlings of other species. The presence of naphthoquinones in *I. glandulifera*, *I. parviflora*, *I. capensis* and *I. nolitangere* tissues has been previously established (Lobstein et al., 2001) and extracts from leaves, stems and roots have been shown to exert inhibitory effects on the germination and development of several plant species (Vrchotová & Krejcova, 2008). Published studies on the effects of germinating *Impatiens* plants on the development of competitors are singularly lacking, however. This chapter aims to address this crucial aspect of *Impatiens* seed and germination biology. The fact that seedling root exudates represent 30-40% of a plant's photosynthetic productivity (Whipps, 1990) and decline with increasing plant age (Bertin, Yang & Weston, 2003) provides further support for an investigation into the allelopathic potential of *I. glandulifera* during germination and shortly thereafter. This is a time when allelopathic effects would be most beneficial to the plant's establishment and coincides with a moment in the plant's lifecycle when allelochemical exudation is likely to be at its peak.

In the case of crop plants, the critical period for yield reduction from weeds is the first month of crop growth (Olofsdotter, 2001) and it seems likely that a similar inhibitory effect might be exerted on newly emerging competitors by the already established *Impatiens* seedlings.

## **6.2 Studies into the allelopathic effects of germinating seedlings on competitors**

Higashinakasu et al (2004) showed that germinating seeds can exert allelopathic effects on the germination and growth of seedlings of other species. In addition to the cereal, legume and weeds described in this study, other species which have demonstrated allelopathic effects when germinating are: watermelon (*Citrullus lanatus*) (Kushima et al., 1998), sunflower ( *Helianthus annuus*) (Ohno et al., 2001), *Arabidopsis thaliana* (Yokotani-Tomita et al., 1998), cress (*Lepidium sativum*), (Hasegawa et al., 1992) and coffee (*Coffea arabica* ) (Friedman & Waller, 1983). These studies concentrated on crop species rather than invasive alien weeds; the literature provides strikingly few examples of the effects of germinating invasive alien weeds on the development of their seedling competitors. Rice (1984), for instance, makes no mention of it in his monograph Allelopathy (2<sup>nd</sup> edition). Most studies involve the effects of leaf leachates from established plants of invasive alien species (Sun et al., 2006), (Yang et al., 2007), (Belz et al., 2007) rather than the exudates or leachates from



germinating seeds and seedlings, or are pot- based studies involving the effects of established plants on the germination and subsequent development of acceptor species (Abhilasha et al., 2008; Maharjan, Shresta & Jha, 2007; Prati & Bossdorf, 2004).

### **6.3 Experimental aims**

As no studies into *Impatiens* seedling allelopathy had been published at the time of this investigation, it was decided to explore some of the existing protocols and modify the chosen one to suit the following questions which arose as a result of previous investigations.

These were:

- a) Are allelopathic exudates produced by germinating *Impatiens* seeds able to inhibit the growth of receiver seedlings?
- b) The seedlings of *I. glandulifera* usually emerge weeks before those of their competitors. If allelopathic inhibition is present, does early germination increase the intensity of its effects? This could occur due to an increase in allelopathic output as the *Impatiens* seedlings develop, or by the accumulation of allelochemicals in the growing medium in which both species are growing.
- c) Are the putative allelochemicals produced by the *Impatiens* seedlings able to diffuse through the growing medium and affect

the growth of receiver seedlings at a distance as was shown in the Plant Box Method described in Chapter 5?

A bioassay design was sought which would enable these questions to be answered, using a simple, easily replicable design. Many of the published studies use aqueous extracts (Dorning & Cipollini, 2006; Guenzi, McCalla & Norstadt, 1967; Kimber, 1967) and were therefore not sufficiently versatile to answer the questions posed above as they did not involve the release and movement of allelochemicals through a common growing medium. Two possible methodologies for the study were investigated for their suitability. These were the Equal Compartment Agar Method (ECAM) (Wu et al., 2000b) and the Relay Seeding Technique of Navarez & Olofsdotter (1996). Both methods involve the growing of the donor and receiver seedlings together for a specified period. In the case of the Relay Seeding Technique, seedlings are grown in petri dishes containing perlite, whereas the ECAM relies on beakers containing agar. After due consideration, it was decided that the ECAM, in modified form, would produce a more versatile bioassay, mainly due to its use of agar as the medium of allelochemical transfer, which allows for accurate placing of seeds at set distances from each other. Another disadvantage of the Relay Seeding Technique was the necessity to trim the rice seedlings if their growth was excessive; this process is known to stimulate allelochemical production in some plant species (Rice, 1984). Agar had been used

successfully in previous experiments as the medium of allelochemical transfer in the Sandwich and Plant Box Methods and some familiarity with its properties had been developed. In addition, agar media had shown themselves to be easily prepared and stored until required, easily dispensed and having standardised properties when made up to the same formulation; these are invaluable characteristics when investigating allelopathic interaction. As the abundance of confounding factors are frequently cited by sceptics as limiting the validity of allelopathy experimental designs, the use of a medium with predictable properties was obviously advantageous. A brief description of the original method is given below, followed by the modifications used in this study.

#### **6.4 The Equal Compartment Agar Method (ECAM)**

The ECAM (Wu et al., 2000b) was originally used as a screening method for assessing seedling allelopathy in wheat (*Triticum aestivum*) accessions. The original design used glass beakers filled with 30 ml of 0.3% water agar and the pre-germinated seedlings of the donor (*Triticum aestivum*) and acceptor species (in this case *Lolium rigidum* was used) placed in rows on either side of a notional midline. To eliminate inter-species light competition, a piece of white card was placed between the two types of seeds, along the midline of the beaker, just above the agar surface, thus creating the two equal compartments; the shared agar medium allowed

root intermingling and free diffusion of allelochemicals. By autoclaving the agar and sterilising the seeds prior to insertion, the direct effects of allelopathy of the wheat on *Lolium rigidum* were explored, without the complicating factors of microbial activity or contamination. Beakers were placed in growth cabinets and different agar concentrations, seed densities and exposure times were used in order to determine the optimum protocols.

#### **6.4.1 Modifications**

For this study, some modifications and simplifications were implemented to the original ECAM design so that it better fitted the purposes outlined above:

- 1) Rather than the glass beakers specified by Wu et al., (2000b), Magenta GA-7 plant culture vessels, as used previously in the Plant Box Method, were adopted. They had the advantage of tough, shatter-resistant construction, square shape for more efficient space utilisation and autoclavable lids.
- 2) The thin white card used by Wu et al., (2000b) was substituted by rotating the vessels one quarter turn in a clockwise direction per day, while at the same advancing their position from front to back of the incubator. This was carried out to equalise light and

temperature variations between treatments within the growth chamber.

- 3) Both lettuce and *Impatiens* are dicots, with leaves which tend to be aligned horizontally. This is in contrast to the monocots *Triticum* and *Lolium* used in the original ECAM experiments, which have a generally vertical leaf orientation. In order to reduce light competition between individuals and species and provide them with sufficient space to align their cotyledons correctly, it was decided to reduce the numbers of seeds of each species from 12 to 6. This reduction in seed number also had the additional benefit of diluting the potential amount of allelochemical released by the donor seedlings (Romeo, 2000), perhaps rendering their levels more realistic and representative of natural conditions than those obtained in the original ECAM.
- 4) The lettuce seeds were placed in two rows, at 15 and 30 mm distances from the closest germinating seeds of the donor *Impatiens*. The measurements of the two rows were recorded separately so that order that differences in their length, reflecting the diffusion rate of any allelochemicals could be detected.

Full details of the experimental protocol are conceptualised in Figures 6.1 and 6.2.

An initial experiment demonstrated that germinating seeds of *I. glandulifera* were able to grow and develop successfully within the Modified Equal Compartment Agar Method (MECAM) system for a period of up to 3 weeks. After this time, the *I. glandulifera* seedlings died quickly, presumably due to nutrient shortages. This was therefore determined to be the maximum time period during which a complete experimental run could take place.

### **6.5 Vessel Preparation**

Magenta® vessels were filled with 30 ml 0.3% water agar, Bacteriological agar no.1 agar (Oxoid – Thermofisher Scientific Inc), which was chosen for its superior diffusion properties. Agar was boiled in a microwave to melt it and then pipetted into the Magentas using a syringe. The lids were sealed and the Magentas were autoclaved at 121°C for 15 minutes in a Boxer autoclave before being allowed to cool to room temperature. If not required immediately, the Magentas were stored sealed in trays at room temperature in a low light environment.

### **6.6 Seed Treatment**

Seeds of *I. glandulifera* and *I. parviflora* were collected from wild or laboratory grown plants and sterilised prior to storage by immersion in a

10% commercial bleach (sodium hypochlorite) solution for 15 minutes. They were then washed three times in sterile distilled water and placed on sterile filter papers in Petri dishes for the period of stratification, which usually took about three months. Petri dishes were sealed using parafilm and placed in the dark at 4°C. They were checked once a week initially and where mould growth became obvious, infected seeds were removed and the remaining seeds washed in chilled sterile distilled water and replaced on clean, sterile filter papers. In cases where the filter papers showed signs of excessive drying, the Petri dishes were opened, moistened with sterilised distilled water and then resealed. Seeds were considered ready for the experimental procedure when emergent radicle length reached 1 mm (Rashid, Asaeda & Uddin, 2009) or, if insufficient numbers of seeds in this condition were available, when splitting or bulging of the testa indicated that radicle emergence was imminent. The use of pre-germinated seeds was recommended by Wardle et al., (1993) as a means of reducing experimental error, increasing the sensitivity of elongation measurements and ensuring that the correct number of seedlings were available for the chosen experimental design and subsequent analyses. In contrast, the lettuce variety used as the acceptor plant ('Great Lakes', (Nicky's Nursery, Broadstairs, Kent, UK) had previously shown rapid and reliable germination under a range of temperatures and it was therefore not considered necessary to pre-

germinate them prior to the experiment. Instead they received the same sterilisation treatment as the *Impatiens* seeds and were then allowed to surface dry in a laminar flow cabinet before use in order to facilitate their easy manipulation.

Vessels were placed in a laminar flow cabinet to maintain sterile conditions whilst opening and seed insertion was carried out.

### **6.7 Arrangement of seeds in Vessels**

Seeds of lettuce and the donor *Impatiens* species were placed, on either side of the notional midline of the base of the Magenta, with both acceptor and donor species arranged in two rows of three individuals. The two lettuce seed rows were positioned at distances of 15 mm and 30 mm from the closest row of *Impatiens*, so that any differences in inhibition related to distance from the putative allelopathic source could be measured. Seeds were inserted with sterile ceramic tipped tweezers as recommended by Fujii for the Plant Box Method (Fujii et al., 2007). In order to prevent cross contamination, they were dipped in 70% ethanol between treatments and then wiped dry on sterile tissue paper. To aid quick and accurate placement of the seeds, a simple template marked on a piece of laminated graph paper was used. Each Magenta vessel was placed upon this and the dots used as a guide for the placement of the seeds in the agar.



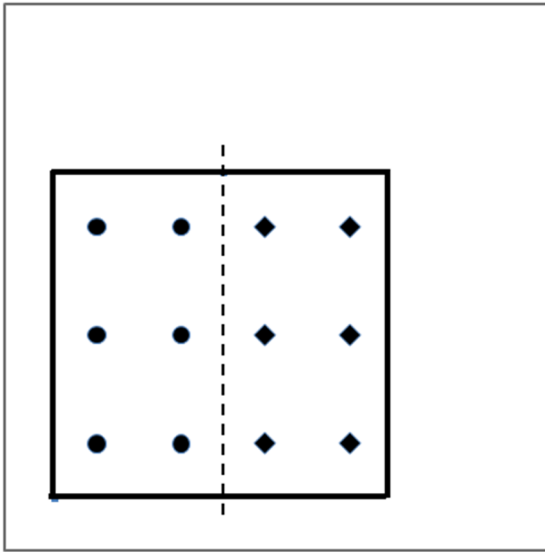


Figure 6.1. Magenta Vessel 60 x 60 mm with location of seeds shown. Circles (●) represent *Impatiens* seeds, diamonds (◆) represent lettuce seeds. Horizontal co-ordinates are (from left to right) 7.5, 22.5, 37.5 and 52.5 mm; vertical positions 10, 30, and 50 mm. Dotted line = notional midline. Distance between first row of lettuce seeds and *Impatiens* seed = 15 mm; distance between second row of lettuce seeds and closest row of *Impatiens* seeds = 30 mm

A single experimental run consisted of five replicates of each of the four treatments, giving a total of 20 vessels per run. Each replicate was positioned randomly within the other treatments on one of five different shelves in a Sanyo growth cabinet Model MLR 351 (Sanyo Electric Co. Ltd, Osaka, Japan). Temperature was set at a constant 20°C and a photoperiod of 12:12 hours light: darkness was chosen to approximate day length occurring during the early growth of the plants under natural conditions outside.

Table 6.1. MECAM treatment protocols for *Impatiens* and lettuce seed insertion and measurement

Treatment	Protocol
Control	No <i>Impatiens</i> seeds added; lettuce seeds sown on Day 0, radicles measured on Day 7
Day 0	Synchronous sowing of <i>Impatiens</i> seeds and lettuce seeds; both species measured on Day 7
Day 7	<i>Impatiens</i> seeds sown on Day 0; lettuce sown on Day 7, both measured on Day 14
Day 14	<i>Impatiens</i> seeds sown on Day 0; lettuce seed sown on Day 14, both measured on Day 21

Photosynthetically active radiation (PAR) readings were recorded using a Skye 200 PAR meter (Skye Instruments Ltd, Llandrindod Wells, Wales, UK) and gave a reading of  $120 \mu\text{molm}^{-2} \text{s}^{-1}$ .

To equalise variation in light and temperature fluctuations Magentas were rotated through  $90^\circ$  and moved one place forwards as shown in Figure 6.2.

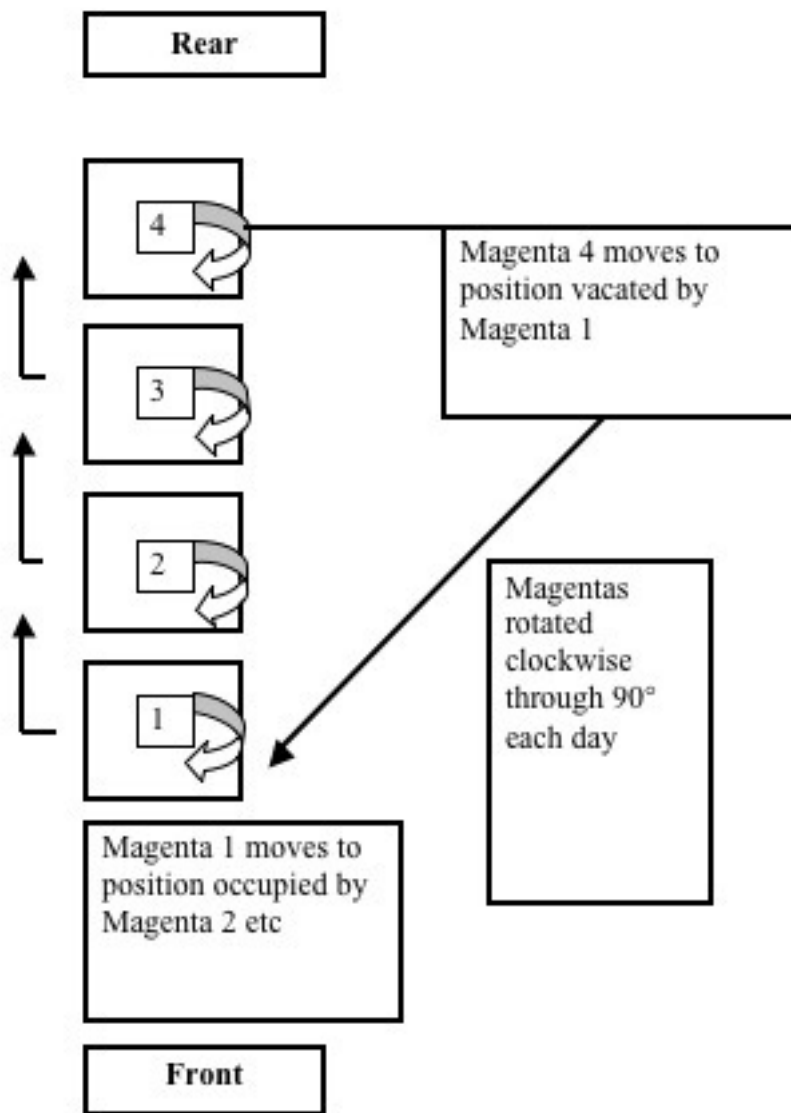


Figure 6.2. Daily movement of Magentas on one incubator shelf in order to equalise influence of light and temperature fluctuations on germinating seedlings

Experiments were repeated on three or four occasions, depending on the availability of suitable seeds and the data pooled. Unfortunately, seeds of *I. noli-tangere* and *I. capensis* were not available during the months over which the MECAM experiments took place and were thus excluded from comparisons between species.

## **6.8 Measurement**

Following removal using ceramic tweezers, seedlings were placed on a laminated sheet of graph paper for measurement. In the case of the *Impatiens* seedlings, which often had markedly branching roots, only the longest root of each donor plant was measured. This method was chosen to facilitate the rapid measurement of the roots as, in most cases, each individual *Impatiens* seedling had a total of five, radiating roots, which required a number of delicate manipulations to measure accurately. The same method was adopted by Wu et al., (2000b) when measuring the root lengths of their chosen acceptor plant, *Lolium rigidum*, which also has a branching root system. In a second experiment into the possible autotoxic inhibition of *I. glandulifera* roots (see Section 6.12.2) a different approach was adopted, with each root being measured to give a combined total for each individual. The main focus of the experiment was on the allelopathic inhibition of root development and as the results of previous experiments in Chapter Four indicated that there was a high degree of correlation between radicle and hypocotyl length, data on the length of lettuce hypocotyls were not collected. Agar from the different treatments was collected in plastic bags and frozen at -20°C for later HPLC analysis.

Percentage inhibition compared to control root elongation was calculated, and at the same time the longest root of each donor seedling (either *I. glandulifera* or *I. parviflora*) was recorded as a means of

determining the amount of putative allelopathic plant tissue capable of producing any phytotoxic effect.

## **6.9 Data Analysis**

Statistical analysis was carried out using SigmaPlot12. Data were subjected to one way ANOVA and post hoc differences between treatment means were calculated using the Holm-Sidak method as specified with this statistical package.

Table 6.2. Experiments carried out using MECAM and their location.

<b>MECAM Experiment</b>	<b>Section where results are located</b>
<i>I. glandulifera</i>	
Germinating: Seeds with 1 mm radicle emerged, or bulging testa	6.10.1
Heat killed: Seeds heated at 60-70°C for 12 hours	6.10.4
Dead, non-sterile: Seeds with visible signs of moulding or decay	6.10.5
Dormant: (Sterilised, fully imbibed seeds stored at 15°C)	6.10.6
<i>I. parviflora</i>	
Germinating: Seeds with 1 mm radicle emerged or bulging testa	6.10.8

## **6.10 Results**

### **6.10.1 Germinating *Impatiens glandulifera***

*I. glandulifera* seeds produced a highly significant inhibition of lettuce radicles at all three sowing dates, when compared to the control, with differences between treatments being highly significant ( $F_{3,461} = 57.41$ ,  $p < 0.001$ ). The amount of lettuce radicle inhibition increased with increasing length of exposure of the agar to germinating *I. glandulifera*, with 15% inhibition at Day 0, 27% at Day 7 and 38% at Day 14 when compared to the control (Fig 6.3). Possible explanations for these results can be found in Section 6.11. An initial data analysis showed unequal variances amongst the treatments: data for Day 0 showed twice the variance found in either the control or any of the other treatments. However, sample sizes were large and close to equal (113-118) and standard deviations were within a ratio of 2:1 largest to smallest.

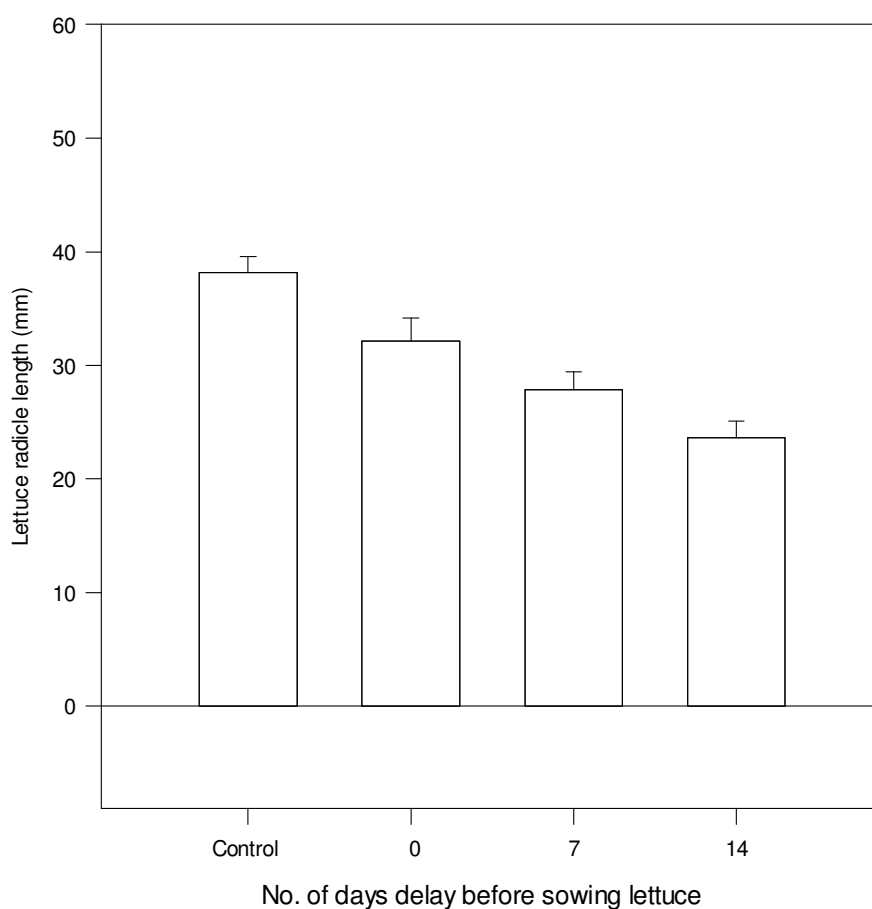


Figure 6.3. Effect of germinating *I. glandulifera* seedlings on the elongation of lettuce radicles using the MECAM experimental system. Error bars represent 95% confidence intervals

There were visible differences between the radicle lengths of the two rows of lettuce seeds, with the radicles being longer in the second row (see Figure 6.4). This suggests that the inhibitory effect of the germinating *I. glandulifera* seeds was slightly reduced by doubling their distance from the lettuce seeds from 15mm to 30mm. However, these differences did not prove significant ( $p>0.05$ )

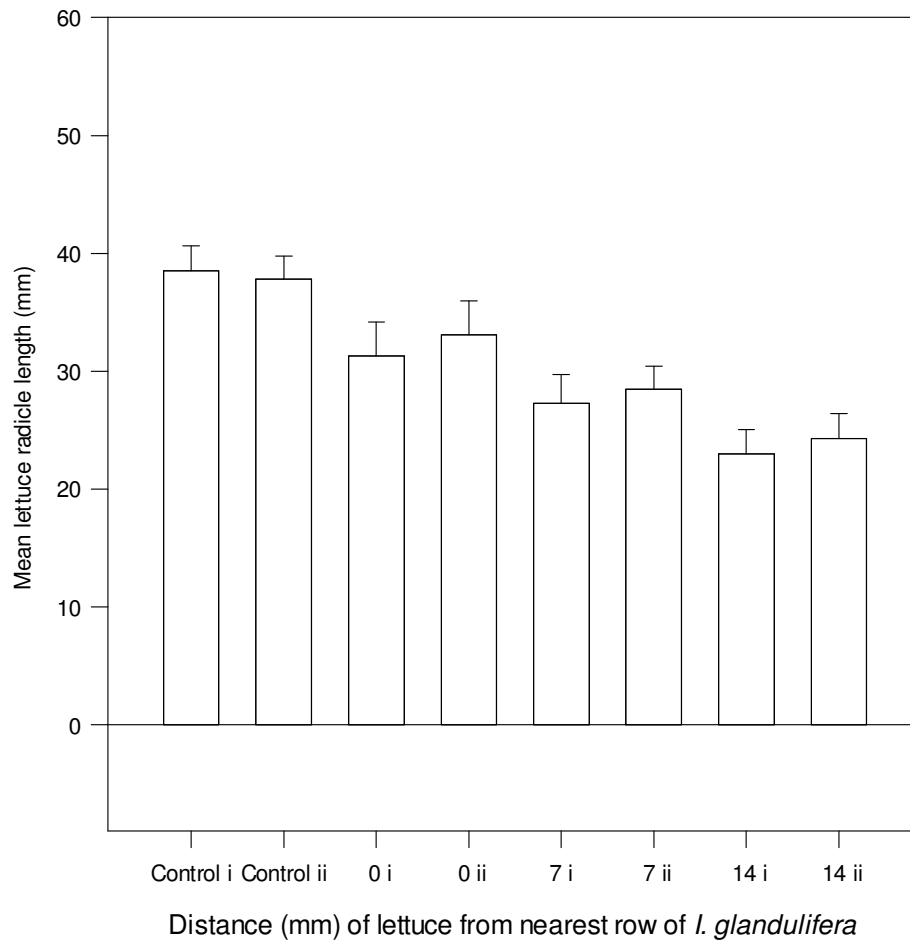


Figure 6.4. The effect of doubling distance between *I. glandulifera* seeds and lettuce seeds from 15 mm to 30 mm on the degree of inhibition shown by lettuce radicles when using the MECAM experimental system. ( i = 15 mm from nearest row of *I. glandulifera* seedlings; ii= 30 mm); 0, 7, 14 refer to the number of days delay before the lettuce was sown into vessels containing germinating *I. glandulifera* seeds. Error bars represent 95% confidence intervals. There were no significant differences within the pairs of the treatments ( $p > 0.05$ ).

