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DOCTOR OF PHILOSOPHY

Improved control of rhododendron ponticum for environmental management

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Award date:
2009

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IMPROVED CONTROL OF *RHODODENDRON PONTICUM*
FOR ENVIRONMENTAL MANAGEMENT

A dissertation submitted to the University of Wales Bangor for the
degree of Doctor of Philosophy

By

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December 2009



‘The bushes puzzled him, they were so big, almost trees, some twice his height, and there seemed so many....With their zigzag branches and long oval leaves finger-like in every direction they seemed to belong to a different climate, to a different land, whose gravity pulled softer than this one.’

A description of Rhododendrons by John Updike (Rabbit Run, 2006)

Abstract

Rhododendron ponticum is an invasive non-native shrub that represents a significant threat to productive land use, biodiversity conservation in priority habitats and potentially other ecosystem services in Britain and Ireland. Management actions that reduce the spread and impacts of invasive species require knowledge of their ecological characteristics as well as best control methods. A series of glasshouse experiments was carried out to investigate the effect of microhabitat on *R. ponticum* germination and seedling survival rates. Light environment in the form of different shade treatments, watering regime and seedbed substrate were manipulated in a factorial design to test their effects and interactions. Short-growing moss, bare soil and sown lawn grass produced a combination of high germination and seedling survival rates, with much lower rates in tall growing moss, leaf litter and grassland turf. Periodic droughting had a large negative effect on *R. ponticum* seed germination, especially in the more exposed seedbed substrates. The presence of additional canopy shade above a vegetated ground layer was very detrimental to *R. ponticum* establishment and this effect was found to be due to a reduction in light quality (ratio of red to far red light) and not light quantity (photosynthetic photon flux density).

The use of chemical control methods with herbicides, in particular glyphosate, dominates current practice in the removal of *R. ponticum* from invaded habitats. In order to optimize the efficacy of glyphosate, a series of experiments were designed to improve knowledge of the effect on glyphosate absorption and translocation, of its dose in combination with application at different times of the year in the field, and of foliar application and light level in the glasshouse. Glyphosate applied to the foliage of *R. ponticum* plants less than 1.5 m in height, was most readily translocated when the plants were more metabolically active. Plants treated in August and May showed greater rates and extent of crown damage than those treated in November and February. ¹⁴C-labelled glyphosate applied to the lower leaf surface of *R. ponticum* plants was rapidly absorbed over the first six hours with maximum absorption after three days. Translocation to other parts of the plant (measured as glyphosate concentration) was greatest to the stem adjacent to the

treated leaves and then to the roots, concentrations were lower in untreated leaves over 30 days. Supplemental lighting did not increase the foliar absorption of the ^{14}C but did increase the amount translocated to the other parts of the plant.

This study has brought a new understanding to the invasion dynamics of *R. ponticum* that can be used in identifying habitats vulnerable to invasion and in developing a post clearance management strategy for preventing reinvasion of sites. It has also brought a basic knowledge to glyphosate absorption and translocation patterns that could have profound implications in improving practical application techniques of invasive woody plant species.

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Acknowledgements

First and foremost I would like to thank my supervisors John Healey and Rob Brook for their continual support, encouragement and sound advice. They have helped me improve my approach, taught me to reevaluate the details to make my conclusions stronger and ultimately have been the most critical factor in making this thesis complete.

Secondly I would like to thank the SENR and ECW technicians: Gareth Williams, John Evans, Jonathan Roberts and Gordon Turner for helping me carry out my experiments and supplying absolute everything I needed to complete this research. Llinos Hughes gets special thanks for being the most willing to help on a practical basis I met during my thesis, she did everything to make my experiments possible. Also thanks to Mark Hughes and Julian Bridges for keeping my experiments on track and continually keeping an eye out to water some thirsty rhododendrons.