### 6.10.2 Increases in root length of *I. glandulifera* seedlings for Days 0, 7 and 14

These were calculated by measuring the longest root of each *I. glandulifera* seedling and calculating the mean for each treatment so that *I. glandulifera* root development for the three sampling dates of the experiment could be ascertained (see Figure 6.5). There were statistical differences between treatments when one-way ANOVA was undertaken ( $F_{2,314} = 56.03$ ,



$p < 0.001$ ). Mean root length doubled from approximately 17 mm for Day 0 (measured after 7 days root growth) to 43mm for Day 7 (14 days root growth). However this doubling was not sustained in the case of measurements for Day 14, where the mean root length was approximately 52 mm. Although this was a statistically significant increase over the Day 7 measurement, the value was lower than might have been expected due to the failure of 20% of the Day 14 treatment *I. glandulifera* seeds to develop radicles of more than 1mm in length (see Table 6.3).

Table 6.3. Percentage emergence of *I. glandulifera* radicles among different treatments (N=120)

Treatment	Day 0	Day 7	Day 14
Number of radicles emerged	115	116	96
Percentage %	95.8	96.7	80

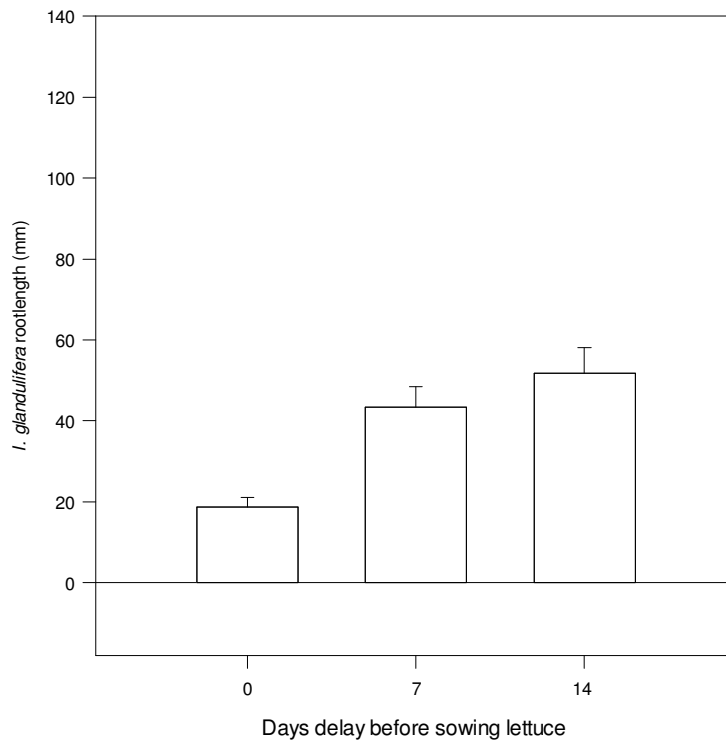


Figure 6.5. Mean root length of *I. glandulifera* seedlings in the MECAM experimental system at 0, 7 and 14 days before sowing lettuce. Error bars represent 95% confidence intervals. All three treatments were significantly different from each other

### 6.10.3 Relationship between *I. glandulifera* root lengths and lettuce radicle inhibition

When mean *I. glandulifera* root length values for days 0, 7 and 14 and lettuce radicle inhibition values were subjected to linear regression, there was a very strong correlation between the two factors indicating that increasing length of the *Impatiens* root system was responsible for the reduction in lettuce radicle length. ( $r = 0.987$ ,  $r^2 = 0.974$ ) (see Figure 6.6).

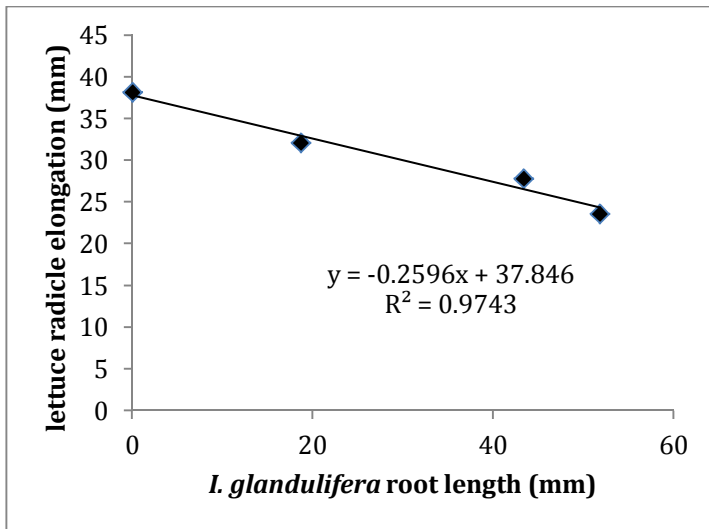
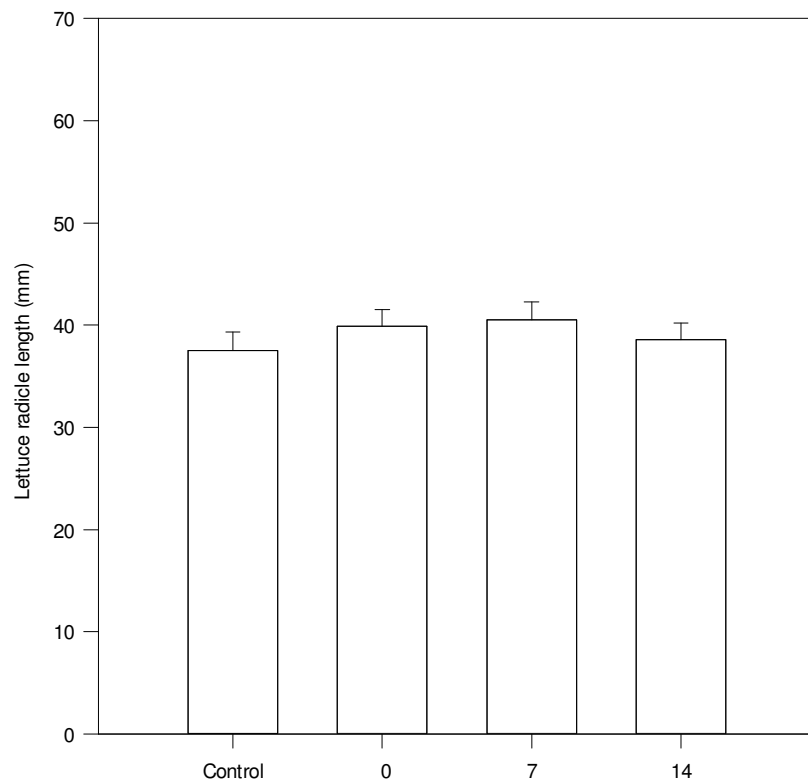


Figure 6.6. Relationship between *I. glandulifera* root lengths at days 0,7 and 14 and lettuce radicle length in the MECAM experimental system.

#### 6.10.4 Dead (heat treated) seeds of *Impatiens glandulifera*

The presence of heat treated seeds caused a small, but non-statistically significant, increase in mean lettuce radicle length compared to the control ( $F_{3,469} = 2.40$ ,  $p = 0.065$ ). There were no significant differences between the treatments, although the power of the test ( $\alpha = 0.05$ : 0.367) was below the desired level, so this negative result should be interpreted with caution. Nevertheless, when plotted in Figure 5.7 the differences between treatments appear to be minimal.



Number of days delay before sowing lettuce into vessels containing heat treated dead *I. glandulifera* seeds

Figure 6.7. Effects of dead (heat treated) *I. glandulifera* seeds on lettuce radicle elongation in the MECAM experimental system. Error bars represent 95% confidence intervals.

#### 6.10.5 Dead, mouldy, non-sterilised seeds of *I. glandulifera*

Data recorded here proved to be non-normal, although variances were equal. Radicle elongation was suppressed by approximately 12% compared to the control, but no statistically significant differences were evident between treatments.

There was a statistically significant difference between the control and treatments Day 0 and Day 7.  $F_{3, 230} = 3.365$ ,  $p = 0.019$ ), but no difference between treatments, nor between the control and Day 14 ( $p > 0.05$ ).

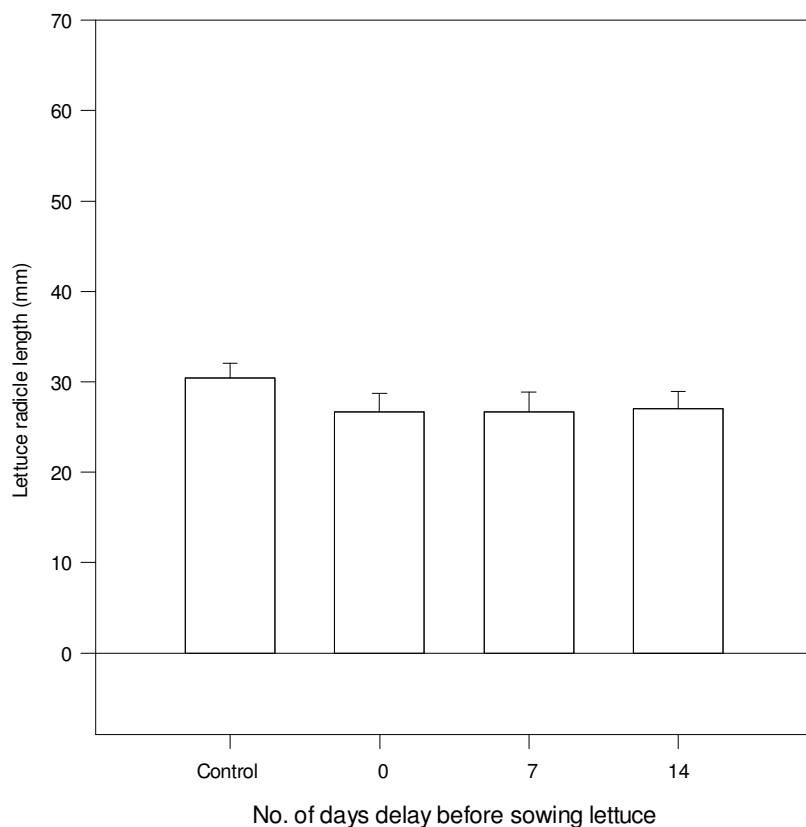


Figure 6.8. Effects of dead (mouldy) *I. glandulifera* seeds on lettuce radicle elongation in the MECAM experimental system. Error bars represent 95% confidence intervals

This small amount of lettuce radicle suppression by the dead seeds may be due direct microbial activity against the emerging radicles, the production of phytotoxic exudates by the decay microorganisms or the release of compounds from the decaying seeds themselves.

#### 6.10.6 Dormant *I. glandulifera* seeds

One-way ANOVA showed that there were statistically significant differences between the control and the treatments ( $F_{3, 462} = 51.890$ ,

$p < 0.001$ ). There was a significant increase in radicle length between the treatments with radicle length increasing with time when compared to the control. Increase in lettuce radicle lengths as a percentage of the control were 32, 38 and 57% for days 0, 7 and 14 respectively. Initial analysis of the data indicated that they were not normally distributed, and variance was, once more, unequal. The ratio of largest to smallest variance was below the 2:1 cut off above which the assumptions of ANOVA are considered to be violated (see Section 6.11). Differences between day 14 and both days 0 and 7 were significant ( $p < 0.001$ ), whereas days 0 and 7 did not show statistically significant differences ( $p = 0.202$ ), see Figure 6.9.

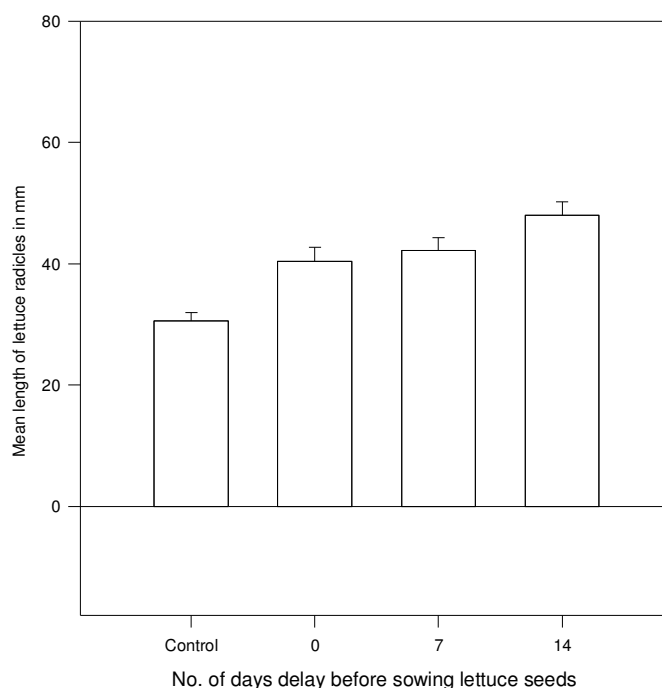


Figure 6.9. Effects of dormant *I. glandulifera* seeds on lettuce radicle elongation in the MECAM experimental system. Error bars represent 95% confidence intervals

### 6.10.7 Dormant *I. glandulifera* seeds: effects of doubling distance on lettuce radicle inhibition

Lettuce radicle lengths were once again visibly affected by increasing distance from the closest row of *I. glandulifera* seeds (see Fig. 6.10). In this instance, in contrast to previous experiments, the inner rows of lettuce showed an increase in radicle length when compared to the outermost rows. However, these effects were not statistically significant when the pairs were compared using one-way ANOVA ( $p > 0.05$ ).

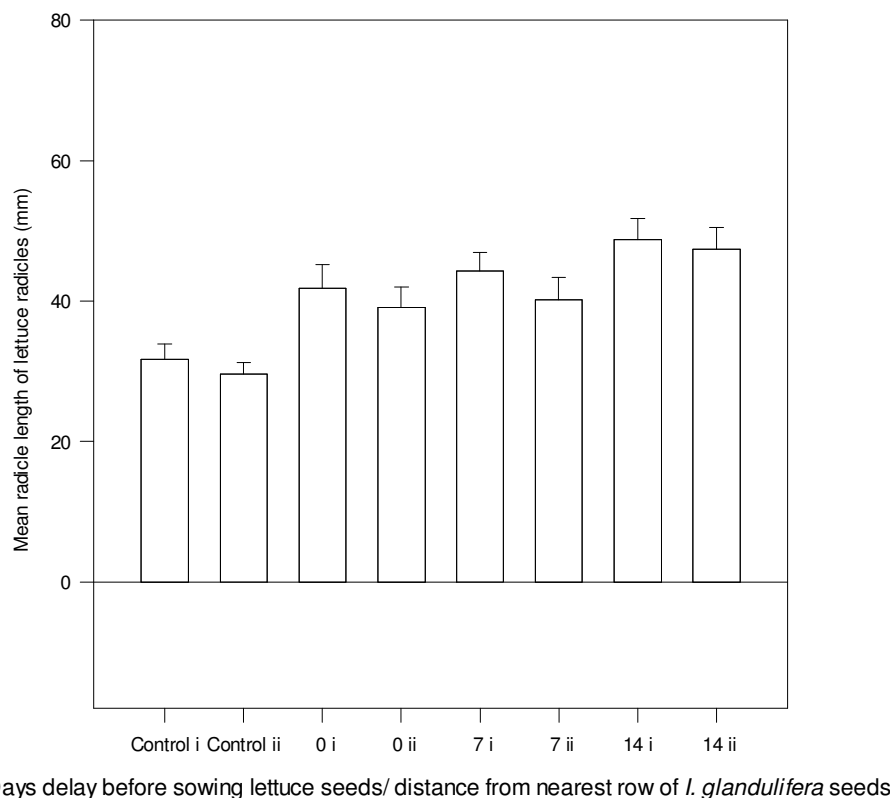


Figure 6.10. Effects of doubling distance (15 mm to 30 mm) between dormant *I. glandulifera* seeds and lettuce seeds on the degree of inhibition shown by lettuce radicles when using the MECAM experimental system (i = 15 mm from nearest row of *I. glandulifera* seedlings; ii = 30 mm); 0, 7, 14 refer to the number of days delay before the lettuce was sown into vessels containing dormant *I. glandulifera* seeds. Error bars represent 95% confidence intervals.

### 6.10.8 Germinating *Impatiens parviflora* seeds

In marked contrast to *I. glandulifera*, *I. parviflora* showed a statistically significant stimulatory effect of 14.6% on radicle elongation at Day 0 when compared with the control; this was reversed in the case of Day 7 (-1.84%) and Day 14 (-6.88%). Neither of these two reductions were statistically significantly different from each other or from the control, however:  $F_{3, 351} = 8.383$  ( $p > 0.05$ ), Figure 6.11.

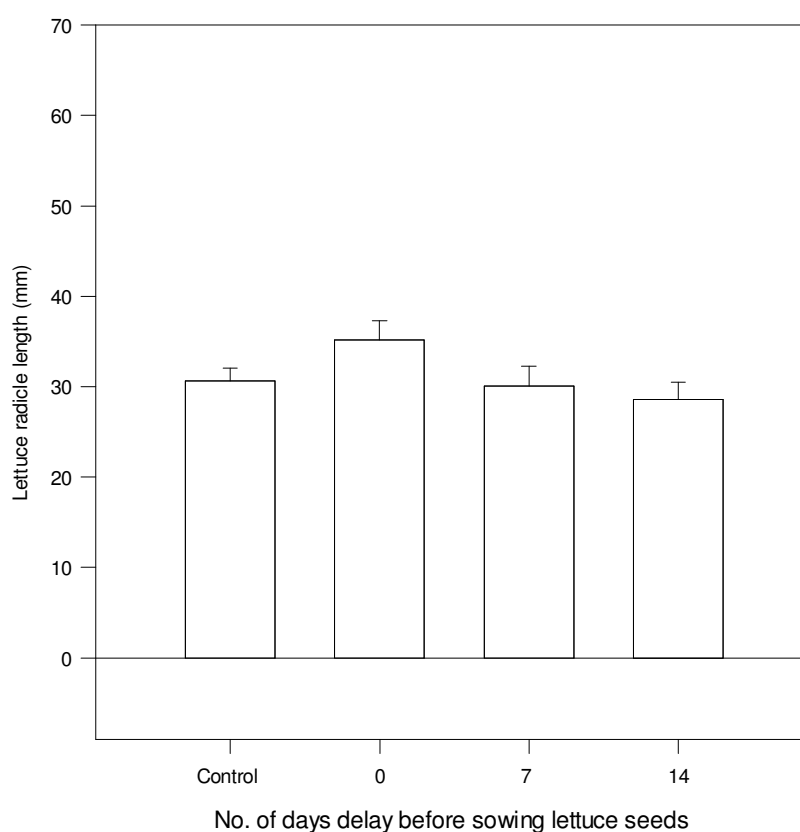
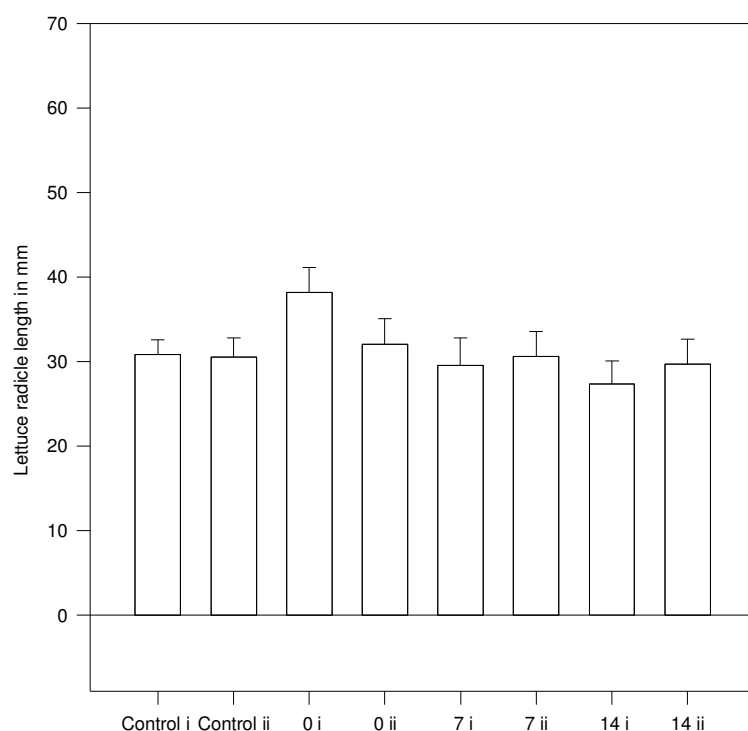


Figure 6.11. Effect of germinating *I. parviflora* seeds on lettuce radicle elongation in the MECAM experimental system. Error bars represent 95% confidence intervals.



### 6.10.9 Effects of distance from nearest row of germinating *I. parviflora* seeds on lettuce radicle elongation

There were no statistically significant differences between the pairs of treatments apart from the case of Day 0, where the lettuce seeds closest to the germinating *I. parviflora* seeds underwent a statistically significant increase in length. Results are displayed in Figure 6.12.



Days delay before sowing lettuce seeds/ distance from nearest row of *I. parviflora* seeds

Figure 6.12. Effect of doubling distance between *I. parviflora* and lettuce seeds from 15 mm to 30 mm on the degree of inhibition shown by lettuce radicles when using the MECAM experimental system (i = 15 mm from nearest row of *I. glandulifera* seedlings; ii = 30 mm); 0, 7, 14 refer to the number of days delay before the lettuce was sown into vessels containing germinating *I. parviflora* seeds. Error bars represent 95% confidence intervals.

#### 6.10.10 Root length of *I. parviflora*

Successful germination and radicle elongation of *I. parviflora* was particularly problematic, with close to half the data values missing for treatments Day 7 and Day 14. (Table 6.4) As a result, when the data were subject to analysis of variance, no significant differences were recorded between root lengths for Days 7 and Day 14 ( $p = 0.08$ ), whereas there were significant differences between Day 0 and Day 7, as well as Day 0 and Day 14 ( $F_{2,181} = 54.273$ ,  $p < 0.001$ ), Figure 6.13.

Table 6.4. Percentage germination of *I. parviflora* seeds (N=90)

Treatment	Day 0	Day 7	Day 14
No. radicles emerged	86	51	47
Percentage %	95.5	56.66	52.2

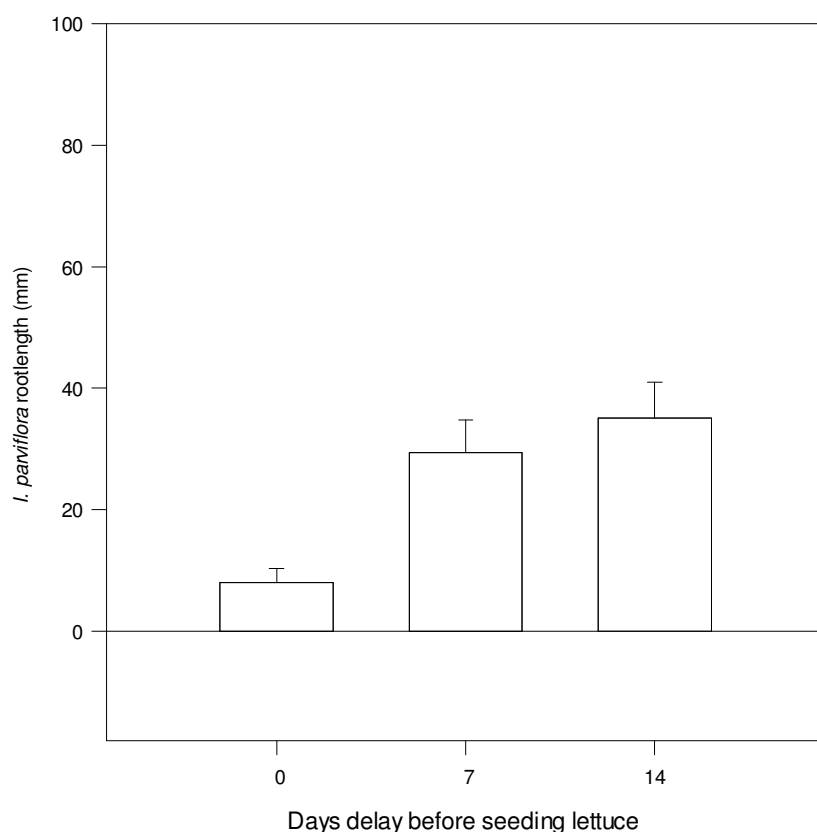


Figure 6.13. Mean root lengths of *I. parviflora* seedlings measured after 7, 14 and 21 days growth in MECAM experimental system. Error bars represent 95% confidence intervals. There was no significant difference between Days 7 and 14 ( $p = 0.08$ ), whereas Day 0 was significantly different from the other two days ( $p < 0.001$ ).

### 6.10.11 Mean root length versus lettuce radicle inhibition in *I. parviflora*

When plotted and subjected to linear regression, a very weak negative correlation was seen between *I. parviflora* root length and the degree of lettuce radicle inhibition (Figure 6.14).

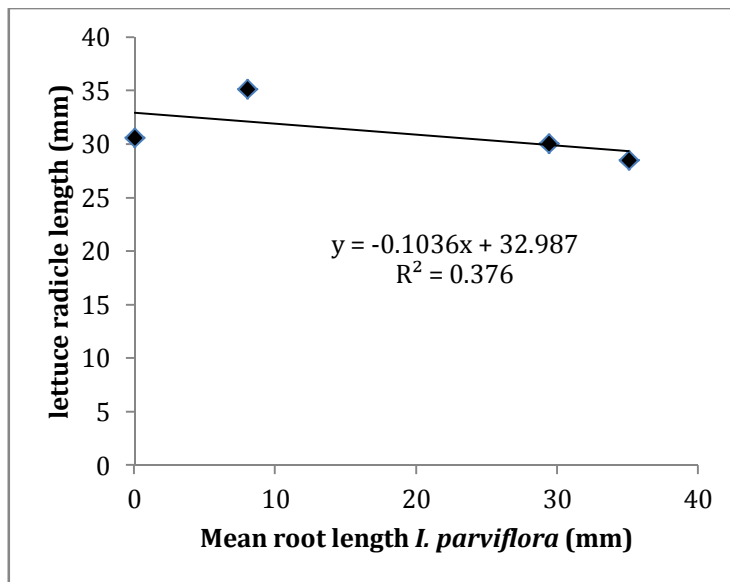


Figure 6.14. Relationship between *I. parviflora* root length and its effects on lettuce radicle elongation in the MECAM experimental system. There was a very weak correlation between the two sets of values.

## 6.11 Discussion

The results above provide strong evidence that germinating *I. glandulifera* seeds were capable of releasing water soluble, allelopathic compounds as part of the germination process. This allelochemical, or mix of allelochemicals, exerted an inhibitory effect on the growth of lettuce radicles. These effects were noticeable from Day 0, indicating that the allelochemicals were able to diffuse rapidly from the germinating *I. glandulifera* seeds and were present in the agar surrounding the lettuce seeds by the time latter began to germinate. Inhibition increased with agar's increasing length of exposure to *Impatiens* exudates before lettuce seeds were sown.

The putative allelochemicals were only produced or liberated during and after the germination process and did not appear to be present in a biologically active form in either dormant or dead seeds. Similar results were obtained by Wu et al (2000b). There are two possible explanations for these results. Firstly, as the process of germination proceeded, the allelochemicals were produced at a constant rate but their rate of breakdown was slower, resulting in a net accumulation of allelochemicals over time. The increasing allelochemical concentrations accumulating in the agar over time led to a concomitant increase in the inhibition of lettuce radicles. The second possibility is that increasing root biomass led to the release of larger quantities of allelochemicals as the root systems developed. Although the samples were small (N=4), there was strong negative correlation between *I. glandulifera* root length and the amount of inhibition experienced by the lettuce radicles, with the coefficient of determination  $r^2 = 0.974$ . This supports the proposition that the increase in root biomass led to the production and release of greater quantities of allelochemicals from this rapidly expanding tissue.

When the radicle lengths of lettuce were compared at the two distances 15 mm and 30 mm from the nearest row of *Impatiens* seedlings, simultaneous sowing (Day 0), showed that the allelochemical (or chemicals) were able to diffuse to a distance beyond 30 mm within a week of sowing. Doubling the distance from the nearest row of *Impatiens*

seedlings did not produce any significant differences in inhibition between the two rows of lettuce radicles. Visible differences could be ascertained by eye, however, suggesting that the innermost rows of seeds (15 mm) experienced slightly more inhibition than the outer one. Similar slight, though non-significant differences were visible for the other treatments. The rate of diffusion exceeded 30 mm within the first 7 days, as in most cases; no significant differences were detected between the radicle lengths of the two rows of germinating lettuce seeds placed at 15 and 30 mm from the nearest row of donor seedlings.

Heat treated *I. glandulifera* seeds had no inhibitory effect on lettuce radicle length. In fact their effect was to slightly, although not significantly, increase them. As this effect showed no significant increase over time, it suggests that the stimulatory chemicals were quickly released from the donor seeds after they were placed in the agar medium and that once liberated, no additional release took place subsequently. By way of contrast, the mouldy seed treatment had a slightly inhibitory effect on the lettuce radicles. This could be due to the breakdown and release of phytotoxic by-products from the seeds as a result of microbial action, the production of phytotoxins by the pathogens themselves, or as the result of direct attacks by pathogenic microorganisms against the germinating lettuce seeds. Some species of saprophytic fungi, for instance, are known for their ability to colonise otherwise healthy seeds

and seedlings (Martin & Loper, 1999). This inhibitory effect was slight, however and easily distinguishable from the allelopathic interactions shown by the live *I. glandulifera* seeds.

In contrast to germinating seeds, dormant *I. glandulifera* seeds showed a stimulatory effect on the growth of lettuce radicles. This may have been due to the presence of a separate, stimulatory compound liberated into the agar by dormant seeds.

This effect did not, however, seem to increase through the three week experimental time course, rather it remained at an elevated level above the control, but with no significant increase between treatments. Another possibility is that the stimulation recorded was due to the release of very low levels of the inhibitory allelochemicals. At very low concentrations, otherwise phytotoxic compounds have been demonstrated to stimulate plant growth, a process known as hormesis (Duke et al., 2006). Hormetic responses have previously been recorded in plant species known to demonstrate allelopathy, including invasive alien plants (Belz & Cedergreen, 2010; Belz et al., 2007; Duke et al., 2006; Molisch, 1937). Synthetic herbicides have also been shown to elicit hormetic effects when applied at appropriate concentrations (Duke et al., 2006). In the case of the experiment carried out with dormant seeds, differences between the two rows of receiver seeds were once again visible, although not significant.

In some cases, radicle emergence of the *Impatiens* seedlings was irregular, which is most likely to have been due to sensitivity to low oxygen levels in the agar medium, or perhaps some other unidentified cause. Radicle extension failures approached 20% in some *I. glandulifera* treatments and were higher in *I. parviflora*, reaching levels close to 45%. Nevertheless, regression analysis suggested that there was a linear relationship between mean root length of *I. glandulifera* and the amount of inhibition experienced by the radicles of the lettuce seedlings.

Even in those treatments where *Impatiens* radicles failed to emerge in the anticipated numbers, a significantly inhibitory effect on lettuce radicle elongation was often present; a 20% failure rate in *Impatiens* radicle elongation did not produce a corresponding reduction in lettuce radicle inhibition, for example. This suggests that the non-emergent seeds were still able to produce and liberate allelochemicals despite their failure to develop radicles, or that the allelochemicals produced by those seeds which germinated successfully were continuing to accumulate in the agar.

The results obtained for *I. parviflora* present a more confused picture, mainly due to the very low germination success rate mentioned above. The disproportionately large values for Day 0 were not supported by the values for Day 7 and Day 14 which were not significantly different when compared with the control.



In some instances, the data as analysed showed deviations from normality and equal variance. ANOVA is known to be robust to moderate violations of equality of variance and non-normality, however (Box, 1953). The results of a number of simulation studies using non-normal distributions have shown that the violation of the normality assumption does not lead to a serious increase in the false positive rate (Type 1 error) (Harwell et al., 1992; Lix, Keselman & Keselman, 1996). Tests of homogeneity tend to be more sensitive when sample sizes are larger, but ANOVA itself is less affected by this heterogeneity as sample size increases. It was therefore decided was considered acceptable to continue with standard one way ANOVA, rather than transform the data or use a non-parametric test such as Kruskal-Wallis.

## **6.12 Investigation into the effects of increasing density on allelopathic potential of germinating *Impatiens glandulifera* seedlings**

### **6.12.1 Introduction and rationale for study**

The tendency of *Impatiens* seedlings to develop in dense, even aged stands suggested that an investigation into the effects of increasing seedling density on their allelopathic potential was warranted.

For the *Impatiens* seedlings to accrue maximum benefit through negative allelopathic interaction with their neighbours, they would need to suppress the growth of competitors whilst experiencing minimal allelopathic inhibition themselves. Autotoxicity is a well-established concept within allelopathy, however (Friedman & Waller, 1985; Singh, Batish & Kohli, 1999) and has been implicated in replant disease in agriculture, horticulture and forestry (Ervin & Wetzel, 2000; Kong et al., 2008; Viator et al., 2006). It has also been recorded in studies involving invasive weeds (Arora & Kohli, 1993; deJong & Klinkhamer, 1985; Kumari & Kohli, 1987). In the case of *Impatiens*, there are no published studies to support or disprove the occurrence of autotoxicity in dense single species stands such as those that occur commonly in *I. glandulifera* invasions.

### **6.12.2 Experimental Design**

The basic protocol was the same used in the previous MECAM experiments and involved daily rotation and movement of the Magentas as previously described. A total of 25 vessels were used per experimental run, with five replicates per treatment, one on each shelf, randomly placed amongst replicates of the other treatments. Lettuce seed placement was identical to the previous experiments. The *Impatiens* seeds and lettuce were sown synchronously and were measured after 7 days. Two

experiments were carried out, one using germinating *I. glandulifera* seeds, the other using dormant *I. glandulifera* seeds.

In this experiment, prior to sowing, the germinating *I. glandulifera* seeds were allowed to develop both radicles and lateral roots. As previously shown, *Impatiens* seedlings have distinctive early root morphology (Beerling & Perrins, 1993; Hatcher, 2003). In the case of *I. glandulifera*, this consists of a primary radicle, with, usually, four lateral roots emerging soon after. The seedlings were placed on the surface of the agar so that their roots were able to spread either through or across it. This avoided the problems with *Impatiens* radicle emergence and development which had been experienced in the previous experiments. To maintain the same distances between the rows of acceptor and the first row of *Impatiens* seeds, the seeds were placed so as to occupy the same area used in the previous experiments.

Due to the irregular development of the lateral roots, wholly accurate placing of the seeds was difficult to achieve. Nevertheless, an attempt was made to ensure that seeds in the first row were placed as close to the correct locations as possible so that the distance of 15 mm was maintained between them and the first row of lettuce seeds. Additional densities were placed as equidistantly as was possible within the area delineated by the standard 6 seed density used in previous experiments. In the case of dormant *I. glandulifera* seeds, more accurate placement was

possible and a template was used to locate seeds in approximately the correct locations.

At the time of harvest, the lengths of each individual root were measured separately and the total measurement per individual calculated. This usually entailed measuring five roots per individual. Using these measurements, mean root length per treatment was ascertained and autotoxic root length reductions to increasing density, if present, could be determined.

Table 6.5. Arrangement and distances of seeds used in density experiment

<b>Number of seeds per vessel</b>	<b>Number of rows of <i>Impatiens</i> seeds</b>	<b>Distance between rows of <i>Impatiens</i> seeds in mm</b>	<b>Approximate distances of <i>Impatiens</i> seeds in mm from first row of lettuce seeds</b>
3	1	n/a	15
6	2	15	15, 30
9	3	7.5	15, 22.5, 30
12	4	5	15, 20, 25, 30

## **6.13 Experiments carried out**

### **6.13.1 Experiment 1: Germinating *I. glandulifera***

Germinating *I. glandulifera* seeds were placed as described above.

### **6.13.2 Experiment 2 Dormant *I. glandulifera* seeds**

An additional experiment was carried out using dormant seeds at the same densities as germinating seeds. Dormant seeds were sown directly following sterilisation.

No seeds of *I. parviflora*, *I. noli-tangere* or *I. capensis* were available at the time of these experiments.

## **6.14 Results**

### **6.14.1 Experiment 1: Germinating *Impatiens glandulifera***

Germinating seeds at the densities used caused increasing inhibition when compared to the control, starting at around 18% for the 3 seed density, nearly doubling to 35% at the six seed density, then starting to flatten out at the 6 and 9 seed densities, with 41 and 47% inhibition respectively. There was a statistically significant difference between the different seed densities and the control when one-way ANOVA was undertaken ( $F_{4, 431} = 99.395$ ,  $p < 0.001$ ). All combinations were statistically significantly different from one another, ( $p < 0.05$ ) apart from the seed densities 9 and 12, which were close to, but not significantly different from one another ( $p = 0.051$ ). The data showed equal variance, although they were non-normal, due to the presence of outliers.

Table 6.6. Effects of increasing *I. glandulifera* seedling number on mean percentage inhibition of lettuce radicles compared to control (100%) ns = not significant (  $p= 0.051$ )

Seed density per Magenta	Mean % radicle inhibition compared to control
3	17.8
6	34.7
9	41.6
12	47 (ns)

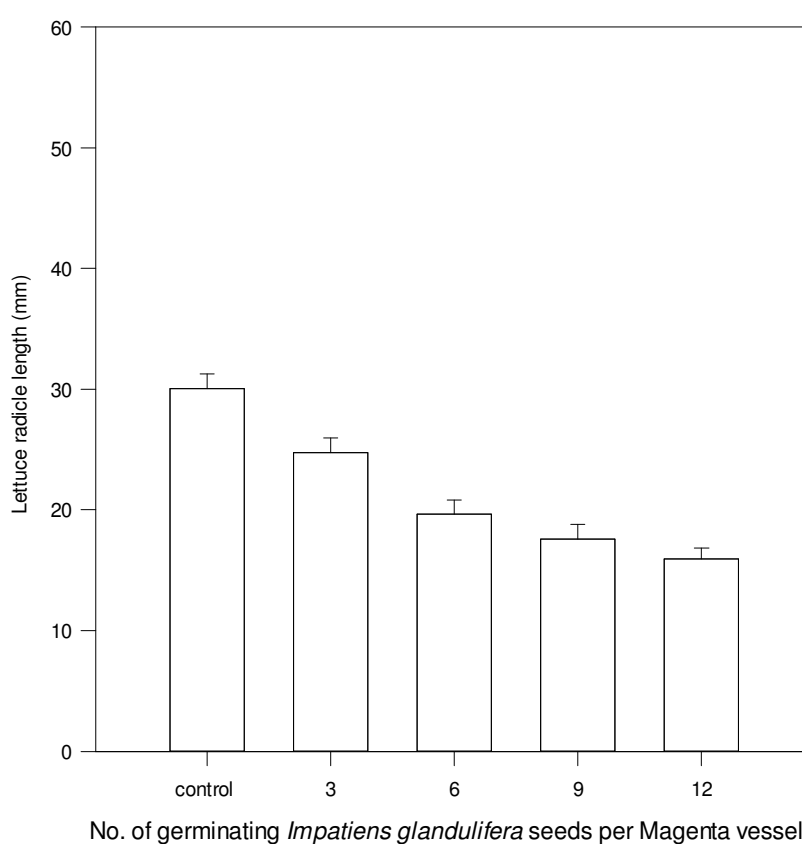


Figure 6.15. Effect of increasing *Impatiens glandulifera* seedling numbers on lettuce radicle inhibition using MECAM. Error bars represent 95% confidence intervals

When plotted as a linear relationship (Fig 6.16), there was a strong correlation between the number of seeds per vessel and the degree of lettuce radicle inhibition ( $r^2 = 0.9383$ ).

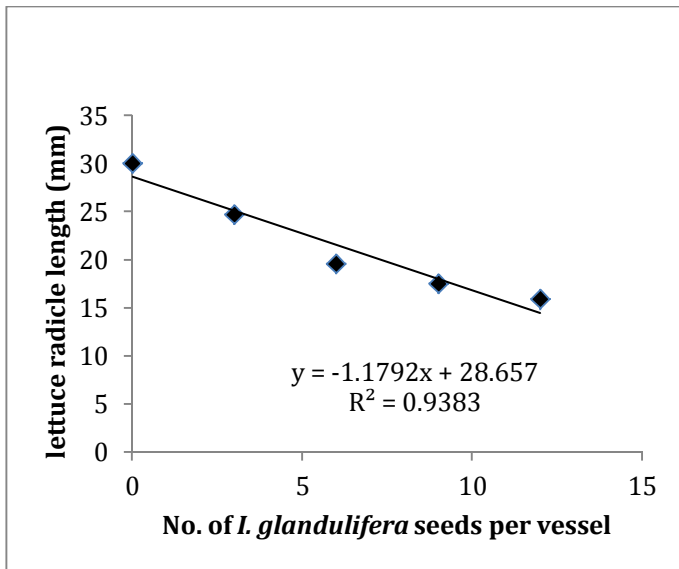


Figure 6.16 Linear relationship between *Impatiens glandulifera* seedling density and lettuce radicle inhibition in MECAM experiments.

#### 6.14.2 Effect of increasing seedling number on *I. glandulifera* root length

The results suggested that increasing densities of *I. glandulifera* seeds did not cause autotoxic inhibition of root elongation, at least for the densities used in this experiment. Means are presented graphically in Figure 6.17. ANOVA showed that there was no statistically significant difference between the means of the treatments ( $F_{3,703} = 1.642$ ,  $p = 0.178$ ).

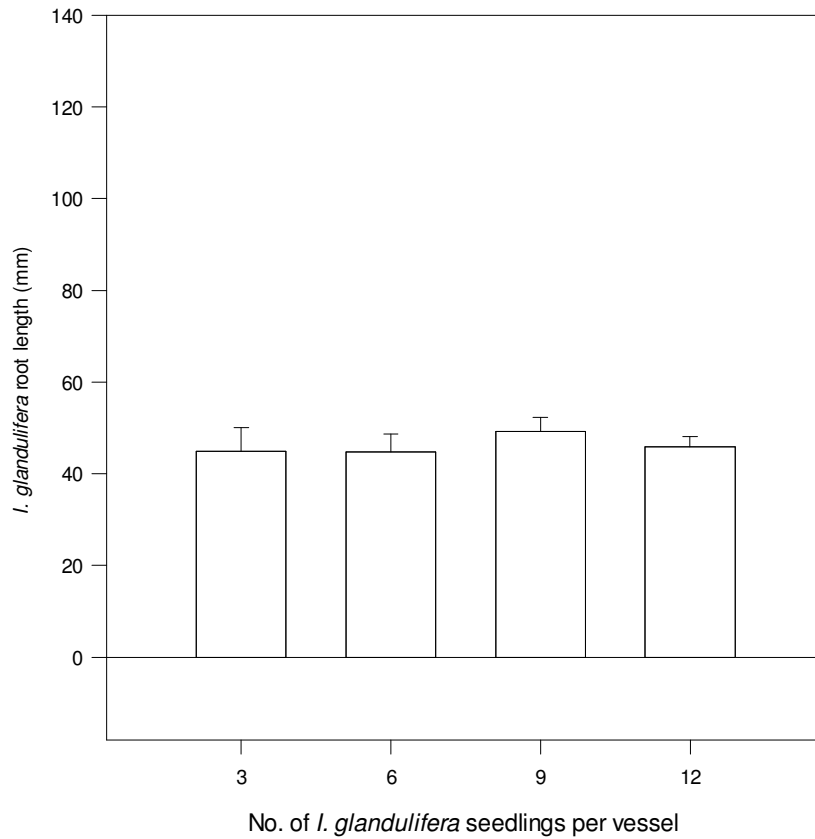


Figure 6.17. Effects of increasing seedling number on auto toxic inhibition of *I. glandulifera* roots using the MECAM system. Error bars represent 95% confidence intervals. There were no significant differences between treatments ( $p=0.178$ )

### 6.14.3 Experiment 2 : Dormant *Impatiens glandulifera* seeds

Dormant *I. glandulifera* seeds produced a highly statistically significant increase in lettuce radicle length when compared to the control ( $F_{4, 284} = 11.820$ ,  $p < 0.001$ ), but there were no statistically significant differences between treatments ( $p > 0.05$ ). The data are presented graphically in Fig 6.18.



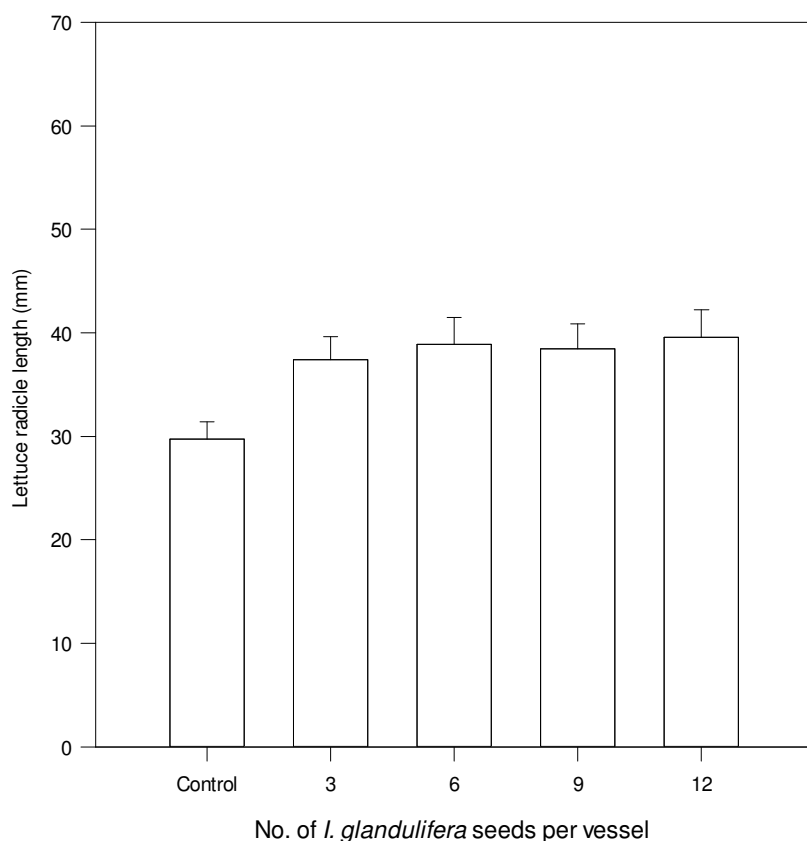


Figure 6.18. Effect of increasing number of dormant *I. glandulifera* seeds on lettuce radicle elongation in the MECAM experimental system. Error bars represent 95% confidence intervals.

## 6.15 Discussion

The improved sowing technique adopted for this second series of experiments successfully eliminated the germination problems previously experienced. It seems safe to conclude, therefore, that these were caused by oxygen deficiencies induced by inappropriate sowing depth.

Germinating *I. glandulifera* seeds showed statistically significant inhibitory activity against root elongation of lettuce and these effects

were more pronounced at higher densities. This suggests once more that the biomass of the root system, as measured by its proxy, root length, was responsible for the inhibition observed.

The root development of *I. glandulifera* showed no autotoxic inhibition when sown at the range of densities chosen. This suggests that while naturally emerging populations of *I. glandulifera* seedlings, often occurring at densities of up to 350 m<sup>-2</sup> (Beerling & Perrins, 1993) will experience intraspecific light, water and nutrient competition, autotoxic root exudates are not likely to be a factor in preventing their establishment. These same root exudates may, however, provide them with a competitive advantage over other species which are affected by this additional factor as well as the usual competitive mechanisms.

Dormant seeds once again showed a stimulatory effect on lettuce radicle elongation when compared to the control, as was previously shown in the first experiment involving dormant and different sowing dates. Increasing densities of seeds did not produce significant increases in lettuce radicle elongation, suggesting a non-density dependent interaction was occurring.

## **6.16 Conclusions**

### **6.16.1 Evaluation of the MECAM**

As a general conclusion, the MECAM system as developed for this study proved to be successful in achieving the original aims, namely the detection of allelopathic potential in germinating *Impatiens* seeds. The Magentas were an easily managed substitute for the glass beakers (rotation notwithstanding).

The attempt to detect the rate of allelochemical diffusion by comparing receiver radicles sown at distances of 15 and 30 mm from the nearest row of donor seeds proved less successful. The results obtained suggested that diffusion exceeded this distance within 7 days and thus significant differences between the two rows were not obtained.

Perhaps the most serious shortcoming experienced with this method was the failure of the donor seeds to germinate properly. It seems likely that this was due to the immersion of the embryo when the seeds were inserted into the agar in a vertical position. Although this technique allowed for accurate seed placement, *Impatiens* seeds proved to be very sensitive to the effects of subsurface sowing, despite occurring in riparian habitats and moist soil (Tickner et al., 2001a). When the seed placement technique was altered for the density experiments, germination failures were reduced to insignificant levels. In repeats of the experiments at a future dates, this revised seeding method, should be adopted so that the

seeds are placed horizontally on surface of the agar, with the embryo above the surface of the agar. Alternatively, the seedlings should be allowed to reach a more advanced stage of development, where the lateral roots have started to develop, as used in the second set of experiments. In this case, the seedlings can be placed in a vertical position, so that at least part of the root collar remains above the agar's surface while the seedlings become established. A similar problem was reported by Wu et al. (2000a) who adopted the same technique to reduce germination difficulties. Once rapid growth had commenced, the roots seemed able to grow through the agar medium successfully.