Thanks go to those people willing to go beyond their job help, for me this includes Paul Hill and Paula Roberts for taking time out to help me understand how to use the equipment. I am grateful for advice and comments from Davey Jones and Mike Hale and for the greatly appreciated computer and IT help I thank my fellow students; James Walmsley, Matthew Ling and Mark Farrell, without whom my thesis wouldn't have been able to take shape.

For the outside support I would like to acknowledge my sincere appreciation to Peter Jackson of *Snowdonia National Park*, Jenny Wong from *Wild Resources*, Colin Edwards *Forest Research and Forestry Commission*, Dave Smith of the *National Trust* and importantly Julian Miller and Andy Kehoe of *Kehoe Countryside*.

I would also like to acknowledge Kehoe Countryside who cosponsored my project with the European Science Foundation and also Bangor University SENRGY for providing me with the funds to bring my project to completion.

Lastly but not least, I would like to say a huge thank you to my friends and family who encouraged me, supported me and trusted me to finish this thesis.

CHAPTER I: General Introduction

1.1 INTRODUCTION

Biological invasions by non-native plant species have been of a growing concern in recent years and are considered to be one of the major driving forces of biodiversity loss with an important interaction with other major components of global change (Mack *et al.*, 2000). Significant economic impacts have arisen from invasive species damage in agriculture and forestry, in addition to having profound environmental consequences. Pimentel *et al.*, (2000) estimated that introduced non-indigenous species in the USA cause major environmental damage and losses totalling approximately US \$137 billion per year. The removal or even reduction of a species is a challenging undertaking with large-scale programmes incurring great costs. For example, the European loosestrife (*Lythrum salicaria*), which was introduced as an ornamental plant to the USA in the early nineteenth century, has been spreading at a rate of 115,000 hectares per year and costs US \$45 million per year in control costs (Pimentel *et al.*, 2000).

The sooner an infestation is detected and control implemented after an introduction, the greater the likelihood of success and the less the costs (Simberloff, 2003). There are, however, some examples where invasive species have been successfully eradicated or managed at low densities in the long-term (Mack & Lonsdale, 2002). The virtual eradication of the common barberry (*Berberis vulgaris*) in the USA was accomplished early in the twentieth century by vigorously searching and eradicating even isolated plants whilst simultaneously destroying larger foci (Mack, 1985). Simberloff (2006) asserts that eradication is a feasible goal but that the biggest hurdle is marshalling the political and social support to organise an effective comprehensive operational structure, including early warning and rapid-response capability. Control of biotic invasions is most effective when it employs a long-term, ecosystem-wide strategy rather than a tactical approach focused on

battling individual invaders (Mack *et al.*, 2000). Although many exotic plants species are introduced into semi-natural habitats, only a fraction (0.1%) becomes aggressively invasive and displaces native species (Williamson & Fitter, 1996). Nevertheless, since its introduction in the late eighteenth century, *Rhododendron ponticum* L. has become one of the most invasive non-native species in Britain and Ireland.

1.2 ECOLOGY OF RHODODENDRON

A comprehensive account of the ecology *Rhododendron ponticum* is described by Cross in his PhD thesis (1973) on invasive populations in Ireland, which is the basis of his subsequent review of *R. ponticum* in the Journal of Ecology, Biological Flora of the British Isles (1975). He also gives an account of *R. ponticum* establishment in the Killarney oakwoods in southwest Ireland (1981). What follows is largely based on these data, unless otherwise stated.

1.2.1 Origin and Distribution

Rhododendron, the largest genus of the Ericaceae family, encompasses more than 1,000 species distributed mainly in mountainous areas of the arctic, northern temperate regions and the mountainous tropics. They are particularly abundant in Asia, whence many of the popular cultivated species and hybrids are derived. *R. ponticum* is an evergreen shrub, with dark green, waxy oblong leaves and striking pinkish-purple flowers. The etymology of the name Rhododendron is derived from the Greek rhodon 'rose' and dendron 'tree'. *Rhododendron ponticum* occurs as a native species in the southern Iberian Peninsula and also around the Black Sea. Within its native range, *R. ponticum* occurs in the area south of the Black Sea (Bulgaria, northern Turkey, the Caucasus and Lebanon) and disjunctly in three small areas in the Iberian Peninsula: in southwest Spain, and central and southern Portugal (Popova, 1972; Cullen, 2005). In Spain and Portugal it is restricted to relict Tertiary populations and has been classed as an endangered species in Andalusia, southern