It was unfortunate that it was not possible to analyse the agar samples collected from the MECAM experiments using HPLC as had been originally intended. The presence of naphthoquinones in the agar, if recorded, would have provided strong support for the argument that these chemicals were responsible for the inhibition observed and would have provided much-needed support for the basic premise of this project. If the experiments were to be repeated, this would be a priority.

### **6.16.2 Limitations of the MECAM**

Clearly, any laboratory bioassay for allelopathy is, at best, an approximation or simplification of the natural situation. This is certainly the case in the MECAM described in this chapter. Although the inhibition

observed was marked, it is quite possible that a soil containing active microorganisms would have given completely different results. There is ample evidence that naphthoquinones in soil can be broken down by microbes and that levels usually, although not always, decline rapidly due to microbial degradation (von Kiparski, Lee & Gillespie, 2007). On the other hand, allelopathic potential was clearly demonstrated under conditions of good light and water availability, a situation not always occurring in the wild. Perhaps under natural conditions, where multiple stressors such as water, nutrient and light competition are intense, allelochemical release might be increased as has been recorded in other species (Rice, 1984). This would lead to greater inhibition than that found within the MECAM.

The choice of lettuce as the acceptor species could be criticised as being of little ecological relevance to wild *Impatiens* stands – what is actually required is a naturally occurring competitor. However, the exigencies of experimental design demanded that a species with rapid, reliable germination was chosen so that dormancy and other confounding factors were eliminated as much as possible from the experimental design. For the purposes of this initial study, lettuce, although not ideal, proved to be very useful; its rapid and reliable germination, known sensitivity to allelochemicals and a single, generally straight radicle which facilitated easy measurement were all notable benefits.

### **6.17 Further experimental work**

These simple experiments only provide a limited picture of the field situation and a number of criticisms could be laid; some of these could be addressed by further studies

#### **6.17.1 Comparison of *I. glandulifera* with *I. noli-tangere* and *I. capensis*.**

The non-availability of seeds of *I. noli-tangere* and *I. capensis* during these experiments was unfortunate. Comparisons of these species with *I. glandulifera* and *I. parviflora* may provide more support for the assertion that variation in the production and type of putative allelopathic exudates influences the differing invasive success of the various species.

#### **6.17.2 Use of alternative acceptor species in place of lettuce**

Research should be undertaken to locate alternatives to the lettuce used as the acceptor species. Fast germinating members of the Brassicaceae, such as radish (*Raphanus sativus*) might be suitable and are representative of the kind of ruderal species with which *I. glandulifera* competes in some habitats. Another possibility would be the use of small rooted shoots of nettle (*Urtica dioica*), a major competitor with *I. glandulifera* in riparian habitats. These could be produced by vegetative propagation or plant tissue culture and as clones would help to eliminate genetically derived

variation to the effects of allelochemicals, which must be a factor when using seeds. The problems surrounding the use of nettle seeds has been described previously in Section 4.10.

### **6.17.3 Persistence of allelochemicals**

In order to ascertain whether the effects demonstrated are a product of accumulation of allelochemicals in the agar or a response to increased production from developing root systems, an additional experiment should be carried out. This would involve removal of the donor seeds prior to insertion of the acceptor seeds. Any effects demonstrated would therefore be limited to allelochemical accumulation prior to sowing of the acceptor seeds.

### **6.17.4 Rates of diffusion**

Sampling times a week apart proved to be too long to ascertain the rate of allelochemical diffusion across a distance of 30 mm. Rather than weekly measurements, an experiment could be conducted which measured acceptor radicle elongation on a daily basis. This would provide a better method of determining the rate of diffusion.

#### **6.17.5 The effects of darkness on allelopathic potential of *Impatiens* roots**

As all around illumination is a somewhat artificial state of affairs for a plant and its root system, experiments should be devised so that the roots are allowed to develop in the dark. This could take the form of some sort of masking on the outside of the Magenta (as was used in the Plant box method experiments described previously).

Although agar has many useful properties when used as cultivation medium, its biggest drawback is the criticism of its artificiality when compared to plants growing in a soil based medium. In order to address this aspect of the allelopathic potential of *Impatiens*, an experiment was undertaken with the express aim of exploring *Impatiens* allelopathy using a soil based medium. This Target-Neighbour experiment can be found in Chapter 7 of this thesis.



## **7. Additional Methods and Developments**

The protocols outlined in previous chapters proved useful in identifying the allelopathic potential of the *Impatiens* species investigated. The absence of soil or other solid growth media in these protocols makes it hard to infer similar allelopathic activity to the behaviour of wild plants, however. The following chapter explores some other experimental methods which were used or developed by the author in an attempt to address some of these concerns. They represent methodological development rather than perfected protocols and are pilot studies rather than fully developed studies. They are described below.

### **7.1 Rhizosphere Soil Method - an overview**

This is an intermediate stage between a soil based and an agar based allelopathy bioassay, which involves the use of soil collected from plant roots and solidified using agar. It was developed by Fujii et al.(2005) and is a modification of the Sandwich Method described in Chapter 4.

#### **7.1.1 Method**

Plants of *I. glandulifera* and *I. parviflora* were grown individually in 1 litre pots using a blend of 1:1 volume of multipurpose compost and John Innes no. 1 compost (Westland, Sinclair Horticulture, Lincoln, UK). The plants were grown under the light racks as described in Chapter 3 Materials and

Methods for a period of 2 months so that the root systems filled the pots, but before the plants required additional feeding due to nutrient depletion. A dry soil sample was collected from a control pot and dried for 24 hours at 105°C. The moisture content of the soil was determined by this method so that when soil samples from the roots were collected, the undried equivalent of 3 g dry soil mass was used in each well.

Prior to sampling, water was withheld from the plants until the compost appeared dry, but before any noticeable water stress such as wilting was visible in the plants. Partially drying the soil in this way facilitated its removal making it easier to shake it from the plant roots.

Plants were knocked out of their pots and any loose soil removed. The plants were then shaken vigorously to produce root soil. Finally the roots remaining were gently brushed and shaken to release the fine rhizosphere soil adhering most closely to the roots themselves. The different soil types were collected separately before sieving through a 1 mm sieve once and then a second time to remove root fragments. Any visible pieces of roots which passed through the two sieving processes were carefully removed using ceramic tweezers.

After the soil samples had been weighed and added to the wells, 5 ml of 0.75% low temperature agar, cooled to around 45°C was then carefully pipetted into the well. This was absorbed by the soil and created a soil-agar layer.

When this initial agar layer had hardened, an additional 3.2 ml of the same agar was pipetted on top and allowed to cool to room temperature. Following hardening of the agar, five lettuce seeds were placed flat on the surface of the agar in the same way used in the Sandwich Method. A control soil plate was set up, using soil from a pot which was placed by under the light racks, kept moist, but without the addition of *Impatiens* plants. If insufficient control soil was available, an agar control, with no soil present, was set up instead.

Following seeding, the plates were sealed using laboratory film, wrapped in aluminium foil to exclude the light and incubated at 20°C for 3 days. At the end of this period, they were removed from the incubator and radicle and hypocotyl lengths were measured as described previously. Data were subjected to one-way ANOVA.

### **7.1.2 Initial experiment using *I. glandulifera***

Soil from *I. glandulifera* plants was collected as described above and the allelopathic inhibition of the root soil, rhizosphere soil and the controls compared.

### **7.1.3 Comparison of rhizosphere soils of *I. glandulifera* and *I. parviflora***

Soil from plants of *I. glandulifera* and *I. parviflora* which had been sown individually in pots at the same time was collected and the rhizosphere, root soil and control soils compared.

### **7.1.4 Time course experiment investigating the effects of root decay on the allelopathic potential of rhizosphere soil from *I. glandulifera***

Decay products from plants are known to be allelopathic under some circumstances (Didyk & Mashkovska, 2007). In order to determine whether similar effects occurred with *I. glandulifera*, an experiment was carried out in which *I. glandulifera* plants were grown for a period of two months in 1 litre pots. At the end of the growth period, the plants were severed just below the first node and the severed portion of the stems removed. The hypocotyl was allowed to remain in the pot. The plants were then placed back under the grow lights and kept watered as the pots dried out. Within a few days of this action, the stems became soft and began to decay indicating that no regrowth would occur and that the above and below ground portions of the plant were dying. An initial harvest was made at the time of cutting (Week 0) and every two weeks

following this date, with the final harvest occurring after 10 weeks, giving a total of six harvests. Root and rhizosphere soil samples were harvested as described above and controls consisted of soil removed from pots filled with identical mix to the sown pots, but without with any seedlings of *I. glandulifera*. One-way ANOVA was carried out. Results are found in Section 7.1.5.3.

## **7.1.5 Results**

### ***Initial experiment***

All treatments showed a high degree of significant differences between means ( $p < 0.001$ ). Rhizosphere soil caused the greatest inhibition of radicle elongation when compared to the control soil, 33.5%, while the hypocotyls were inhibited by 61.5% when compared to the value obtained for the control soil. Root soil caused less inhibitory effects than the rhizosphere soil, with 7% inhibition of radicles and 49% inhibition of hypocotyls. The results suggest that the soil intimately associated with live roots caused more pronounced allelopathic activity than material sourced from further away. See Figure 7.1.

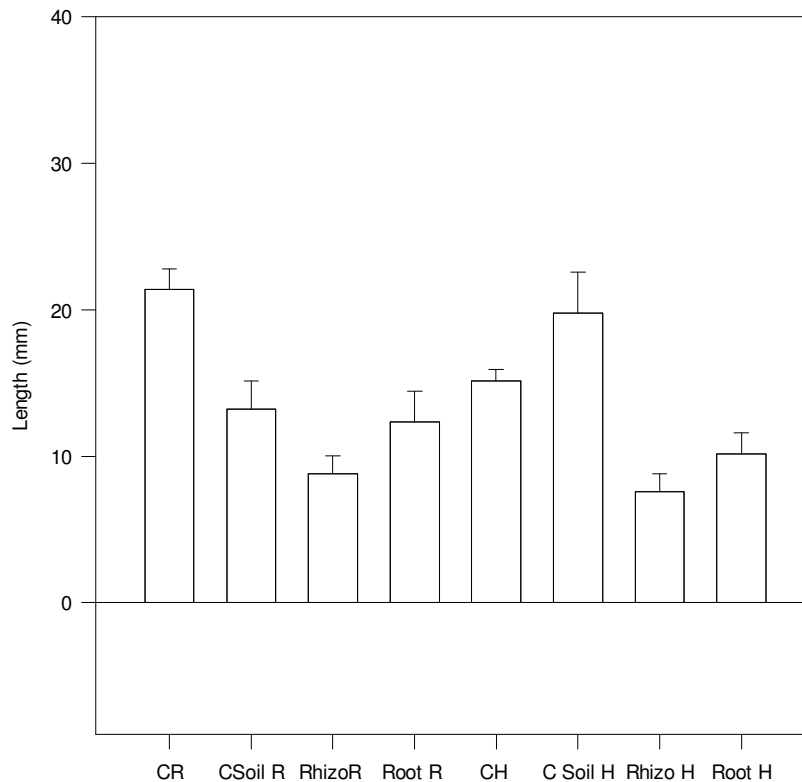


Figure 7.1. Effects of rhizosphere and root soil on the elongation of lettuce radicles and hypocotyls. C = control, R = radicle, H = hypocotyl. CR and CH are agar controls without the addition of any soil. Error bars represent 95% confidence intervals. N= 45.

### ***Comparison of rhizosphere soils of *I. glandulifera* and *I. parviflora****

All treatments showed significant differences from one another ( $p < 0.05$ ), apart from the two controls, which did not show a statistically significant difference ( $p = 0.151$ ). Rhizosphere soil from the roots of *I. glandulifera* showed a 30% inhibition compared to the agar control and a 21% inhibition compared to the control soil. Rhizosphere soil from *I. parviflora*, by contrast, showed close to an 18% increase when compared to the

control agar and close to 16% increase in the case of the control soil.

Results are presented graphically in Figure 7.2.

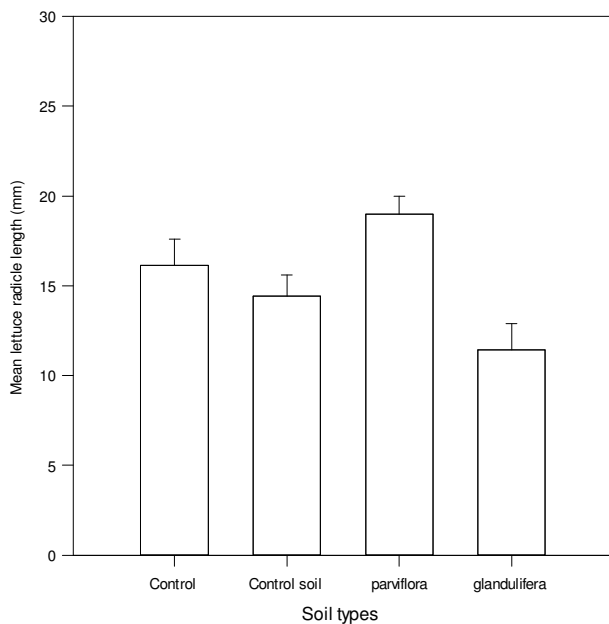


Figure 7.2. Effects of rhizosphere soils from roots of *I. glandulifera* and *I. parviflora* on lettuce radicle elongation. Error bars represent 95% confidence intervals,. N = 45.

### *Time course experiment*

#### Radicle length

At Week 0, both the rhizosphere soil and the root soil showed a significant difference from the values obtained from the control soil, ( $p=0.002$ ) with inhibition of 34% compared to the control soil. Root soil showed 7% inhibition; this was not a statistically significant difference, however ( $p=0.428$ ). There was a statistically significant difference between the rhizosphere and root soils ( $p=0.035$ ), with the rhizosphere soil showing a 29% reduction in length when compared to the root soil.

Differences between the rhizosphere soil and the root soil were only statistically significant for Week 0 ( $p < 0.001$ ) when radicles were measured. In all other cases, the differences between them were not statistically significant ( $p > 0.05$ ).

At weeks 2,4,6,8 and 10 both the rhizosphere and root soil values were higher than those of the control soil, the difference being statistically significant (either  $p < 0.001$  or  $p < 0.05$ ). There were no statistically significant differences between the rhizosphere and root soils at any of these sampling dates, however ( $p > 0.05$ ).

#### Effects on hypocotyls

In all cases the control soil produced higher mean values than either the rhizosphere or the root soil; these differences were statistically significant for both treatments at Week 4, ( $p < 0.001$ ) and week 10 ( $p < 0.05$ ). There were no statistically significant differences between the rhizosphere and root soils at any harvest date ( $p > 0.05$ ).



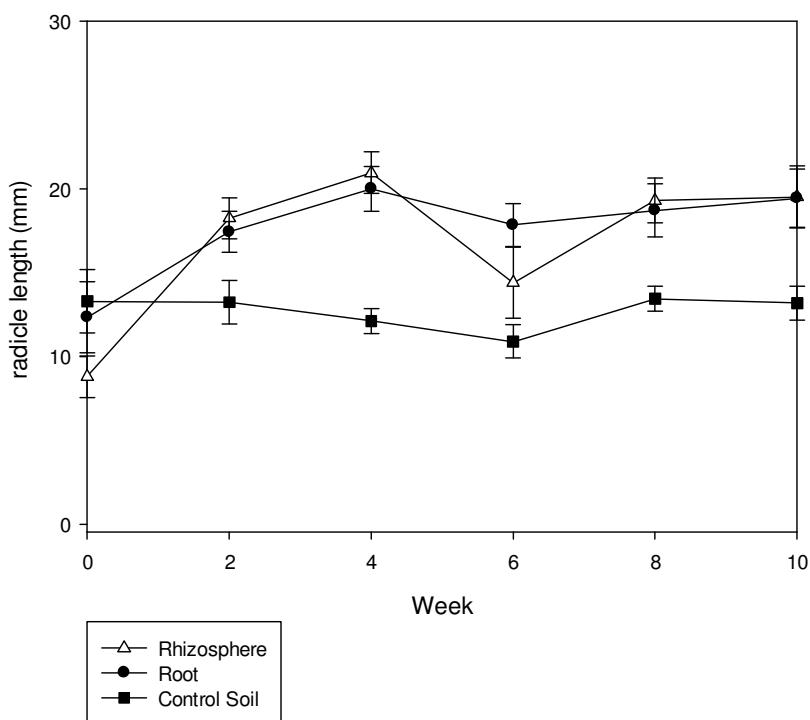


Figure 7.3. Effect of rhizosphere and root soil collected from *I. glandulifera* over a ten week period on lettuce radicle elongation. Error bars show standard error of the mean. N=60.

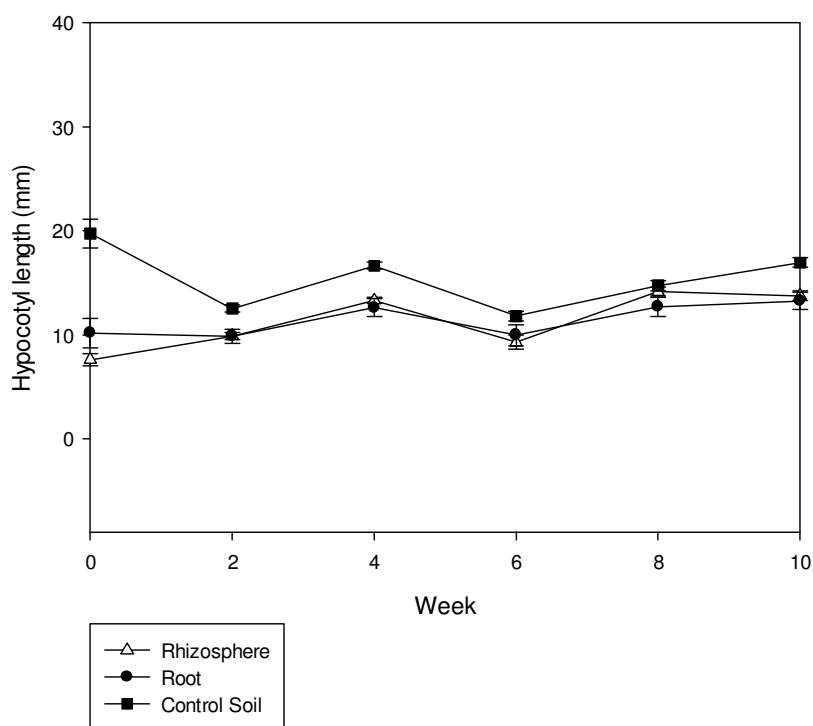


Figure 7.4. Effect of rhizosphere and root soil collected from *I. glandulifera* over a ten week period on lettuce hypocotyl elongation. Error bars show standard error of the mean. N=60.

### 7.1.6 Discussion

The Rhizosphere Method proved effective in distinguishing the allelopathic potential of rhizosphere soil from root soil. The effects observed indicated that freshly harvested soil from the surface of roots of *I. glandulifera* caused a greater inhibition of lettuce than the “root soil” - soil less closely associated with the surface of the roots. The differences in the inhibitory effects between *I. glandulifera* and *I. parviflora* were also distinguished effectively.

In the case of the induced dying off of the plants caused by severing the stems, the inhibitory effect of rhizosphere soil was short-lived. In fact both the rhizosphere and root soil produced a stimulatory effect on radicle length after the first harvest (Week 0) with the values obtained significantly higher than those of the control soil obtained from Week 2 (second harvest) onwards. This rapid decline in inhibitory potential could have been caused by the microbial degradation of the compounds responsible for the original inhibition into harmless ones, or as a result of the release of nutrients from the decay of the roots. Another possibility is that of a hormetic effect where the declining levels of the putative allelochemicals stimulated rather than inhibited the growth of the lettuce radicles. In any case, the results obtained suggested that the inhibitory allelochemicals produced by *Impatiens* species were quickly neutralised. A similar effect was recorded by Didyk and Mashkovska

(2007) in a study of three *Tagetes* species. The stimulatory effect, whatever its cause, persisted throughout the experiment from the second harvest at two weeks, to the final one which took place eight weeks later. By this later stage, the roots themselves had decayed to a large extent and the collection of the rhizosphere soil was becoming increasingly difficult.

The effect on hypocotyl elongation was somewhat different, with the values obtained for both the rhizosphere and root soils below that of the control throughout the run of the experiment.

As the ambient temperatures experienced by the plants and their roots were probably much higher than those found outside during the dying off process in the autumn, it is possible that the breakdown and neutralisation proceeded much faster than would normally be the case. An experiment to investigate this could be carried out using pot grown plants in a standardised medium outdoors. These would be harvested at regular intervals through the late autumn and winter as the plants died back and the soil obtained from them prepared in the Rhizosphere Method. Previous studies using the Rhizosphere Method have limited themselves to single measurements and have not attempted to address this aspect of rhizosphere soil allelopathy.

## **7.2 Pipette Tip Stair-Step Method**

Seedling exudate allelopathy was investigated using the MECAM described in Chapter 6. The results provided good evidence for allelopathic effects. In the light of those results, it was felt that an attempt should be made to explore the potential for allelopathic interaction using a medium more closely resembling soil. In soil, interstices are periodically filled and flushed by rainfall, oxygen levels are likely to be higher and the resultant microbial activity is more intense than that found within agar media. This suggested that an attempt be undertaken to design a simple experiment to explore these effects.

One method of separating the effects of root exudates from direct competitive effects is the Stair-step Method. The basic design involves the positioning of pots on an A frame construction with two shelves or steps, somewhat resembling a step-ladder. The donor pots are placed on the top shelf and a plastic tube is attached to the bottom of the pots so that all liquids pass through it. The distal end of the tube sits on or close to the surface of the pots on the lower shelf where the receiver plants are situated. Irrigation water is poured into the donor pots and drains into the base of the pot. Root exudates present are flushed from the donor pot and pass along the linking tube to the receiver pot below. In some cases the water is recycled via a pump. Variations on this basic method have

been used successfully used in a number of allelopathy studies (Bell & Koeppe, 1972; Mallik, 1987; Viard-Cretat et al., 2009).

As space was very limited and a comparison was sought with the kind of allelopathic activity shown by seedlings in the MECAM experiments, it was decided to radically reduce the pot volume used in the studies mentioned above and use seedlings rather than larger plants. A small pilot experiment had shown that *I. glandulifera* seedlings were capable of developing satisfactorily in 10 ml pipette tips over a period of several weeks. The small volume of the tips combined with the deep rooting area they provided, allowed plants to be spaced quite closely together. The aim of the second experiment was to use a small scale experimental rig which would enable the root systems of the donor and receiver plants to be physically separated but without the necessity of a large scale bench construction to support them. In the pilot experiment a 1:1 mix of perlite and vermiculite was used, this being commonly recommended for use in hydroponic systems. As the *I. glandulifera* plants grew satisfactorily in this medium, the same mix was used for the larger experiment.

### **7.2.1 Method**

The experimental design consisted of three frames with dimensions of 50 x 45 cm, each holding 18 pairs of pipette tips. Each of the donor plant tips

was aligned with its corresponding receiver tip so that unimpeded flow of water could occur between the two. The pipette tips were attached to the frame by means of loops of plastic coated horticultural wire attached to the top bar or a double galvanised wire strung across the frames at a height of 25 cm from the bottom so that the bottom row was approximately 6cm below the tips of the donor pipettes. 6 cm lengths of 3 mm diameter hydroponic dripper tube were affixed to the bottoms of the upper pipettes using a small quantity of silicone sealant around the outside to ensure a firm seal. The distal ends of the tubes were placed close to the surface of the corresponding receiver tubes. In some instances, due to natural curvature of the dripper tube material, it was necessary to remove a small section or cut a slightly longer tube so that the fit was accurate. Three treatments were prepared: a control with no *I. glandulifera* seedling present, and two treatments using *I. glandulifera* seeds, one in which the pipette tip was covered in black plastic to exclude light and the other left uncovered, so that the effects of light on allelopathic exudation could be investigated. The three racks were lined up in a row against the back wall of the light racks and were repositioned every two days so that each rack came to occupy the space previously filled by another, with the aim of equalising variations in light experienced.

For this experiment, rather than lettuce as the receiver plant, radish (*Raphanus sativus*) 'Juba' (Nicky's Nursery, Broadstairs, Kent, UK) was chosen. The advantages of radish over the lettuce were its larger seed size and its rapid development of a larger root system combined with a more upright habit of growth. This made it less likely to be swamped or washed away by the flow from the donor pots. When fully filled with the perlite: vermiculite mix, the rooting volume available to each seedling of either *I. glandulifera* or radish was around 12 ml. Waste water from the lower pipette tips was discharged into shallow plastic trays which were periodically emptied. Temperature and light regimes were identical to those used in previous experiments and described in Chapter 3, Materials and Methods.

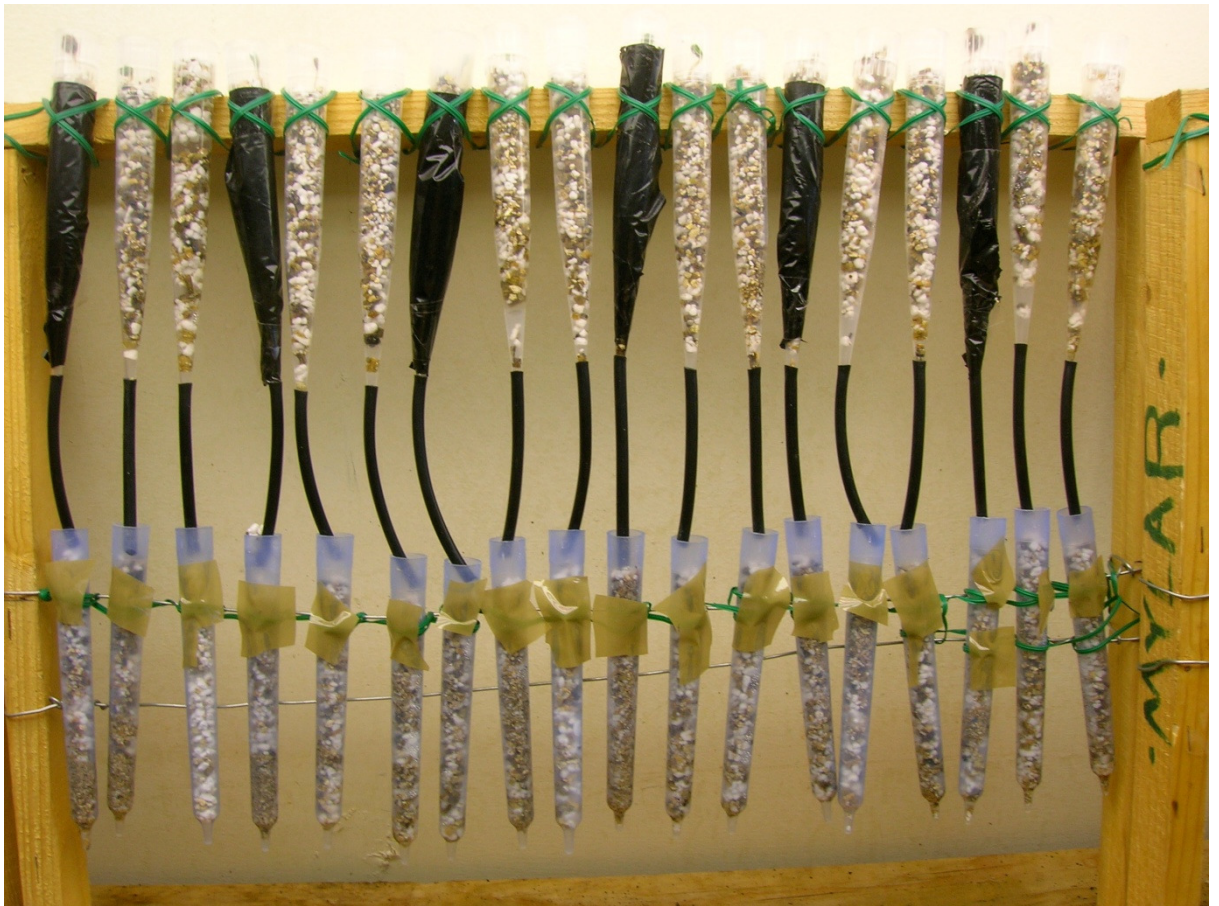


Plate 7.1. Pipette Tip Stair step Experiment. Arrangement of pipette tips and hydroponic tubing on the wooden frame. Each rack carried 18 individual experiments as described in the text.

Pre-germinated seeds of *I. glandulifera* were removed from the refrigerator and placed carefully into the tops of the upper pipette tips. They were covered with an additional 5 mm of the perlite: vermiculite mix and watered using the half strength orchid feed used previously in the cultivation of plants for the Plant Box Method described in Chapter 3. In this instance, the pH was adjusted to 6.5 as the radish requires a higher pH than the *Impatiens* plants.



Seven days after the *I. glandulifera* seeds were planted, single radish seeds were sown directly into the lower pipettes and covered in the same manner as the *I. glandulifera* seedlings. Plants were watered on a daily basis using 10 ml of the half strength orchid feed, this having been previously established to be sufficient to produce a steady flow of irrigation water through the pairs of tips. All plants were harvested one month after the radish seeds were sown. At harvest, the plants were clipped at the surface of the growing medium and each individual was loosely wrapped in foil before drying at 60°C for 24 hours. Data analysis was carried using Kruskal-Wallis ANOVA.

### 7.2.2 Results

In general, most of the *I. glandulifera* seedlings and the radishes developed satisfactorily, although in the case of the exposed *I. glandulifera* seedling treatment, 5 out of the 18 *I. glandulifera* seedlings did not grow. By contrast, only one radish seedling failed to develop successfully, this being a receiver of water which had passed through a covered *I. glandulifera* seedling. When the data were analysed using Kruskal-Wallis ANOVA, there was no statistically significant difference between any of the treatments ( $H=6.246$ ,  $p = 0.182$ ). The apparatus as used did not produce any evidence of allelopathic inhibition caused by the root systems of *I. glandulifera* against those of the radishes, nor were there any

differences between the covered and uncovered root systems in terms of the production of inhibitory allelochemicals.

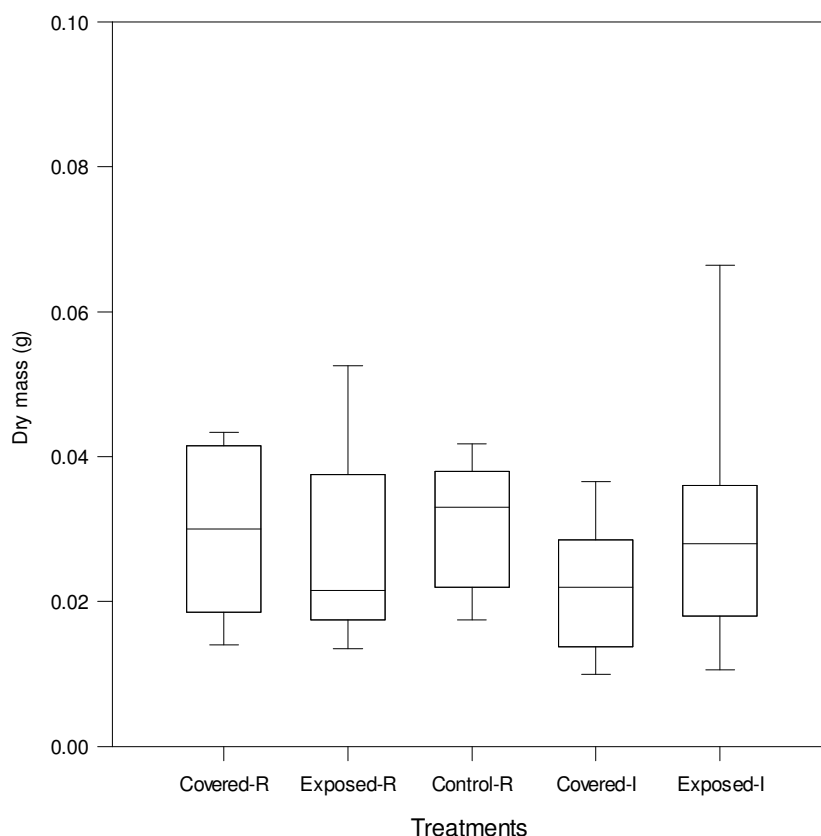


Figure 7.5. Box plots of radish and *I. glandulifera* mass using the pipette tip stair-step method. There were no significant differences when the treatments were analysed using Kruskal-Wallis ANOVA ( $p = 0.182$ ). Covered and exposed refer to the presence or absence of opaque black plastic around the pipette containing the *I. glandulifera* roots. R = radish, I = *I. glandulifera*. N= 18. Whiskers represent 5<sup>th</sup> and 95<sup>th</sup> percentiles.

### 7.2.3 Discussion

This initial finding suggested that allelopathic interactions between the root exudates of the spatially separated root systems of *I. glandulifera* and the radish seedlings did not occur under the conditions used. The relatively high failure rate of the *I. glandulifera* seedlings could be

indicative of problems with the growing medium. It is possible that the very large interstices created by the perlite might have been sufficient to cause excessive drying of the seeds between irrigations and this then prevented their establishment. This may have also impacted on root development in the other *I. glandulifera* plants, thus reducing their production of root exudates. Another possibility is that the excellent aeration provided by the growing medium facilitated the development of a large and active microbial flora and these were able to utilise or break down root exudates into harmless forms.

The experiment was particularly time limited. Due to the requirements of other users, it was necessary to vacate the space at the end of the month long experimental period. An extended experimental run, with multiple harvests over a number of weeks, might have provided a more complete picture of the effects. It is also possible that the dilution factor of the feed was too great for the quantities of exudates being produced by the small root systems which managed to develop during the experiment.

Although the results obtained did not provide evidence of allelopathy, the pipette tip design might prove to be a simple, cheap and useful method of assessing the effects of seedling root exudates. A range of different growing media, pH regimes and species could be used.

## **7.3 Target- Neighbour Experiment**

### **7.3.1 Introduction**

One of the more promising ways of distinguish allelopathy and resource competition has been outlined by Weidenhamer (2006). It is known that when plants are grown at varying densities, the total yield per area increases linearly until plants begin to compete with one another for resources. Above a certain density yield remains constant but individual plant mass declines (Kira, Ogawa & Sakazaki, 1953). Where yields remain constant, the slope of the log mean plant mass and log density line approximates -1. This is considered to be one of the few universally applicable laws of plant population ecology (Harper, 1977; White & Harper, 1970).

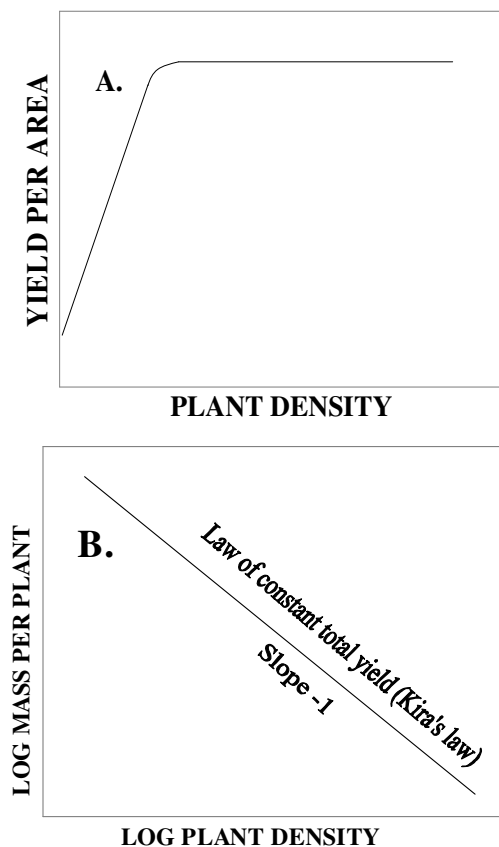


Figure 7.6. Effects of increasing competition on A ) yield per unit area and B) log mean mass per plant where total yield is constant. From Weidenhamer et al. 1989. Used with permission of Blackwell Science Ltd.

In the case of allelopathic responses, Weidenhamer (2006) argued that if allelopathic effects were present, the mean mass of the individual plant would increase with increasing density as each individual received a smaller dose of the allelochemical and would therefore experience less inhibition. The result would lead to a deviation from the usual -1 law of constant yield with a positive slope rather than a negative one. At very low densities allelopathic effects would limit plant size; at very high densities resource competition would again be a dominant controlling factor, so the expected maximum mean individual mass would occur at

intermediate densities. This method, if successful, would enable resource competition and allelopathy to be distinguished from one another.

Density dependent phytotoxic effects have been reported from herbicides (Hoffman & Lavy, 1978; Skipper, Gilmour & Furtick, 1967) as well as from naturally occurring compounds found in soil (Weidenhamer, Hartnett & Romeo, 1989). Experimental systems using a target neighbour design include that of Thijs et al. (1994), where the herbicide atrazine was added to pots containing corn and soya beans grown at different densities. To date, no studies investigating this approach on the effects of root exudates from invasive alien species have appeared in the literature. This pilot study was an attempt to explore the possibilities and limitations of such an approach. Weidenhamer (2006) suggested growing plants at a range of densities between 1, 2, 4, 8 and 16 so that allelopathic effects be determined.

### **7.3.2 Method**

A first trial experiment using *I. glandulifera* with differing densities of nettles (*Urtica dioica*), a common competitor, was abandoned when the nettle seeds showed very slow and erratic germination, a problem previously described in Section 4.10. A substitute species was sought and Phacelia (*Phacelia tanacetifolia*) was selected. Although not a natural competitor of *I. glandulifera*, it was chosen due to its reputation as a green

manure crop, where it is valued for its rapid germination and vigorous weed-suppressing growth. It matures rapidly, reaching maximum root development within about two months of sowing (Anon, 2009). It was felt that this might make it a suitable competitor for sowing at variable densities in pots with *I. glandulifera*.

In February 2010, some space became available in an unheated greenhouse at Portland Villas and this was used to carry out the experiment. Growing conditions are described in Section 3.4.1.

108 1 litre pots were filled with 1:1 mix multipurpose compost and John Innes no.1 compost (Sinclair Horticulture, Lincoln, UK) which was used previously to grow plants under the light racks as described in Chapter 3, Materials and Methods. Half the pots were amended with 10 gkg<sup>-1</sup> AC, a figure close to that used by other studies (Callaway & Aschehoug, 2000). The AC was added to adsorb any putative allelochemicals that might be exuded by the roots of the *I. glandulifera* plants. The effect of this, according to Weidenhamer (2006), would be a return of the line of the slope to -1, as competition rather than density dependent allelopathy assumes greater importance.

Pre-germinated *I. glandulifera* seeds were removed from the refrigerator and placed in the centre of the 1 litre pots, at a depth of 1 cm. They were then carefully watered with a fine rose watering can and placed on capillary matting on the greenhouse staging. Pots were

watered as required and randomised on a weekly basis so that edge effects were minimised. Three weeks later, pre-germinated *Phacelia* seeds were planted into the pots at the densities of 1,2,4 and 8 *Phacelia* seeds per pot with three replicates per treatment for each of the three harvest dates: 3,6,9 weeks after sowing the *Phacelia*. This gave a factorial design of seedling density combined with the presence/ absence of AC. 18 single *Phacelia* plants grown in 1 litre pots were planted as controls, half the pots containing AC, the other half unamended. *I. glandulifera* controls consisted of 18 plants sown contemporaneously with the other *I. glandulifera* seeds, but not sown with *Phacelia*, again with half in AC amended pots, the remaining 9 without amendment.

Other than regular watering and weekly randomisation, the plants received no other attention or fertilisation.

Each pot was assigned a number; pots were selected for harvest by randomly choosing the pot number. Plants were clipped at soil level, loosely wrapped in aluminium foil and dried in an oven at 60°C for 24 hours and their dry masses recorded. Data analysis was carried out using ANOVA or Kruskal-Wallis ANOVA, depending on the normality of the data being analysed. SigmaPlot 12 statistical software was used for the analyses.



## **7.4 Results**

### **7.4.1 Combined mean mass per pot of *I. glandulifera* and *P. tanacetifolia***

There were no significant differences between any of the treatments when they were compared using Kruskal-Wallis ANOVA at any of the three harvest dates ( $p > 0.05$ ). There was no apparent indication of a statistically significant increase in values for increasing pot densities at Weeks 3 and 6. However the very low power of the tests at Weeks 3 and 6 ( $\alpha = 0.05$ : 0.05, desired value = 0.800) may throw these results into doubt. A visible increase was particularly noticeable in the case of week 9 (see figure 7.8 below).

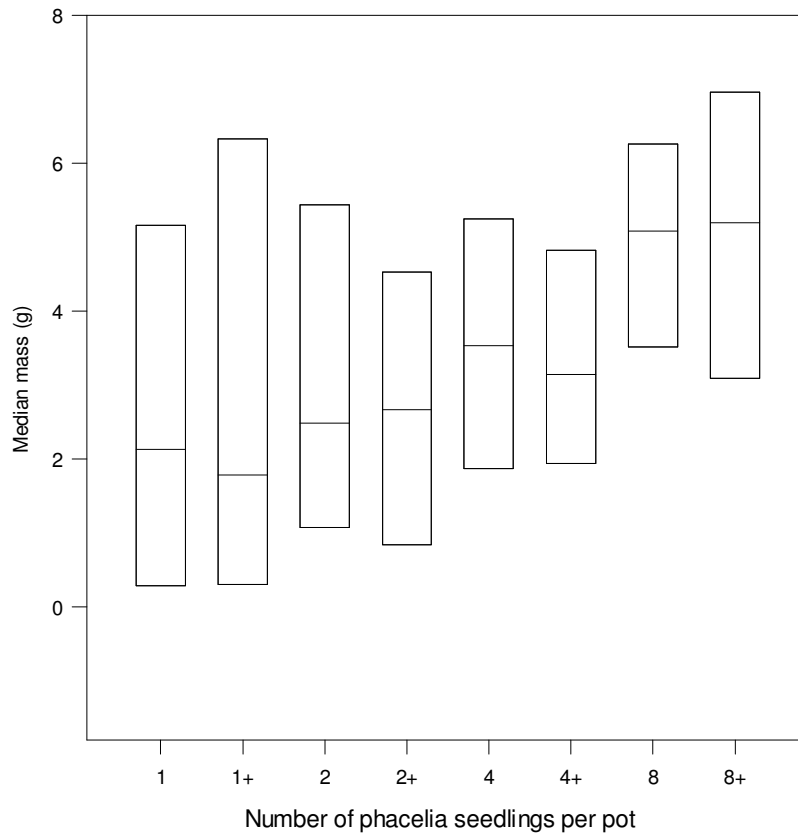


Figure 7.7. Non statistically significant increase in median combined mass per pot of phacelia and *I. glandulifera* at Week 9, using Target-neighbour experimental system. ( $p=0.337$ )  $N=6$

#### 7.4.2 Comparison of *I. glandulifera* grown in competition with Phacelia with controls grown singly

There were no significant differences between any of the treatments ( $p>0.05$ ) at weeks 3, 6 and 9, suggesting that the *I. glandulifera* plants were not experiencing any significant inhibition as a result of the Phacelia's presence at any of the densities, although power of the tests in week 6 and 9 was well below the desired value of 0.800, so that caution should be exercised in interpreting these effects as non-statistically significant.

There were no statistically significant effects caused by the addition of activated charcoal to the growing media, either.

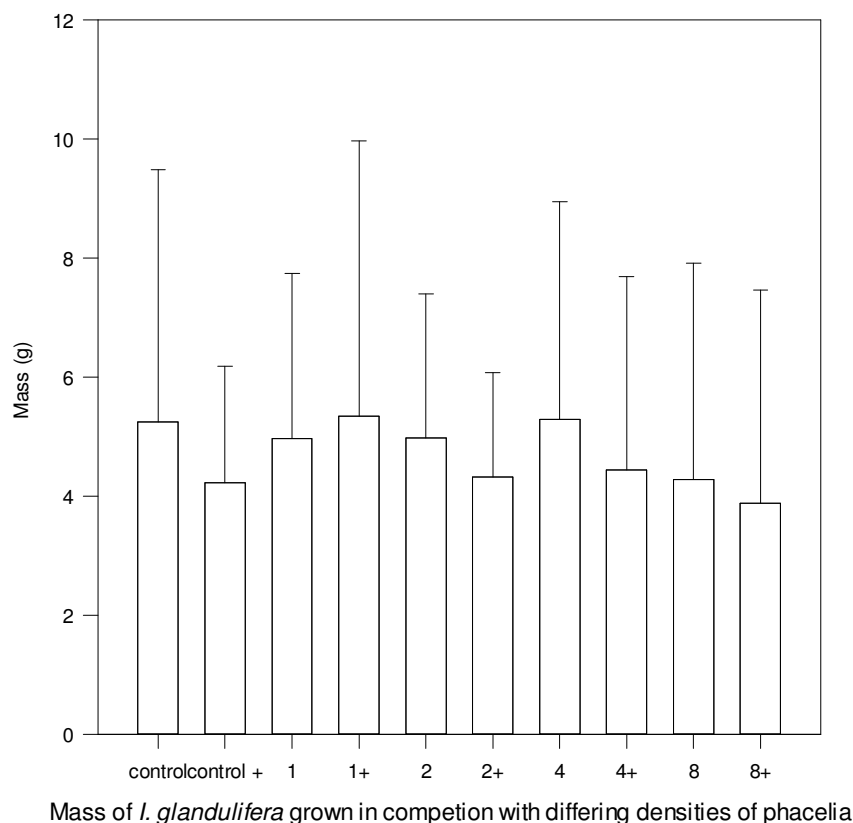


Figure 7.8. Effects of increasing densities of phacelia on the growth of single *I. glandulifera* seedlings using Target-neighbour experimental system. Data from Week 9, final harvest. Error bars show 95% confidence intervals. There was no significant difference between treatments ( $p>0.05$ ).  $N=3$ .

### 7.4.3 Comparison of differing densities of Phacelia grown in competition with *I. glandulifera*

There were no statistically significant differences between the control Phacelia plants, grown without competition from *I. glandulifera* and those of any other treatments at the first harvest at 3 weeks ( $p>0.05$ ) By week 6 the situation was reversed, with the controls showing highly statistically

significant increases in mass when compared to all other treatments ( $p < 0.001$ ), whereas those *Phacelia* plants growing in competition with *I. glandulifera* showed no statistically significant differences between treatments ( $p > 0.05$ ), irrespective of the density of sowing. The single plant controls were more than twice the mass of the 8 seedling treatments, with values of around 4 g as opposed to the 1.5 g values obtained from the 8 per pot seedlings. The two controls did not show any statistically significant difference.

At week 9, an upward trend in mean mass per pot was visible, with an increase between the single plants and increasing densities up to 4 per pot. Increases ranged from about 0.3 g for the 1 *Phacelia* per pot density to around 2 g for the 4 seedling per pot density. These differences were not statistically significant, however ( $p > 0.5$ ). The differences between the 8 seedling densities and the lower densities were statistically significant, with  $p < 0.001$  for the single density plants and 2 per pot densities and  $p < 0.05$  for the differences between the 8 per pot densities and the 4 per pot densities. The two controls, were not statistically significantly different from each other ( $p = 0.978$ ) with a mass of around 12 g.

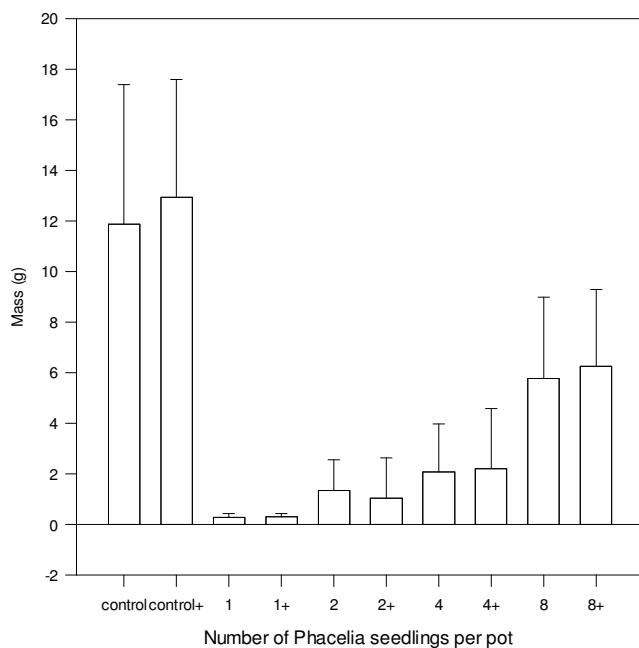


Figure 7.9. Effects of increasing density of Phacelia seedlings grown in competition with *I. glandulifera* on mean phacelia mass per pot using Target-neighbour experimental system. Data from Week 9. The + symbol indicates the addition of activated charcoal to the growing medium. N=3.

#### 7.4.4 Log mean plant mass versus log density

##### Week 3

A Comparison of the two Week 3 harvest graphs shows radically different trends in the slope of the two lines obtained. The unamended plot produced a line suggestive of an allelopathic effect, with the slope tending towards positive and if the initial value for 1 plant per pot is removed, the line was almost perfectly linear ( $r^2 = 0.9987$ ). The AC amended pots produced a line with a strongly negative slope, resembling the -1 thinning law as described previously.