Spain (Mejías, *et al.*, 2002). In contrast, it has become an aggressive invader since its introduction in northwest Europe and has now become naturalised and poses a threat to native flora in Britain, Ireland, Belgium, and France (Cross, 1975). First introduced into Britain in 1763, it was extensively planted in gardens, estates and parks as an ornamental and for game cover (Elton, 1958). It has since become widely established where conditions were favourable and expanded its range into woodlands, heaths, bogs, and sand dunes (Thomson *et al.*, 1993). Invasive populations in northwest Europe originated from the subspecies *baeticum* populations from the Iberian Peninsula (Erfmeier & Bruelheide, 2005). There is also some evidence for introgression with the more frost tolerant North American species *R. catawbiense* and *R. maximum* (Milne & Abbott, 2000). The subspecies *ponticum* has also become widely abundant in several forests of Turkey where it threatens natural tree regeneration (Esen *et al.*, 2006).

1.2.2 Topographical range and growth habit

Topographical range

Rhododendron ponticum displays a wide geographical and altitudinal range within its native area of distribution. However, it shows a strong preference for damp, shady habitats, especially in drier climates such as its native Iberia where relict populations are restricted to moister sites (Mejías *et al.*, 2002). The climatic extremes of temperature and drought that can limit establishment in its native habitat are rarely experienced in Britain and Ireland. It is therefore very well adapted to this temperate maritime climate, especially the wetter and more humid conditions of the west and north. Like most of the ericaceous species, *R. ponticum* is a calcifuge and grows best on well-drained, acidic podsolised soils, although it can also occur on other substrates, including wet clays, brown earths, alluvium and peat. It can thrive in most British soil conditions except in areas subject to prolonged drought or water-logging or in high pH soils. It grows best in soils with a pH <4.5 but can be found growing in a range between 3.3 and 6.4. *Rhododendron ponticum* is a very shade tolerant plant and can establish in light levels as low as 2% daylight, but growth is

much reduced in deep shade. It is most vigorous in hyperoceanic woodlands, but also occurs in other woodlands, coniferous plantations, heathlands, bogs and sand dunes (Fuller & Boorman, 1977; Rotherham, 1986). Although the altitudinal range of *R. ponticum* reaches up to 2000m in the Black Sea Region, it is mostly limited to 500m above sea level in Britain and Ireland (Cross, 1975). The few plants that have been recorded growing above 400m in exposed sites have appeared unhealthy with signs of leaf damage and dieback (Jackson, 2008).

Flowering, seed dispersal, germination and seedling growth

Rhododendron ponticum flowering does not usually commence until it is 10- to 12-years-old and occurs mainly in May to June with seeds being released in February or March in Britain and Ireland (Stout, 2007). Plants flower most profusely in the open or under a light canopy. Mature naturalised *R. ponticum* plants produces a massive floral display of large (> 60 mm corolla diameter), conspicuous bright pink/purple flowers that secrete large volumes of nectar. Although flowers are self-compatible for pollination, higher levels of seed set occur following outcrossing, which usually occurs by generalist pollinators (Stout, 2007). A mature *R. ponticum* plant in full light is capable of producing up to 1 million seeds annually. *Rhododendron ponticum* produces small, very light seeds which are primarily dispersed by wind and which travel mostly less than 10 metres from the parent plant (Stephenson *et al.*, 2007). Although *R. ponticum* seeds are positively photoblastic (require light to germinate), once established, seedlings are capable of surviving under very low levels of natural light. Seedling distribution in woodlands has been associated with a thin surface cover of bryophyte species, rocky outcrops and breaks in slope (Cross, 1981) or thin layers of leaf litter on decaying timber (Stephenson *et al.*, 2006). Suitable conditions or 'safe sites' for seedling establishment in heathlands and bogs are found at the base of *Molinia* tussocks and on compact *Sphagnum* hummocks (Cross, 2004; Jackson, 2008). Seedling growth is initially very slow but progresses more rapidly with age. A fully mature *R. ponticum* plant usually reaches a height of 5 m under a tree canopy but can grow to 6 – 8 m if its upper shoots are physically supported by a tree. In the open it can grow up to 4 m and in very exposed situations may not exceed 2 m. Limited vegetative reproduction can also occur

where branches come in contact with the ground. If damaged by fire or cut the plant sprouts vigorously from its well-developed root system or underground 'lignotuber' (Çolak, 2004).

1.2.3 Facilitative and limiting factors to *Rhododendron ponticum* invasion

The invasion success of *R. ponticum* in Britain and Ireland is due to several reasons: the attributes of the species, the invasibility of the environment and socio-economic factors. Extensive planting of the species in the nineteenth and early twentieth centuries was in part due to the gardening fashion of the time but also the ease with which it was propagated. Improved hardiness achieved through cross breeding (section 1.2.1 above) and relatively cheap price made it a popular choice for widespread planting as game cover in woodlands and as grafting stock for other *Rhododendron* species (Dehnen-Schmutz *et al.*, 2004). There are several species attributes that are key to its invasion success: prolific production of viable seeds; ability to tolerate shade; ability to outcompete native vegetation by casting a deep shade; and wide niche-breadth in terms of climatic tolerance, and soil and habitat requirements (Shaw, 1984). It also lacks natural predators and the leaves, which contain toxins, unpalatable to grazing animals. Exudates released from the roots and leaves might have an allelopathic affect on other plants (Rotherham & Read, 1988). As with most ericaceous species, symbiotic mycorrhizal relationships cause an enhancement in growth of *R. ponticum* especially in poor edaphic conditions through the increased absorption of phosphorus and other nutrients from soils (Pearson & Read, 1973a,b; Rotherham & Read 1988). One of the few limitations to expansion of its range is the availability of safe sites for establishment given the vulnerability of its small young seedlings, though this is partially overcome by prolific seed production. The abundance of seedlings and young plants has been positively correlated with the frequency and density of safe sites. Whilst safe sites develop naturally, disturbance by livestock and humans, direct or indirect, contributes substantially to their occurrence. The two most important disturbance factors are grazing pressure and mechanical disturbance resulting mostly from forestry practices (Edwards, 2004; Jackson, 2008).

1.3 THE IMPACT OF *RHODODENDRON PONTICUM*

1.3.1 Impact on native plants and animals

Invasive species are considered to be one of the most significant threats to global biodiversity, in that they can have severe impacts on the local flora and fauna as well as economic effects on agriculture, horticulture and forestry. The risk of invasion is large with continued distribution by humans of species outside their native range together with the effects of rapid climate change. Of the many non-native species that have been introduced over the centuries, only a small minority have become invasive. *Rhododendron* invades threatened habitats such as oak woodlands, blanket bogs and heathlands. These three habitats are of such high conservation value that they have been designated as priority habitats under both the UK Biodiversity Action Plan (UK BAP, 1992) (as a response to the Convention on Biological Diversity, 1992) and as Special Areas of Conservation (SAC) under the EU Habitats Directive (1992). In Ireland it also invades several Annex 1 habitats listed under the EU Habitats Directive, including old oak woodland with *Ilex* and *Blechnum*, North Atlantic wet heaths, dry heaths and blanket bogs. *Rhododendron ponticum* is by far the biggest threat to the native ancient oak woodlands of Killarney that are renowned for their richly diverse and abundant lichen and bryophyte communities, where it is spreading with vigour (Higgins & Barron, 1996). On Lundy Island, *R. ponticum* is considered to be the major threat to the endemic plant the Lundy cabbage (*Coincya wrightii*), a BAP listed species (Compton & Key, 2004). The Lundy cabbage also supports at least one endemic insect species, the Bronze Lundy cabbage flea beetle (*Psylloides luridipennis*) and two other beetles that differ from mainland relatives. In each of these habitats, *R. ponticum* forms dense thickets, which effectively inhibit growth of ground vegetation and suppress the regeneration of trees.