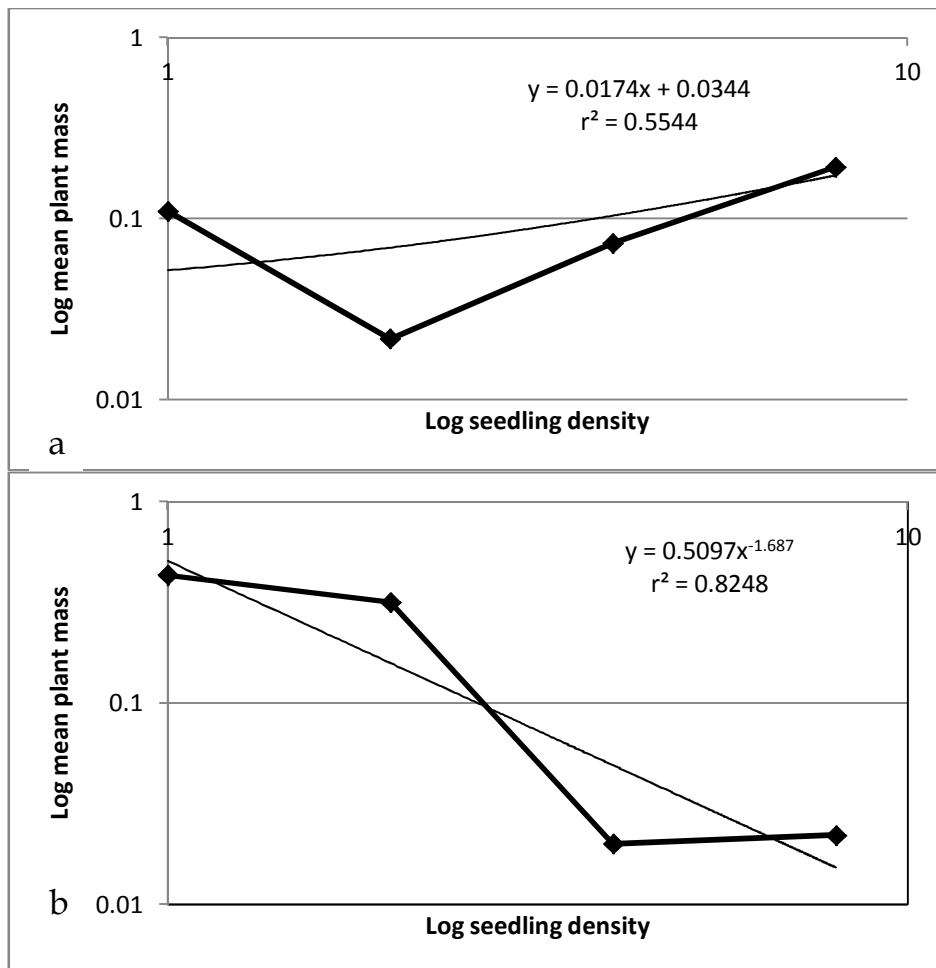


Figure 7.10. Plots of log phacelia seedling density and log plant mass using arget-neighbour experimental system. Week 3 harvest a) no AC, b) with AC. Trend lines and equations fitted to the data.

## Week 6

At 6 weeks, the trends appeared to be in the direction of a negative slope, although the data were not conclusive as evidenced by the fit of the trend lines and their coefficients of determination. Unlike Week 3, however, both sets of data showed very similar shapes, with maximum values at 2 seedling per pot densities.

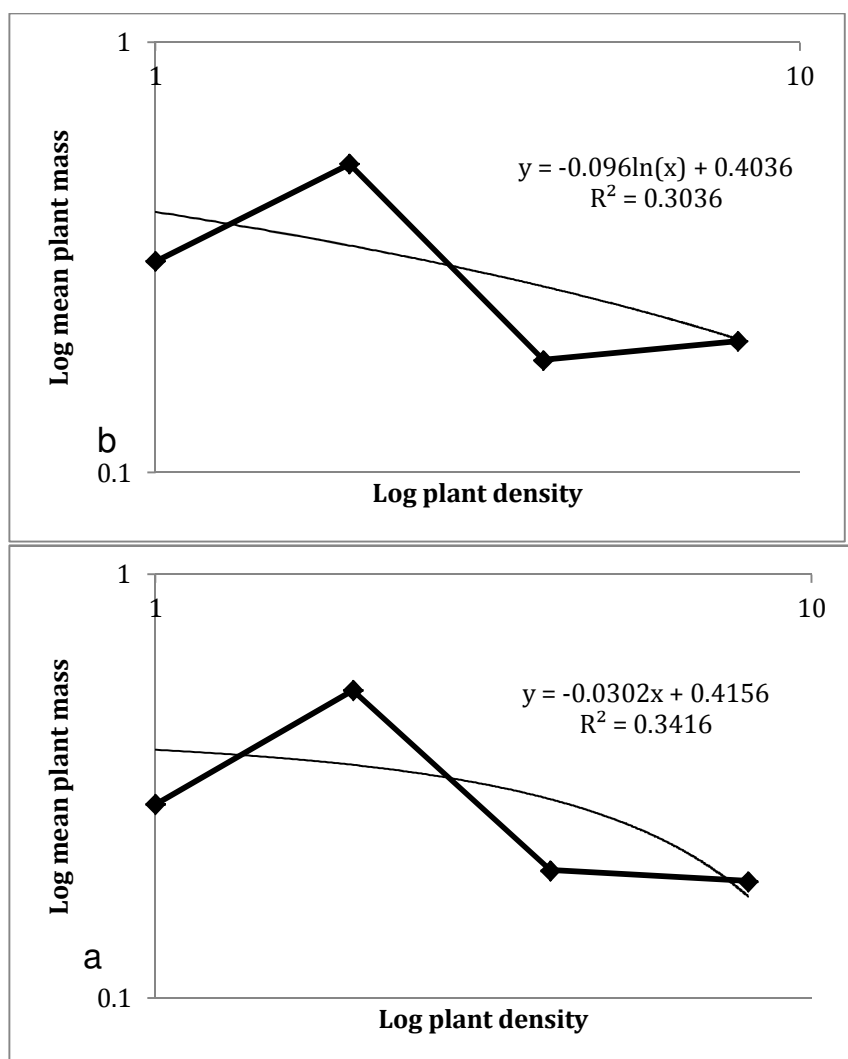


Figure 7.11 Plots of log phacelia seedling density and log plant mass using Target-neighbour experimental system. Week 6 harvest a) no AC, b) with AC. Trend lines and equations fitted to the data

### Week 9

Here the situation was reversed, with both the untreated and activated charcoal treated pots showing a pronounced upward trend, with  $r^2$  values of 0.872 for the unamended pots and 0.9369 for the activated charcoal amended pots.

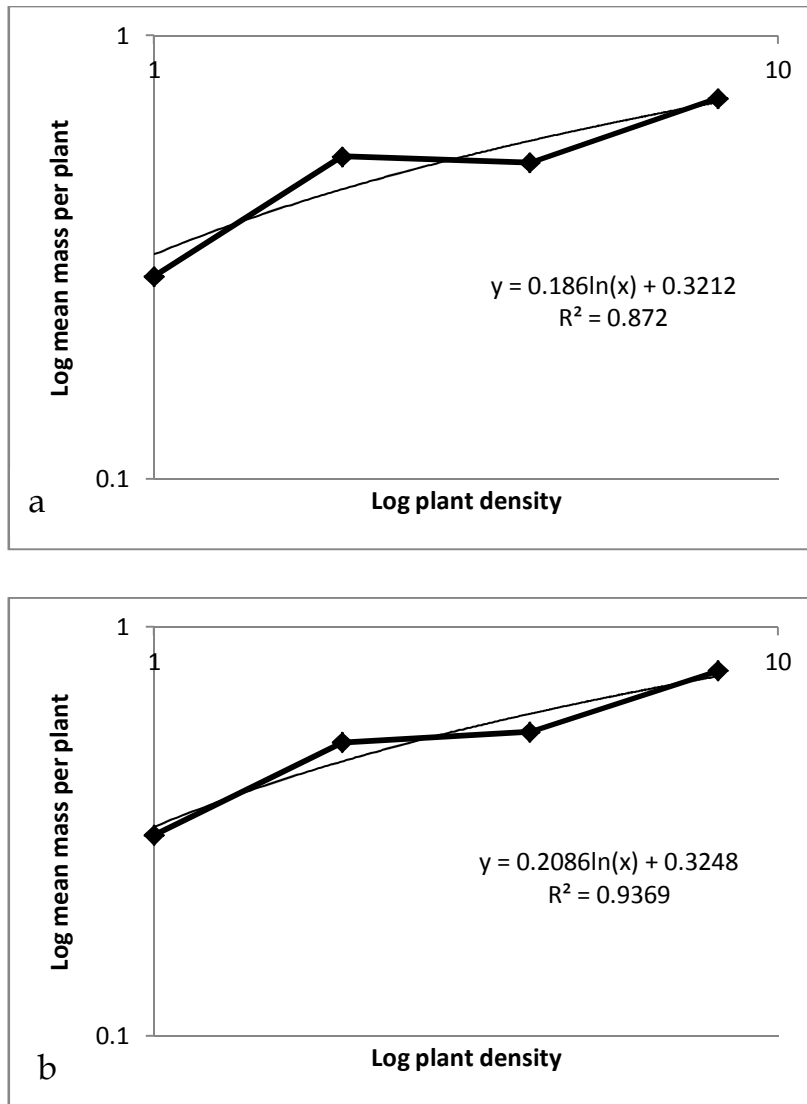


Figure 7.12. Plots of log phacelia seedling density and log plant mass using Target-neighbour experimental system. Week 9 harvest a) no AC, b) with AC. Trend lines and equations fitted to the data.

## 7.5 Discussion

The results showed that sowing the *I. glandulifera* three weeks in advance of the Phacelia was sufficient time for the *I. glandulifera* to establish dominance over it, irrespective of the density of Phacelia used. When *I. glandulifera* and the Phacelia were grown separately, however, the dry matter production of the Phacelia equalled *I. glandulifera* at Weeks 3 and



6, with no statistically significant differences between treatments, despite its later sowing ( $p>0.05$ ). At the 9 week harvest, the Phacelia dry mass was more than twice that of the *I. glandulifera* (around 12 g for the Phacelia and 5 g for the *I. glandulifera*), with the differences being statistically significant between the two species ( $p<0.05$ ), although there were no statistically significant differences between the non-amended and AC treatments for each species ( $p>0.05$ ). The low biomass of *Impatiens* species has been noted previously (Scharfy et al., 2011) and may reflect the production of a succulent fleshy stem with little lignification when compared to the much woodier stems produced by Phacelia.

Due to space restrictions, it was not possible to grow Phacelia seedlings on their own at the full range of densities. Comparisons between Phacelia growing in competition with *I. glandulifera* and their counterparts at the same density was not therefore possible and comparisons had to be made with single seedling per pot controls.

Activated charcoal did not appear to play an important role in ameliorating allelopathic effects in this study. There were no statistically significant differences between treatments as a result of its addition to the growing medium as might have been expected if absorption of allelochemicals was taking place. The only case in which it appeared to exert an effect was in the log mass/ log density experiments described

below. It is possible that the addition of AC altered the nutrient status and absorbency of the growing medium and did not function as a means of removing allelopathic root exudates as had been intended. Some doubt has been cast on its efficacy in this role (Lau et al., 2008).

The results obtained from the log density log mean mass plots were inconsistent and unclear, with contradictory directions of slope at Week 3, where the addition of activated charcoal produced a negative slope as opposed to the positive slope found in the unamended pots. This result was consistent with Weidenhamer's prediction of the behaviour of activated charcoal amended soils, but was not repeated subsequently in weeks 6 and 9 as might have been expected if the effect was more than an artefact of the data. There was an apparent tendency towards a negative slope at week 6 for both treatments, whereas a definite positive slope was present at Week 9, with high  $r^2$  values, again suggestive of the effect of increasing density leading to reduced individual dose of inhibitory chemicals and commensurate stimulation in plant growth as described by Weidenhamer (2006). As mean yield per pot showed a clear but non-statistically significant increase at Week 9 for the lower densities and statistically significant increases at the 8 per pot density, caution is advised against inferring allelopathic interactions on the basis of the results obtained here. Further studies should seek to establish at what

density independent final yield per area is established and use this as the starting point for further investigations.

The experiment as it was conceived was limited in its scope due to the space restrictions imposed by the size of the greenhouse. Only three replicates were possible for each treatment; more would have helped. However this technique, with appropriate improvements, could prove to be a useful method of exploring the density dependent aspect of allelopathic effects. To date, the author is unaware of any published studies which have used a target-neighbour design specifically to explore the density dependent allelopathic interactions of invasive plants, although one study looked at the effects of different hydrological conditions on the competitive interactions of a marsh plant, *Schoenoplectus fluviatilis* and its competitors (Davis, Bidwell & Hickman, 2009).

Further studies using ecologically relevant species at a wider range of densities and for an extended period would be beneficial in assessing the efficacy of target-neighbour designs as a means of distinguishing resource competition and allelopathy as proposed by Weidenhamer (2006). Nettle plants of a suitable size could be grown from cuttings or plant tissue culture to eliminate genetic variation between individuals, thereby reducing one of the confounding factors. The results of previous studies carried out as part of this research established that the allelopathic effects of *I. glandulifera* were limited, in the main, to root inhibition rather

than preventing germination. The use of plantlets or seedlings would therefore be unlikely to influence the results obtained. As *I. glandulifera* frequently grows in sites where grasses are common and often competes effectively against them, a further experiment using a reliably germinating grass species as the neighbour plant would also be indicated. In addition to the simple pairwise interaction carried out here, it would be possible to include additional neighbour species and compare the differential effects of *Impatiens* root exudates, if any, on their relative growth.

Although conclusive proof of allelopathy was not established by this method, the results obtained indicate that it could form the basis of a useful tool for distinguishing the effects of resource competition and allelopathy. Other studies such as that of Thijs et al (1994) concentrated on the addition of herbicides to the experimental system rather than on the production of allelochemicals by the target plant itself. This study, therefore, represents a first step towards a more ecologically relevant approach to the study of allelopathic interactions between species growing together under reasonably natural conditions. By linking allelopathic activity to well-established plant laws, such a method might help persuade allelopathy's many sceptics that it deserves more serious investigation as one of the potential factors involved in the spread of invasive alien plants.

## **8. General Discussion and Conclusions**

### **8.1 Introduction**

The investigations carried out in previous chapters aimed to explore the allelopathic potential of the alien invasive plant species *Impatiens glandulifera* and other *Impatiens* species. Although review papers such as those of Field et al. (2006) discuss the subject of allelopathy in some depth, practical studies into allelopathy in the British situation are singularly lacking. In fact, no published studies investigating the role of allelopathy in the spread of invasive weeds in Great Britain are known to the author. Nearly ten years have passed since Fitter (2003) reviewed Bais et al.'s (2003) study of *Centaurea* allelopathy. Fitter used the title "Making Allelopathy Respectable" and suggested that other researchers take up the challenge of exploring allelopathy as one of several mechanisms of plant interference and a possible contributory factor in the spread of invasive alien species. Since that date there has been a flurry of interest in Continental Europe, Asia and North America, but little in the UK. Plant secondary chemistry has been suggested as one possible early screening method for assessing the likelihood that particular plants may become invasive (Ehrenfeld, 2006).

The studies carried out in the course of this research sit within that historical context and represent a preliminary exploration of the possibility of allelopathy as a factor in the establishment and spread of

alien invasive species of *Impatiens*. The thrust of the research was, in fact, twofold: methodological development to provide techniques suitable to detect allelopathic effects and secondly to provide supporting evidence of allelopathic potential from the *Impatiens* material grown. As far as the author is aware, there were no similar studies taking place in the UK at the time this research was being carried out.

## **8.2 Evaluation of methodological development undertaken during this study**

The bioassays used in the research undertaken here were more sophisticated than the simple application of solutions of plant extracts which have frequently featured in allelopathy studies. They had the advantage of low cost and were relatively simple to carry out effectively with basic training, yet were able to produce interesting results. They represent a progression from the measurement of the effects of dried *Impatiens* plant material to more complex investigations involving living plants, soil and attempts to separate the effects of resource competition and allelopathy. As might be expected in a preliminary investigation project of this type, not all methods proved to be equally successful.

In the case of existing protocols, modifications were made to investigate additional aspects of allelopathy to those originally intended by their developers; examples found in this study are the Sandwich, Rhizosphere,

Plant Box and MECAM experiments. Experimental protocols developed specifically for this study were the Pipette Stair-Step and the Target-Neighbour experiments. An evaluation of the performance of the different methods is given below.

### **8.2.1. Sandwich Method**

As well as being used successfully in its original form for assessing the effects of leaf leachates on lettuce seed germination, the Sandwich Method showed itself to be very versatile with the basic protocol easily adapted to a number of previously unexplored possibilities. For instance, it proved to be effective over a much wider range of concentrations (1 – 128 mg) than the original 10 and 50 mg specified and could therefore be easily be adapted to ones previously determined to be ecologically relevant to the species being investigated. It was capable of detecting differences between finely powdered and coarsely flaked leaf material. It was also used to distinguish the allelopathic potential of three *Impatiens* species. It enabled comparisons to be made between different plant organs and their allelopathic potential. Similarly, a modified protocol using the two naphthoquinones, lawsone and 2MNQ, was able to distinguish between their inhibitory effects at different concentrations. The addition of AC to the agar medium showed that the effects of leachates from *Impatiens* material could be changed or reduced by their

adsorption by AC and the effects of this recorded. The Sandwich Method can be described as a good basic allelopathy bioassay and its virtues lie in its versatility, simplicity and minimal cost outlay. Its main limitation is its reliance on a single test species, lettuce, which in the case of the *Impatiens* species studied here, has very limited ecological relevance. Lettuce does have several advantages however, namely its rapid and reliable germination and the production of a single radicle with few little or no branching during the time frame of the experiments. This makes for rapid and easy measurement.

Further studies into the phytotoxic effects of pure naphthoquinones could be undertaken, either singly or in blends; this work would be informed by the data obtained on concentrations in *Impatiens* tissue via HPLC and would give a more complete picture of their role as potential allelochemicals.

### **8.2.2 The Plant Box Method**

This proved to be a useful tool for demonstrating the effects of living *Impatiens* root systems on lettuce radicle inhibition. As such, it provided clear evidence of distance dependent inhibition of lettuce radicles by the exudation of water soluble allelochemicals from *Impatiens* root systems. This combined with the staining of the agar, made it a particularly visually impressive bioassay. Its main limitation was the uninvestigated nature of the changes caused to the roots and root hairs by the removal of



the sand growing medium and subsequent encapsulation of the whole root system in the agar. It is known for example that the development of root hairs in *Sorghum* and the rate of exudation of the allelochemical sorgolone from them can be modified by the medium in which they grow (Yang et al., 2004). The same reservations expressed about the use of a single test species in the Sandwich Method are equally applicable to the Plant Box Method.

The incorporation of AC into the agar medium of the Plant Box Method might demonstrate a reduction in allelopathic inhibition of lettuce radicles, or pure naphthoquinones on cotton wool could be substituted for plant roots and their effects on radicle inhibition compared at various concentrations to give an accurate picture of their rate of diffusion. Further experiments could include comparisons of *I. noli-tangere* and *I. capensis* with *I. glandulifera* and *I. parviflora*.

### **8.2.3 The Rhizosphere Method**

This proved successful in detecting differences in allelopathic potential from different soil fractions. It was able to record both stimulatory and inhibitory effects and proved capable of distinguishing the effects produced by different species. It was sufficiently versatile to be used to determine changes in allelopathic potential produced by the decomposition of roots and their exudates in soils over a period of weeks,

the first time such a study has been undertaken using this bioassay. A further development would be to use this method to assess soil samples from naturally occurring stands of *I. glandulifera* (and other *Impatiens* species if possible) and compare their effects over a complete growing season.

#### **8.2.4 The MECAM**

The MECAM examined allelopathy at the seedling stage, possibly the most critical time in the life of a plant when it is at its most vulnerable and when its root exudate production is at its peak. It demonstrated that even small *Impatiens* seedlings had the potential to suppress the growth of competitors, something which has not been demonstrated previously. It was also possible to distinguish the differing effects of germinating, dormant and dead seeds and the effects of differing sowing densities on the degree of inhibition produced, indicating that the MECAM was a useful addition to the allelopathy researcher's toolkit. Unlike the Plant Box Method, donor and receiver plants roots were cultured together in the same agar medium and the differences in mass between the donor and receiver plants were much less pronounced. This is perhaps a closer approximation of the situation pertaining in natural stands of *Impatiens* in the spring when seedlings of different species emerge and begin to compete. Its limitations included the use of a single test species lettuce

and the exposure of root systems to light, which was avoided in the Plant Box method by wrapping the Magentas in black plastic. The distance between the two rows of lettuce seeds was insufficient for significant differences in diffusion and subsequent inhibition to be manifested, suggesting that more frequent sampling would be necessary for this to be a worthwhile subsidiary test. *Impatiens* seeds proved themselves to be sensitive to placement in the agar and if incorrectly positioned so that the embryo was submerged, germination and development was reduced and seed mortality rates were high. When the sowing technique was modified so that the embryo was placed at the agar-air boundary, rapid germination and radicle elongation occurred and mortality rates were much lower.

Further work could involve the two untested species, *I. noli-tangere* and *I. capensis* which would help to complete the picture of their allelopathic potential throughout their lifecycles and provide useful comparative data to that already gathered. Naphthoquinone exudation is known to be affected by factors such as pH (Babula et al., 2006) and a series of experiments using a range of pH adjusted media might provide some useful insights into the changes in exudation rates elicited by different pH levels.

### 8.2.5 The Pipette Stair-Step Method and the Target-Neighbour experiment

These were both attempts to explore ways of eliminating the confounding factor of resource limitation in allelopathic interactions. In the case of the Pipette Tip stair-Step Method this was attempted by physically separating the root systems of donor and receiver plants, while allowing the free flow of water and leachates from the *I. glandulifera* seedlings through the rhizosphere of the radish seedlings; it was hoped that allelopathic effects could be distinguished from those of direct competition for light, water and nutrients by this means. The arrangement used, consisting of 10 ml pipettes was simple and practical to construct and although the results obtained gave no indication of differences between treatments, further development work on species selection, rooting substrate and fertilisation rates could be carried out.

Similarly, the Target-Neighbour experiment was in essence, a pilot study and the inconclusive results it produced were not unexpected; this was perhaps the most sophisticated experimental design, however, involving the mixed cultivation of donor and receiver species in the same pot in an attempt to mimic the rooting conditions existing in natural stands of *Impatiens* plants and their competitors. Although studies of this type have been suggested by researchers in the field (Weidenhamer, 2006), the author is unaware that any have been carried out to date. Further

research and experimentation is warranted to establish the densities at which constant final yield begins to take effect. A well-designed experiment using this information could provide allelopathy researchers with an effective means of confirming or eliminating allelopathy as one of the interference mechanisms used by plants under reasonably realistic conditions.

### **8.3 Germination and cultivation issues**

Several unplanned and unforeseen interruptions to the programme of study as originally envisaged meant that the original aims as laid out in Chapter 2 were not entirely fulfilled.

Perhaps the most unexpected challenge this study presented was that of achieving reliable germination and growth of the four original target species, namely *I. glandulifera*, *I. parviflora*, *I. capensis* and *I. noli-tangere*. Germination of *I. noli-tangere* proved unreliable and *I. capensis* a complete failure. This mirrors the findings of Perrins, Fitter & Williamson (1993) who experienced similar failures in germination with both *I. noli-tangere* and *I. capensis*. The nature of the differences in germinability between species was investigated by Perglová et al. (2009). Their results were very different: *I. capensis* germinated well both outdoors and in the laboratory. In fact it had the highest percentage germination of all the species in the

common garden experiment and its seeds remained viable in the soil for up to three years.

*I. glandulifera* was not immune to large scale germination failures either. Even commercial supplies were adversely affected by extremely low germination rates, to the point at which the collection of fresh seeds from wild stands was the only option available to secure viable seeds in the quantities required. If properly cleaned and stored at 4°C, with some attention taken to remove mouldy seeds, both *I. glandulifera* and *I. parviflora* proved to be reliable germinators once their dormancy requirements had been met.

The failure of *I. noli-tangere* to thrive post germination was particularly interesting in the light of its very limited distribution in the UK. Both *I. glandulifera* and *I. parviflora* made satisfactory growth under seemingly identical conditions, assuming watering was not excessive. This suggests an increased intolerance on the part of *I. noli-tangere* to some aspect of the cultivation methods used. Although cultivation problems were also experienced with *I. glandulifera* and *I. parviflora*, neither showed the same combination of symptoms as *I. noli-tangere*, namely the pronounced chlorosis of young leaves and distinctive black stem mottling.

The very limited experimental work which was carried out using *I. noli-tangere* suggested a lower allelopathic potential than either *I. parviflora* or *I. glandulifera*. In the light of this fact, it would be particularly useful to compare the allelopathic activity of the two most closely related species *I. noli-tangere* and *I. capensis*, which show very different distribution patterns in the UK. This was, in fact, one of the original intentions of the research.

#### **8.4 Evidence for *Impatiens* allelopathy produced by these studies**

Rice (1984) listed four main processes of allelochemical release, namely leaching, exudation, decomposition and volatilisation;

The bioassays used or developed for this study enabled three out of the four to be explored:

- 1) Leachate production was investigated using the Sandwich Method and its various modifications.
- 2) Exudation was investigated using the Plant Box Method, MECAM, the Rhizosphere Method, the Pipette Tip Stair-Step method and the Target-Neighbour experiment.
- 3) Decomposition was explored using the Rhizosphere Method.

The findings are summarised below:

#### 8.4.1 Effects of leachates

All the *Impatiens* materials tested using the Sandwich Method and its derivatives proved to display inhibitory effects on lettuce seedling development. There were differences between species, with *I. glandulifera* leaf material showing the greatest allelopathic inhibition at the 10 mg concentration than either *I. parviflora* or *I. noli-tangere*. When different organs were assessed in terms of their allelopathic effects, they were ranked using their OAP, a method developed for this study. Pods were the most allelopathic, perhaps suggesting that the putative allelochemicals, although present in biologically active quantities throughout the plant, have a primary function as protection against seed predation and herbivory (Weissenberg et al., 1997). Leaves and stems were also highly allelopathic. The fact that stems are relatively persistent over winter and make up a considerable percentage of the plant's biomass makes their high allelopathic potential noteworthy and suggests that their accumulation as a thick litter could have an effect on the growth of autumn and spring germinating seedlings, as a result of both shading and the leaching of allelochemicals. The reduction in lettuce seedling inhibition produced by the inclusion of AC in the agar medium suggested that the allelochemicals released by *Impatiens* leaf material were small molecular weight organic molecules which were subsequently neutralised by the AC (Cookson Jr., 1978).



#### 8.4.2 Root Exudates

The Plant Box Method produced striking evidence of lettuce radicle inhibition; in the case of *I. glandulifera* this exceeded 50% in all samples when compared to the controls; similar results were obtained for *I. parviflora*. Germination remained relatively unaffected, with no significant reduction when compared to the controls. Also striking was the very clear distance-dependent effects on lettuce radicle elongation caused by the *Impatiens* root system, with those in close proximity showing a high degree of stunting, with the degree of inhibition decreased linearly as distance from the *Impatiens* root system increased. This was accompanied by staining of the agar with an orange pigment, resembling the naphthoquinone lawsone, with most the intense staining occurring in close proximity to the *Impatiens* root systems.

The MECAM examined the effects of root exudates of germinating seeds, a previously unexplored aspect of *Impatiens* biology. Results showed significant inhibition of lettuce radicles, which increased as exposure time to the exudates increased. Increasing *I. glandulifera* seedling number also produced significant reductions in lettuce radicle length. Dormant seeds,

by contrast, stimulated growth. Dead seeds did not produce significant changes to the growth of the test plants.

The two methods used showed that *Impatiens* plants were able to suppress their competitors at both the mature and juvenile stage and that the *Impatiens* germination process itself was sufficient to inhibit the growth of lettuce seedlings. At the *Impatiens* densities tested, autotoxic root inhibition was not a significant factor, suggesting that dense stands of *Impatiens* seedlings are not adversely affected by their own root exudates and inter-individual interference is therefore limited to light, water and nutrient competition.

The Rhizosphere Method showed that soil adhering to *Impatiens* roots had significant differences in its allelopathic effects from bulk soil. *I. glandulifera* produced a 30% inhibition of lettuce seedlings whereas *I. parviflora* produced a stimulatory effect. Whether this difference represents a real and repeatable difference has yet to be established.

### **8.4.3 Decomposition Products**

In addition to its use as a means of identifying the effects of root exudates in a soil-based medium, the Rhizosphere Method was carried out over a ten week period to investigate the effects of decomposition of *I.*

*glandulifera* root systems and root exudates contained in soil on their ability to inhibit the growth of lettuce seedlings. Results indicated that allelochemical effects declined rapidly after plant death had occurred and a stimulatory effect was observed, perhaps indicating a surge in nutrient availability. Further studies are required to verify these findings.

### **8.5 Overall conclusions regarding *Impatiens* alleopathy and suggested avenues of further research**

This research project established that *I. glandulifera* shows the ability, under specific conditions, to display allelopathic potential in both living and dead material. From the data gathered in the course of this study, allelopathic effects were present in the plant from the time of germination until senescence and there were some indications that *I. glandulifera* was more allelopathic than other *Impatiens* species occurring in the UK. Although conclusive evidence of allelopathy in natural conditions has not been established by the studies described here, the case for a larger scale investigation into this aspect of invasive *Impatiens* ecology is certainly supported by the results obtained. Table 8.1 suggests a number of possible follow up studies, organised into phases. These are discussed more fully below. For a much more complete picture of the potential role of allelopathy in the spread of invasive alien *Impatiens* species, a biogeographical approach as proposed by Hierro et al. (2005) would be

highly desirable. As the authors note, very little work has been carried out on invasive alien plants in their native habitats, where they are not usually considered to be a threat to the ecosystems in which they occur. The timing and completion of such studies would be contingent on obtaining sufficient funding and would require collaboration between institutions in countries where *I. glandulifera* is invasive as well as in the countries of *I. glandulifera*'s native Himalayan range.

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Table 8.1 Suggested avenues of further research

Phase 1	Phase 2	Phase 3
HPLC analysis of plant materials and agar samples already collected	Investigations into the rate of breakdown of lawsone and 2MNQ in soils where <i>I. glandulifera</i> occurs	Comparison of bacterial profiles in the soil of invaded non-invaded <i>I. glandulifera</i> sites using PCR-DGGE
Establishment of a living collection of <i>Impatiens</i> species in a common garden.	Identification of volatiles produced by <i>I. glandulifera</i> flowers and roots and other organs using GC-MS	Mycorrhizal studies on native and introduced British <i>Impatiens</i> species and their effects on AMF infection of native trees and herbaceous plants
Comparison of <i>I. nolitangere</i> and <i>I. capensis</i> with other species using allelopathy protocols already developed.		Comparison of <i>I. glandulifera</i> in its native and introduced ranges

The greatest limitation to the studies undertaken here was the inability to link the allelopathic activity displayed by the various *Impatiens* materials with occurrence and quantities of naphthoquinones present in the *Impatiens* plant material as had been intended when the project was initiated (see Appendix 1) This was due to sustained equipment failure, enforced absence on the part of the author and subsequent delays in the commissioning of the replacement HPLC instruments. A thorough investigation into the naphthoquinone content

of plant material and agar samples would certainly be a high priority in any follow-up studies carried out. Although naphthoquinones have been suggested as a likely source of the allelopathic effects demonstrated, there are no doubt other potential candidates contained within the plants. A desk top study followed by HPLC analyses might identify these.

The establishment of a suitable site where all four species could be cultivated and fresh seed stocks regularly collected would be very beneficial. Due to the invasive nature of some of the species involved, the site chosen should be isolated from any semi-natural woodland or watercourses. As all four species are well able to germinate and grow outdoors, it might be possible to collect self-sown seedlings as they appear and then transplant them into a controlled situation indoors. Under most circumstances *Impatiens* seedlings appear to adapt readily to transplantation, perhaps due to the rapid development of adventitious roots from the hypocotyls. A common garden experiment would also be useful in enabling the collection of phenological data on comparative emergence, flowering and other traits as was carried out by Perglová et al. (2009) and Skálová et al. (2011).

## **8.6 *Impatiens* Flowers as a possible source of volatile allelochemicals**

The flowers of *I. glandulifera* have a powerful and potent smell, but the identity of the compounds responsible for this do not yet appear to have been identified. Cut and damaged roots also produce a strong odour and in some instances the author has detected a similar odour emanating from cut stems. Factors affecting the production of volatile organic compounds such as herbivory and wounding have already established that volatile communications are common in plants and have also been implicated in allelopathic interactions (Barney, Hay & Weston, 2005; Vaughn & Boydston, 1997). This could be another as yet unexplored potential source of allelopathic effects and could be investigated using GC-MS.

## **8.7 Allelopathy and microbial interactions**

Apart from the direct plant-on-plant allelopathic effects obtained in this study, there is increasing evidence to suggest that interactions not just between plants, but ones mediated by microbes, may be of more general importance in plant invasions than has hitherto been realised. For example, Batten et al. (2006) reported that phospholipid fatty acid analysis (PLFA) of the rhizospheres of two invasive weed species *Centaurea solstitialis* and *Aegilops truncialis* indicated that the invasive

weeds altered the microbial communities where they occurred. Native and invaded microbial communities were significantly different, with increasing divergence from the original microbial community as the time since invasion increased. Fritz et al (2008) reported permanent changes to the microbial make-up of the soil in which *I. glandulifera* had been growing. The impact of these changes on the growth and establishment of competitor plants have not been investigated but are worthy of further research, using molecular techniques such as PCR-DGGE (Kardol et al., 2007). Mummey & Rillig (2006) found that invasion of grassland by the alien mycorrhizal forb *Centaurea maculosa* caused significant alteration to AMF community diversity and that mycorrhizal abundance was also reduced following invasion. These types of complex, indirect interaction may be far more common than the straightforward inhibition as originally envisaged by the pioneers of allelopathy research.

A further example of the subtlety of the factors involved can be found in the work of Klironomos (2002), who found that pathogenic organisms were more likely to reduce the growth of seedlings of rare native plants grown in soil that had previously supported the same plants than was the case with exotic species. This suggests that soil pathogens may favour the growth of invasive species at the expense of native ones. Presumably, after a suitable period of time has elapsed, the exotic species will go on to develop a corresponding suite of pathogenic



organisms and their relative invasiveness will be reduced. *I. noli-tangere* is a rare native which may have built up a large pathogen burden, whereas the relative newcomer *I. glandulifera*, has yet to do so. An experiment could be devised where soils from recently colonised and long established *I. glandulifera* sites could be included in potting mixes and their effects on plant growth observed. The results could then be compared with *I. noli-tangere* using a similar experimental design. The role of soil biota and plant invasion is necessarily complex; a good review of the subject can be found in Reinhart & Callaway (2006).

Juglone, the best studied naphthoquinone as regards allelopathy (Ercisli et al., 2005; Rietveld, 1983; von Kiparski, Lee & Gillespie, 2007) has been shown to suppress the activity of the actinorhizal symbiont, *Frankia*, which is found on the roots of alder trees (*Alnus glutinosa*) (Vogel & Dawson, 1985). The effect varied according to strain of *Frankia* present. In some areas, *I. glandulifera* occurs in riparian woodland situations with alders. The inhibitory effect of *I. glandulifera* against tree seedlings in wet woodlands has been noted (Maule et al., 2000) and this may be due in part to the effects of chemical inhibition, as well as the usual competition for light, nutrients and water. Another possible effect might be on the rates of mycorrhizal infection of the tree seedlings and other competitor herbaceous plants.

The Eurasian invasive plant *Alliaria petiolata* has been shown to slow the rate of arbuscular mycorrhizal infection of native tree seedlings in North America. This leads to reduced tree seedling growth rates, thereby facilitating its invasion of relatively undisturbed forest habitats (Stinson et al., 2006). The allelochemicals produced by *A. petiolata* degrade mycorrhizal fungi and persist in the soil, preventing mycorrhizal recolonisation even when mycorrhizal fungi are added to it (Stinson, Klironomos & Acland, 2005). This change favours early successional species over late successional woody species and may alter the successional dynamics of the invaded woodland (Stinson, Klironomos & Acland, 2005). Naphthoquinones are known to have anti-fungal properties (Curreli et al., 2001) and may work in a way analogous to the isothiocyanates believed to be responsible for *A. petiolata*'s inhibitory effects.

In another facet of *Alliaria* allelopathy, Bossdorf et al. (2004) reported that native plants of *A. petiolata* were able to outperform plants from the invasive range when both were grown together in an experimental set up. They hypothesised that competitive ability may involve fitness costs which are eliminated by natural selection in the invasive range, where interspecific competition such as herbivory and pathogen attack are reduced - the so called Evolutionary Reduced Competitive Ability (ERCA) hypothesis. This, they suggested, may allow

other processes such as allelopathy to be more fully expressed in the invasive range. The spectacular success of *I. glandulifera* in its introduced as opposed to its native range would provide an excellent opportunity to test this hypothesis.

The determination of rates of mycorrhizal colonisation in *I. glandulifera* and its competitors, both woody and herbaceous, might provide further evidence of the changes caused by *I. glandulifera* invasion. Perhaps the most critical information required is the rate at which the naphthoquinones (or other allelochemicals produced by *Impatiens*) break down in the soil. The relatively low solubility of juglone and its ability to accumulate in the soil to phytotoxic levels under certain conditions has been noted by researchers (von Kiparski, Lee & Gillespie, 2007) and it has been known to persist in soil for up to a year after the removal of the walnut tree producing it (Crist & Sherf, 1973). If the naphthoquinones found in *I. glandulifera* or other *Impatiens* species can be shown to behave in a similar way, it would support the assertion that *I. glandulifera*'s competitors might experience negative chemical interference due to the production and accumulation of allelochemicals in the soil. The evidence gained from the Rhizosphere Method experiment described in Chapter 7 seems to suggest a very limited persistence of root exudates in the soil and in fact these produced stimulatory effect in many cases as the roots decayed. This may reflect the enhanced rate of microbial activity caused

by the relatively high soil temperatures obtained when the plants were grown in a temperature controlled room and may not be representative of the effects produced by the cooler temperatures found in the soil under natural stands of *I. glandulifera*.

Even in the case of their direct action against plants, the *modus operandi* of allelochemicals is more complicated than it might first appear. Juglone, for example, has been shown to have non-linear hormetic effects on post-germination mustard seedlings, when the latter were stressed with methanol (Chobot & Hadacek, 2009). At high doses, without the methanol stress factor, the juglone was inhibitory to the mustard seedlings in the usual way, due to the production of reactive oxygen species (ROS). It is possible that the naphthoquinones found in *Impatiens glandulifera* and other species of *Impatiens* could work in similar ways, that is they may be able to act as both inhibitors and stimulators of plant growth. This might be an explanation for the effects seen when dormant *I. glandulifera* seeds were used in the MECAM; these stimulated the growth of lettuce radicles, unlike germinating seeds which showed marked inhibitory effects on radicle elongation.

The variety and quantities of naphthoquinone in the various alien invasive and native *Impatiens* species shows considerable variation (Lobstein et al., 2001). As biochemically potent molecules are the exception rather than the rule, Firn & Jones (2003) proposed that plants

must employ methods to ensure that their secondary metabolism products are sufficiently diverse to produce some compounds which are biologically active. The so-called Screening Hypothesis predicts that promiscuous enzymes generate a range of compounds, some of which may go on to affect the growth of other plants. The range of naphthoquinones found in the *Impatiens* species studied in this research could be a product of slight differences in the structure of the enzymes which create secondary metabolites. A biochemical study of the two very closely related species *I. capensis* and *I. noli-tangere* would shed light on the differences in secondary metabolism which are present and the resultant differences in naphthoquinone production that ensues.

## **8.8 Conclusions**

It is likely that allelopathic effects, if present in *Impatiens*, would be increased or diminished by interactions with other factors. Allelopathic inhibition (or stimulation) would, therefore, be expected to vary from site to site and season to season, depending on the prevailing biotic and abiotic conditions. It is also likely that allelopathy is the result of the synergistic effects of allelochemicals rather than single compounds and these mixes produce significant effects at lower concentrations than one compound on its own (Rasmussen & Einhellig, 1977). Similar criticisms can be levelled at other factors affecting plant growth, which, like

allelopathy, seldom operate in isolation. However, if allelopathic suppression of native plant species is one of a suite of strategies used by an invasive alien like *I. glandulifera*, it is conceivable that, under some circumstances, it could tip the balance in favour of the invader. This allelopathic effect might be expected to be strongest against “naïve” species, which have not yet developed resistance to the allelochemicals released by *I. glandulifera* in the manner proposed by the Novel Weapons Hypothesis (Callaway & Ridenour, 2004).

The case against *I. glandulifera* as a threat to riparian habitats has been refuted by some authors (Hejda & Pysek, 2006; Hulme & Bremner, 2006). This weakens the justification for expensive control campaigns in these areas, where, ironically, the plants are at their most visible and are particularly likely to be considered a threat.

Although an understanding of the extrinsic factors that regulate soil microbial stability is still at a rudimentary level, it is known that soil microbes are key drivers of ecosystem function and therefore microbial instability is likely to alter soil carbon storage and plant nutrient availability (Orwin, Wardle & Greenfield, 2006). It is possible that *I. glandulifera*, as a recent invader, may be able to alter the microbial flora in the sites where it occurs to the detriment of native plant species. If evidence supporting this hypothesis could be established, it would help justify the considerable financial investment currently allocated in control

programmes. In conclusion, the studies undertaken here support the case for further investigation into the potential role of allelopathy in the ecology of invasive *Impatiens*.

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## Appendix 1. Initial investigation into the naphthoquinone content of *Impatiens glandulifera* using HPLC

### Appendix 1.1 Introduction

HPLC (High performance liquid chromatography) is a chromatographic technique in which a pressurised solvent mixture (referred to as the mobile phase) is used as a diluent for the substances to be analysed. The mobile phase and the analytes are passed through a sorbent column and the time taken for each component to react with the column is referred to as its “retention time” and is characteristic for each compound. Retention times vary according to pressure, mobile phase composition and temperature and this enable mixtures of analytes to be separated and identified. When plotted, each compound produces a characteristic peak on the chromatogram and the area produced under the peaks provides an accurate measure of the concentration of that compound in the mobile phase and enables concentrations in the original material to be determined. For an overview of HPLC practices and applications, see Dong (2006).

Himalayan balsam (*Impatiens glandulifera*) contains several naphthoquinones, which are known to have phytotoxic and anti-microbial effects. It is possible that these chemicals, either directly or



indirectly, might be facilitating the plant's spread by suppressing the growth of competitors, or altering the microbial balance in favour of the invader.

Research carried out at Plymouth University laboratory using bioassays indicated that *I. glandulifera* exerted a significant inhibitory effect on the growth of lettuce seedlings, both in a dried form and as root exudates.

This may be due to the presence of 2-methoxy-1,4 naphthoquinone (2 MNQ), which is found in large quantities in *I. glandulifera* and appears to be more phytotoxic than 2-hydroxy-1,4-naphthoquinone, commonly known as lawsone, another naphthoquinone which is present in *I. glandulifera* (Lobstein et al., 2001). Other research suggests that plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is also present in *I. glandulifera* (Babula et al., 2006).

## **Appendix 1.2 Research Aims**

1) Compare the naphthoquinone content in the roots, leaves and stems of *Impatiens glandulifera* at different life stages: seedling, pre-flowering, flowering and senescence. Establish whether the roots in particular contain large quantities of naphthoquinones as has been mentioned by other researchers, (Vrchotová, Šerá & Tríska, 2005) although no published data are available to confirm this.

- 2) Compare the naphthoquinone content of the species *I. glandulifera* and the other species of *Impatiens* which occur wild in the UK, namely *I. parviflora*, *I. capensis* and *I. noli-tangere*.
- 3) Identify and measure the quantities of the naphthoquinones released by live *Impatiens* plants and dried *Impatiens* material into agar when bioassays were carried out. Establish whether any correlation between the naphthoquinone content of the agar and the degree of inhibition shown by the lettuce was observed.
- 4) Test for the presence of naphthoquinones in soils used to grow *Impatiens* plants, including rhizosphere experiments and look for correlation between the quantities of naphthoquinone, if any, present in the soil and the degree of inhibition shown by the lettuce seedlings.

### **Appendix 1.3 Materials and methods**

Following consultation with Dr Claire Williams from the Chemistry Department of Plymouth University, a Phenomenex Luna 3  $\mu\text{m}$  C18 reversed phase column, 50 x 2 mm, 100 Å, was purchased (Phenomenex, Macclesfield, Cheshire, UK), this column configuration having been determined as suitable for use with the naphthoquinones under investigation. The instrument used consisted of a Dionex GP40 gradient pump and a Spectrasystem UV6000LP detector (Thermo Scientific, Sunnyvale, California, USA).

Mobile phase liquids (HPLC grade methanol, 0.1M acetic acid and acetonitrile were purchased (Sigma Aldrich, St Louis, Missouri, USA) and 100µg/ml standards of lawsone, 2-methoxy-1,4 naphthoquinone and plumbagin were prepared using commercially available pure naphthoquinones (Sigma Aldrich, St Louis, Missouri, USA).

In order to separate the peaks of the naphthoquinones under investigation, three different ratios of mobile phase components were used in conjunction with, when noted below, a column heater and guard column.

- a) 33:67 acetic acid, acetonitrile
- b) 33:67 acetic acid: acetonitrile, column heated to 42°C
- c) 40:60 acetic acid: acetonitrile
- d) 50:50 acetic acid: acetonitrile
- e) 50:50 acetic acid: acetonitrile, guard column fitted

A flow rate of 0.75 ml min<sup>-1</sup>, with a pressure of 174 Bar as recommended by Babula et al. (2006) was used, unless otherwise noted.

## **Appendix 1.4 Results**

a) 33:67 acetic acid: acetonitrile

Table 9.1. Retention times for lawsone and 2MNQ produced with 33 : 67 acetic acid : acetonitrile mobile phase

<b>Flow rate (mlmin-1)</b>	<b>Compounds analysed (concentration 100 µgml-1)</b>	<b>Retention times (minutes)</b>
0.75	Lawsone	4.51
		4.53
	2MNQ	5.22
		5.08
	Lawsone: 2MNQ	4.97: 5.08
		5.22: 5.79
0.65		5.22: 5.79
		5.41: 5.91

The retention times of lawsone and 2MNQ were too close for the individual peaks to be distinguished, so the column was placed in a column heater at 42°C in order to decrease the viscosity of the mobile phase.

b) 33:67 acetic acid: acetonitrile, column heated to 42°C

Table 9.2 Effect of column heater (42°C) on 2MNQ and lawsone retention times using 33:67 acetic acid:acetonitrile mobile phase.

Flow rate (mlmin <sup>-1</sup> )	Compounds analysed (concentration 100 µgml <sup>-1</sup> )	Retention times (minutes)
0.75	Lawsone	3.13
		3.13
		3.13
		3.14
		3.12
		3.14
		3.02
	Methanol blank	2.89
		2.92
		2.92
	2MNQ	3.63
		3.62
		3.62

Although this resulted in a reduced retention time for both lawsone and 2MNQ, the peaks were still not sufficiently separated.

c) 40: 60 ratio acetic acid: acetonitrile

Table 9.3. Effects of 40:60 acetic acid:acetonitrile mobile phase on retention times of lawsone and 2MNQ.

Flow rate (mlmin <sup>-1</sup> )	Compounds analysed	Retention time (minutes)
0.75	Lawsone 50 µgml <sup>-1</sup>	3.38
		3.32
		3.31
	2MNQ 100 µgml <sup>-1</sup>	4.09
		4.08
		4.08
	Lawsone: 2MNQ 100 µgml <sup>-1</sup>	3.37: 4.10
		3.42: 4.08
		3.44: 4.10

d) 50: 50 acetic acid: acetonitrile

Table 9.4. effects of 50:50 acetic acid:acetonitrile mobile phase on retention times of lawsone and 2MNQ.

Flow rate (mlmin <sup>-1</sup> )	Compounds analysed (concentration 100 µgml <sup>-1</sup> )	Retention time (minutes)
0.75	Lawsone: 2MNQ	4.19: 5.41
		4.16: 5.38
		4.19: 5.42
	Plumbagin	9.90
		10.03
		9.91
	Lawsone: 2MNQ: plumbagin	4.10: 5.28: 9.74
		4.12: 5.32: 9.79
		4.22: 5.44: 10.03

e) 50: 50 acetic acid: acetonitrile, phenyl guard column fitted

Table 9.5 effects of 50:50 acetic acid:acetonitrile mobile phase on retention times of lawsone, 2MNQ and plumbagin.

Flow rate (mlmin <sup>-1</sup> )	Compounds analysed (concentration 100 µgml <sup>-1</sup> )	Retention time (minutes)
0.75	Lawsone: 2MNQ: plumbagin	4.22: 5.44: 10.03
		4.18: 5.41: 9.97

The guard column was fitted to protect the column from impurities present in the *I. glandulifera* samples which were to be analysed subsequently. These can shorten the column's life. It had very little effect on retention times. Similarly, a reduction in filter sizes from 0.45 µm. to 0.2 µm did not produce a noteworthy change in retention times.

### **Appendix 1.5 Sample analysis**

A sample of *I. glandulifera* root material was sonicated in HPLC grade methanol for 30 mins and then filtered through 0.2µm Teflon filter to remove particulate impurities prior to injection. Peaks occurred at 4.06, 4.30 and 5.47 minutes, but compounds were not identified definitively.

Instrument availability was intermittent and very limited, due to the requirements of prior users and further work on the analysis of plant material, agar and soil samples was prevented when the instruments

were decommissioned. They were not replaced during the duration of this research project, so further work had to be abandoned.



## Appendix 2 - An Initial Investigation into the Population Biology of *Impatiens glandulifera* at Berryman's Marsh, Dartington Hall Estate 2007

The aim of this research was to gain some basic information on the population biology of *Impatiens glandulifera* at sites in Devon, such as the percentage of plants which survived to flower and fruit successfully and any differences detected in overall plant performance according to habitat. Initially a comparison with another site was planned, but this had to be abandoned when the *I. glandulifera* on site was scheduled for clearance by a conservation organisation.

Berryman's marsh, managed as a nature reserve on the west bank of the River Dart, Dartington Hall Estate, near Totnes, Devon. It is composed of a low lying area along the banks of the River Dart approximately 200 meters metres beyond the weir that marks the tidal limit of the river. The marsh is approximately 5 metres above sea level and is prone winter flooding.

A species list for the site was taken in late April using a series of 100 metre transects (or less depending on the width of the site) at 20 metre intervals from the river bank. This date was selected as it allowed the recording of vernal woodland species as well as those occurring in the open marsh. Quadrats were placed every 4 metres or more frequently if

vegetation changes were obvious. A total of 96 quadrats were examined. Plants were identified using Stace 1999 (Field Flora of the British Isles, Cambridge University Press) and recorded below. The rough terrain and dense nature of the vegetation meant that under-recording of species was highly likely.

Following the survey, seven (initially eight, one was washed away by flooding in early May soon after establishment) 1 m<sup>2</sup> permanent quadrats were established, these being considered to represent the majority of the invaded habitats in which HB plants would be likely to grow at the site.

The experiment was terminated in late September 2007, when all plants were harvested, pod counts recorded and basic data analysis undertaken.

Table 10.1. Description of quadrats 1- 7.

Quadrat number	Situation and type
1	Full sunlight -bank of ditch, bare soil pH = 6.6
2	Full sunlight - dense growth of <i>Ranunculus ficaria</i> pH=6.6
3	Full sunlight - “grassland” - growth of <i>Alopecurus</i> pH = 6.4
4	Partial shade of alder, pH = 5.3
5	Shade under oak and Sitka spruce pH = 5.4
6	Shade under oak and spruce pH = 5.6
7	Partial shade under oak pH = 6.1

Each quadrat was divided into 25 20cm<sup>3</sup> squares and ten squares from each quadrat were randomly selected. A total of 40 seedlings from these “mini quadrats” were tagged in each quadrat.

Plant height and survival were recorded at weekly intervals beginning in late April 2007 as rapid increases in height began. Plant height was recorded as the height of the terminal bud prior to flowering. During and after flowering, the distal tip of the inflorescence was used as a measure of height. Some specimens developed large side branches, bearing numerous flowers and pods. Flower and fruit production from these were combined with the totals from the terminal inflorescence to give an aggregated total per individual. A previous study (not included

here) had established the difficulty of recording the individual rate of seed production due to the explosively dehiscent nature of the seed pods and the difficulties of individually bagging flowers without denying access to pollinating insects. A surrogate measure, the counting of the number of pedicels remaining after dehiscence of the seed pods was adopted as this could still be ascertained following seed dispersal. For the purposes of this study any flowers or flower buds present at harvest were included as viable pods. Mortality was recorded, along with the date of flower bud emergence, first flowering, lodging and other events.

Table 10.2 Species recorded in 96 1 m<sup>2</sup> quadrats at Berryman's Marsh.

<i>Acer pseudoplatanus</i> L.	<i>Holcus lanatus</i> L.	<i>Salix capraea</i> L.
<i>Aconitum napellus</i> L.	<i>Juncus inflexus</i> L.	<i>Salix alba</i> L.
<i>Adoxa moschatellina</i> L.	<i>Lamiastrum galebdolon</i> (L.) Ehrend & Polatschek	<i>Salix viminalis</i> L.
<i>Aegopodium podagraria</i> L.	<i>Oenanthe crocata</i> L.	<i>Salix triandra</i> L.
<i>Alliaria petiolata</i> (M. Bieb) Cavara & Grande	<i>Phragmites australis</i> (Cav.) Steud.	<i>Alopecurus pratensis</i> L.
<i>Allium ursinum</i> L.	<i>Quercus petraea</i> (Matt.) Liebl.	<i>Poa annua</i> L.
<i>Anemone nemorosa</i> L.	<i>Ranunculus acris</i> L.	<i>Poa trivialis</i> L.
<i>Angelica sylvestris</i> L.	<i>Heracleum mantegazzianum</i> Sommier & Levier	<i>Deschampsia caespitosa</i> (L.) P.Beauv.
<i>Arrhenatherum elatius</i> P.Beauv	<i>Ranunculus flammula</i> L.	<i>Calystegia sepium</i> (L.)R.Br
<i>Arum maculatum</i> L.	<i>Ranunculus repens</i> L.	<i>Salix cinerea</i> L.
<i>Aster x salignus</i> Willd.	<i>Heracleum sphondylium</i> L.	<i>Taraxacum</i> sp F.H.Wigg
<i>Cirsium arvense</i> (L.) Scop.	<i>Ranunculus ficaria</i> L.	<i>Trifolium pratense</i> L.
<i>Conopodium majus</i> W.D.J.Koch	<i>Rubus fruticosus</i> L. agg	<i>Persicaria amphibia</i> (L.) Delarbre
<i>Dactylis glomerata</i> L.	<i>Rumex obtusifolius</i> L.	
<i>Epilobium hirsutum</i> L.	<i>Stachys sylvatica</i> L.	
<i>Filipendula ulmaria</i> (L.) Maxim.	<i>Stellaria media</i> (L.) Vill.	
<i>Fraxinus excelsior</i> L.	<i>Urtica dioica</i> L.	
<i>Galanthus plicatus</i> M.Bieb.	<i>Veronica montana</i> L.	
<i>Galium aparine</i> L.	<i>Claytonia sibirica</i> L.	
<i>Glechoma hederacea</i> L.	<i>Lythrum salicaria</i> L.	
<i>Hedera helix</i> L.	<i>Impatiens glandulifera</i> Royle	

## **Appendix 2.1 Description of quadrats**

Quadrat 1. Located on a stream bank was vandalised between weeks 7 and 8, with plants being slashed and trampled. This made the subsequent collection of data problematical. Only one of the initial 40

tagged seedlings survived until the harvest date. This large and extensively branched individual produced a pod count of 1096.

Quadrat 2 had HB seedlings growing in a dense ground cover of lesser celandine, *Ranunculus ficaria*, a vernal species. Tagged seedlings grew more slowly than Quadrat 1. In late spring the *Ranunculus* died back, leaving bare ground beneath the HB plants.

Quadrat 3. Located in a dense sward of grass (*Alopecurus* sp). The seedlings tagged showed a lower initial height and slower rate of growth than the other illuminated quadrats. It is possible that the particularly wet growing season helped limit the effects of water stress and competition from the grasses present and that in a drier year mortality would have been much higher.

In Quadrat 4 approximately half of the quadrat's area was dominated by a dense growth of *Holcus lanatus*. Any HB seedlings located amongst the grass were soon swamped. Those located on the shadier side were able to develop and flower in some cases.

Quadrats 5 and 6 were located in an area which became heavily shaded as the tree canopy closed in May.

Quadrat 7, although shaded, received reflected light from the river.

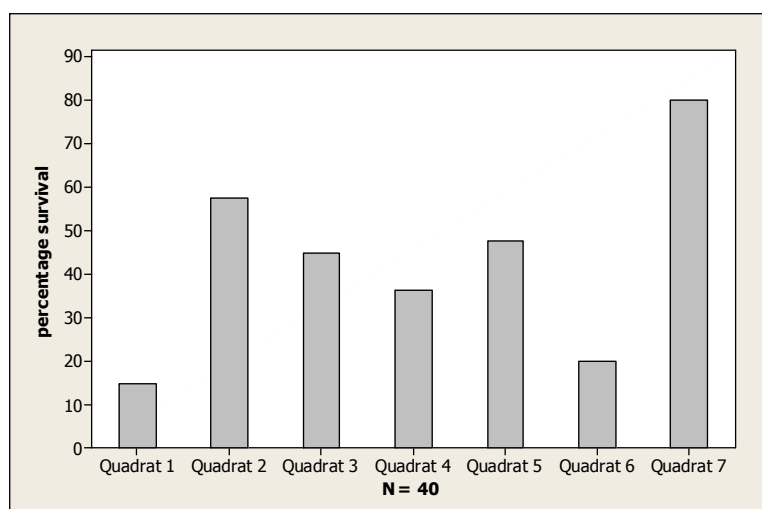


Figure 10.1. Bar chart of percentage survival to flowering of tagged individuals in woodland and riparian sites. Quadrats 1, 2 and 3 were fully illuminated, whereas 4, 5, 6 and 7 were located in shaded areas. 40 individuals were randomly tagged per quadrat.

Percentage survival to flowering was measured as the number of plants out of the 40 tagged in each quadrat which produced flowers and is shown in Figure 1. Subsequent to the initiation of flowering, a number of these plants died, so their contribution to final pod counts was not included. Quadrat 1 was vandalised, with plants being slashed and trampled, making the subsequent collection of data difficult. Percentage survival to flowering by habitat (shaded, fully illuminated) is shown in Table 10.3.

Table 10.3. Mean percentage survival to flowering of *I. glandulifera* seedlings in shaded/unshaded quadrats (N = 40).

Habitat	Mean percentage survival to flowering
Fully illuminated (Q1,2,3)	39.2
Shaded (Q4,5,6,7)	46.0

2007 was a particularly wet and windy season and large numbers of plants lodged and subsequently died as a result of wind and rain damage. For this study lodging was defined as occurring when plants had bent over to the point where they were incapable of remaining upright without additional support. The wet conditions encouraged the development of stem and root rots in the lodged plants, although some individuals went on to root at the nodes, grow upwards and flower successfully. Details are recorded in Table 10.4.

Table 10.4 Percentage lodging of tagged *I. glandulifera* seedlings shown in 7 quadrats (N = 40)

Quadrat	Percentage Lodging %
1	20
2	10
3	0
4	33
5	45
6	83
7	25

The 20% lodging figure for Quadrat 1 is probably lower than would have occurred if the plants had not been slashed and trampled, which may have eliminated many plants that would have subsequently lodged. Quadrat 2, located in a patch of *Ranunculus ficaria* produced slower growing plants which had to compete with the *Ranunculus* until late spring, when the latter died back. Quadrat 3, located in a grassy area



suffered no lodging, probably because the plants were much shorter due to competition with grasses. Quadrat 6, suffered 82.5% losses due to lodging. The other shaded quadrats experienced much lower levels of lodging. Figure 2 shows a graphic representation of the data in the form of box plots. The horizontal lines through the boxes represent the median values for each habitat. It is clear that shaded quadrats suffered higher percentage losses to lodging, with whiskers representing the largest observed values that are not outliers recorded from the shaded quadrats.

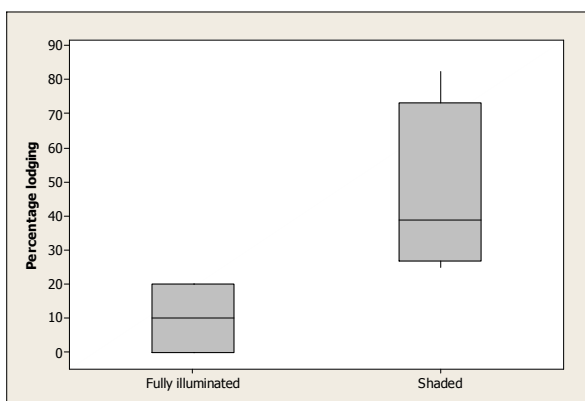


Figure 10.2. Percentage lodging in fully illuminated and shaded quadrats. Fully illuminated N = 3,; shaded N = 4.

Data produced by pooling percentage lodging per quadrat into mean lodging per habitat (Table 10.5) show that fully illuminated sites experienced approximately one quarter of the lodging of those in shade, although this figure should be treated with caution because of the effects of vandalism on Quadrat 1 and the possible loss of plants that would have lodged subsequently. Plants in shaded areas tended to be taller and

thinner than those in fully illuminated areas and this may account for their increased tendency to lodge.

Table 10.5 Mean percentage lodging of *I. glandulifera* per habitat – shaded, fully illuminated.

Habitat	Mean Percentage Lodging
Fully illuminated (Q1, 2, 3)	10 (N= 120) Std Dev = 10
Shaded (Q4,5,6,7)	46.3 (N= 160) Std Dev = 25.54

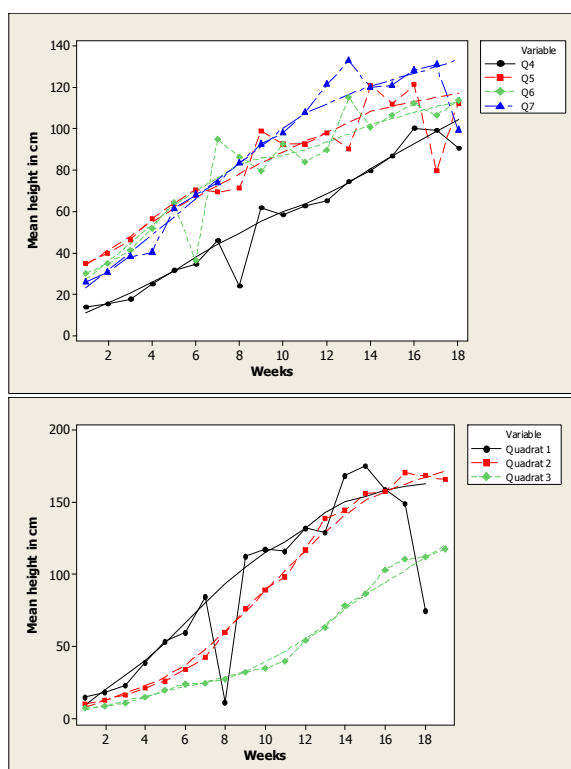


Figure 10.3. Mean height increases of plants in a) shaded quadrats (4,5,6,7) and b) fully illuminated quadrats with LOWESS smoother used.

Mean weekly height increases for the fully illuminated quadrats show the effect of competition on the growth of the plants. Quadrat 1, occurring on fresh alluvial material, showed the fastest increase in height, until vandalism occurred. The competitive effects of *Ranunculus ficaria* and

grasses can be seen in the curves of quadrats 2 and 3 respectively. The same trends are shown in Figure 4 where flowering start dates and numbers are easily distinguished between quadrats 2 and 3. Shaded quadrats show a similar range of values, with the least successful quadrat in terms of height and flowering being Quadrat 4 and the most successful Quadrat 7.

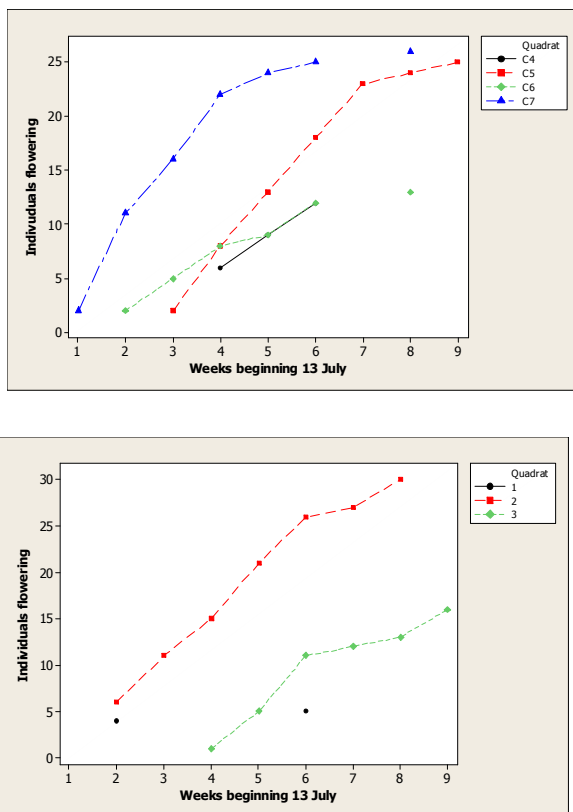


Figure 10.4. Number of individuals flowering in a) fully illuminated quadrats and b) shaded quadrats

Week 1 = 13 July.

Plants were harvested on week 19 as the site was scheduled to be mown soon after this date. Pods, pedicels and flowers were counted. No attempt was made to count seeds in pods, nor were pods, pedicels and flowers distinguished in the counts. Frequency distributions are shown in Figure

10.5. It can be seen that fully illuminated quadrats have an approximately normal distribution.

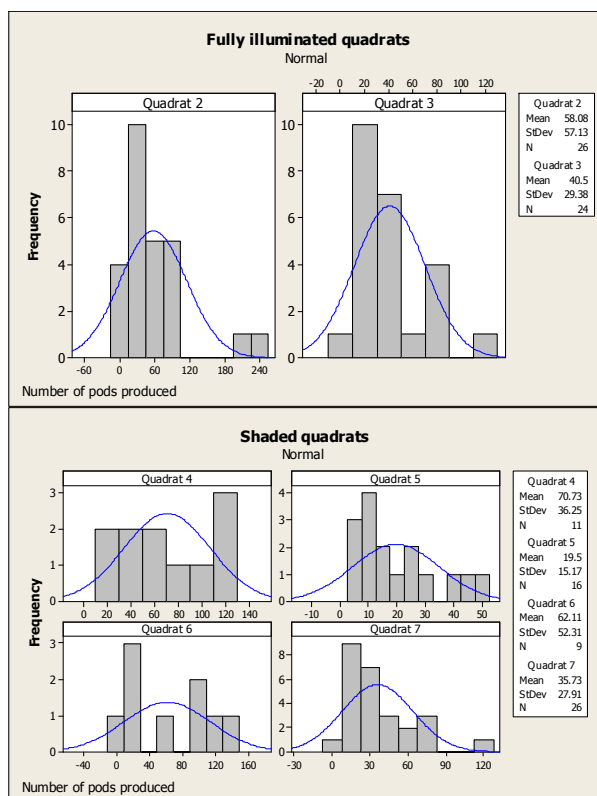


Figure 10.5. Frequency distribution of pod production in a) fully illuminated and b) shaded quadrats.

Mean values of pod production in the fully illuminated quadrats were close to double those found in the shaded quadrats. Mean and median values for shaded quadrats were close (Table 10.6), indicating a close to symmetrical distribution. This may reflect the lack of side branches bearing flowers in the shaded quadrats. Pod production as shown by the box plots in Figure 10.6 shows clear separation of fecundity by habitat.

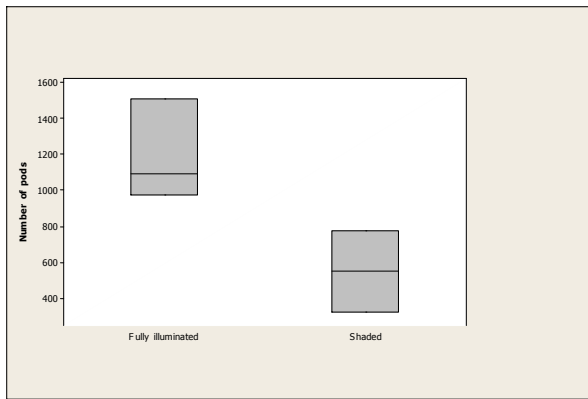


Figure 10.6. Pod production in fully illuminated (N = 51) and shaded quadrats (N = 63)

Table 10.6. Mean and median values for pod production in fully illuminated and shaded quadrats

Habitat	No of individuals	Mean	Median	Std Dev
Fully illuminated	N = 51	1192.25	1096	281.726
Shaded	N = 63	645.25	664.5	265.119

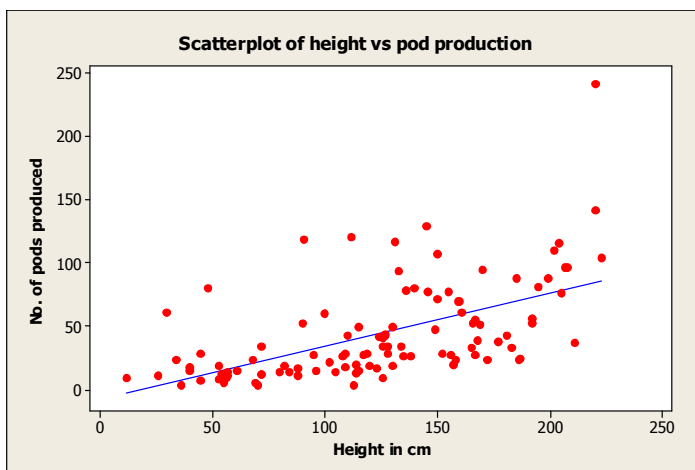


Figure 10.7. Scatterplot of height versus pod production (data from Quadrat 1 has been excluded)  $y = 87.1 + 0.852x$ ,  $r^2 = 31.1\%$  Pearson correlation = 0.558, P-Value = 0.000

As shown in Figure 8, the coefficient of determination  $r^2$  has a value of 31.1%, thus slightly less than one third of the variability shown can be explained by the regression line. Absence of correlation between height and seed pod production is presumably due to the spindly growth of plants in the shaded quadrats and the absence of productive side branches as were present in plants from the fully illuminated quadrats.

Previous work has shown strong correlation between plant height and plant performance in *Impatiens glandulifera* as measured by indicators such as total dry weight and leaf area. No correlation was discovered between plant height and production of seed pods in this study however.

## **Appendix 2.2 Conclusions**

From the limited amount of data analysis carried out it appears that:

- 1) Shaded quadrats have a higher probability of lodging and produce fewer pods. The most likely cause is the spindly growth caused by low light levels producing thin stemmed, top heavy plants.  
  
Further work might involve a study of the number of side branches produced by plants and their contribution to the overall pod production.
- 2) Competition from other species, at least under the conditions prevailing during 2007, may produce smaller, sturdier plants that are

less prone to lodging than those in areas where competition is not as intense.

Although a further study was planned for the following season, the site was not available.

### Appendix 3 – Published Works

Smith, O.P., Alien invasion: Himalayan balsam (*Impatiens glandulifera* Royle) in Britain

Oral presentation O6, at NIAES International Symposium, Tsukuba, Japan, October 22-23, 2007 *Invasive Alien Species in Monsoon Asia: Status and Control*.

**Abstract.** There are four species of the genus *Impatiens* growing wild in Britain. All are annuals. Three of the species are introduced. *I. glandulifera* Royle and *I. parviflora* come from the Himalayan region; *I. capensis* Meerb comes from Eastern North America; *I. noli-tangere* is a rare native. *I. glandulifera* was brought from the Himalayas in 1839 and is now considered to be one of the top three weeds in Britain in terms of its visual impact and is the only annual species in the group. It has colonised riparian habitats and woodlands throughout the British Isles. It is possible that part of its success may be due to allelopathy.

Smith, O.P., Fujii, Y. An investigation into the allelopathic potential of two invasive alien species of balsam (*Impatiens*) found in the UK, using the plant box and sandwich methods.

Poster presentation 137. 5<sup>th</sup> World Congress on Allelopathy: “Growing Awareness of the Role of Allelopathy in Ecological, Agricultural, and Environmental Processes”. Saratoga Springs, New York, USA, 21-25 September 2008. Page 75 of conference proceedings.

**Abstract.** The genus *Impatiens* in the UK includes one native and several introduced species. The native species, *I. noli-tangere* is uncommon, with a limited distribution. Two Asian species, *I. parviflora* and *I. glandulifera* are the most common, with *I. glandulifera* being considered one of the top three weeds in terms of its visual impact. The sandwich method produced 40% inhibition of radicle growth in the case of *I. glandulifera* at a rate of 1 mg of dried leaf/ ml agar and 70% at 5 mg dried leaf/ ml agar. In the case of *I. parviflora* the corresponding figures were 10% and 40%. In some instances the dried leaves produced an orange pigment that diffused into the agar. This was more pronounced in the case of *I. glandulifera* than *I. parviflora*. In the Plant Box Method, living roots embedded in low temperature agar produced a degree of inhibition in lettuce radicle elongation, with both *I. glandulifera* and *I. parviflora* causing approximately 80% inhibition of radicle elongation. Orange staining of



the agar again took place, with this being more pronounced in *I. glandulifera* than *I. parviflora*. The orange staining may be due to exudation of lawsone, one of the naphthoquinones found in both these *Impatiens* species. Naphthoquinones are a group of biologically active compounds which are known to have anti-microbial and allelopathic properties. Both species are powerful inhibitors of lettuce seedling development. These results suggest further investigation into the role of allelopathy in the spread of alien invasive balsam species is warranted.

## **Appendix 4 - Modules, courses and training attended**

The following modules were successfully completed as the taught component of the PhD award:

<b>BIO 5110</b> Plant Taxonomy and Identification	<b>BIO 5113</b> Research Methods in Biology	<b>BIO 5114</b> Research Skills in Biology
<b>EAR 5102</b> Multivariate Analysis for Environmental Science Research	<b>ENV 5101</b> Laboratory-Based Teaching Methods & Practice	<b>BIO 5204</b> Tropical and Temperate Plant Conservation
<b>BIO 5102</b> Applications in Electron Microscopy	<b>EAR 5105</b> Science and the Environment	

### **Training Received**

*Laboratory of Dr Yoshiharu Fujii, National Institute of Agro-environmental Sciences, Tsukuba, Japan. October 2007*

Given extensive training in the correct procedures required to carry out Sandwich, Plant Box and Rhizosphere Methods

*Plymouth University 2008-11*

Autoclave training course. Safe use, correct operation of the autoclaves used in the preparation of agar and other media.

Pipettor cleaning and maintenance course

Safe handling of liquid nitrogen

*In-house short courses attended at Plymouth during the duration of PhD research:*

Speed Reading	Managing Email Effectively	Project Management
Negotiation Skills	Introduction to Microsoft Project	Intermediate Microsoft Project
Microsoft Visio	Microsoft Front Page	Using Adobe Acrobat PDF Files
Time Management	Intranet for Postgraduate Students	Impact Factor: Writing for Research Publication
Preparing for the Viva	Applying for Research Funding	Plagiarism/ Turnitin
Using LateX 1	Using LateX 2	CV Writing and Development
Presenting Research on a DVD	Word for Long Documents	Pebble Pad
Social media for scientists	EndNote for Beginners	EndNote Intermediate
Presenting to an Audience		

### **Other relevant experience**

Demonstrator on Ecology, Biology and Extended Science Courses, with duties including leading practical sessions, and marking tests.

Lead lecturer on Plant Taxonomy module 2006-2007, with responsibilities for lecture preparation and delivery; marking and assessment of tests and coursework.