Few plants can tolerate the deep shade cast by dense *R. ponticum* canopies. In the northern red oak (*Quercus rubra*) forests in the southern Appalachian region of USA, the closely related *R. maximum* inhibits the recruitment of native canopy trees by casting a deep shade (Nilsen *et al.*, 2001; Lei *et al.*, 2002). *Rhododendron*

ponticum and (*R. flavum*) also pose a large problem in the beech forests of the Black Sea Region of Turkey where it reduces *Fagus orientalis* (Oriental beech) regeneration and seriously threatens local floral diversity (Esen & Zedaker, 2004). Although *R. ponticum* thickets can be an excellent roosting and breeding habitat for birds, Batten (1976) found that there were substantially lower populations of breeding birds in *R. ponticum* infested woods in Killarney. However, the effect is not all negative with insects benefitting from the presence of *R. ponticum* and its large nectar production, with more than 30 native Irish insects including bumblebees observed to visit the flowers (Stout, 2007).

1.3.2 Role of *Rhododendron ponticum* in the spread of ‘Sudden Oak Death’

Phytophthora ramorum* and *Phytophthora kernoviae

Phytophthora ramorum is a serious fungal-like (oomycete) pathogen that causes major damage to trees and a range of other ornamental and native plants. *Phytophthora ramorum* became popularly known as ‘sudden oak death’ in the USA where it caused widespread devastation to populations of ‘tanoak’ (*Lithocarpus densiflorus*) in California and Oregon (Ashby, 2004). In Europe *P. ramorum* was first recorded in 1993 both in Germany and the Netherlands but was confined to *Rhododendron* and *Viburnum* and has since been found in many other European countries, including Great Britain in 2002 (Forestry Commission, 2008). Although the disease has mainly affected ornamental plant species in nurseries and public gardens, British woodlands are also considered at risk as a number of tree species have been found to be susceptible to the pathogens (Tracy, 2004). By 2008 a total of 28 tree species had been confirmed with the disease including several species of oak, European beech (*Fagus sylvatica*), horse-chestnut (*Aesculus hippocastanum*), sweet chestnut (*Castanea sativa*), sycamore (*Acer pseudoplatanus*) and roble beech (*Nothofagus obliqua*) (Tracy, 2004). Another *Phytophthora* species, now named *P. kernoviae*, was discovered in Great Britain in 2003 (Brasier *et al.*, 2005). Both *Phytophthora* species result in bleeding stem lesions on forest trees, and foliar necrosis of ornamentals. Unusually for a *Phytophthora* species, *P. kernoviae* infection of rhododendron causes defoliation and dieback of shoots occasionally

resulting in bush death (Beales *et al.*, 2006). The principal host of both the *Phytophthora* species in Britain is believed to be *R. ponticum* which has been found on the majority of sites where *P. ramorum* was recorded on trees (Tracy, 2009). As *R. ponticum* is the most widespread rhododendron species in the wild, DEFRA (Department for Environment, Food, and Rural Affairs) and the Forestry Commission suggest that the eradication of rhododendron is the most effective control measure to reduce disease spread in the wider environment (woodland, gardens and parks) (Anon., DEFRA 2008).

1.3.3 Impact on farming, forestry and tourism

Rhododendron ponticum invasion causes a loss of grazing area and infested land is deemed non-agricultural and ineligible for agricultural subsidies in Wales (Wong, 2004) Ingestion of the vegetation is poisonous to sheep (Black, 1991), cattle and goats (Humphreys *et al.*, 1983) due to the presence of toxic chemicals, particularly diterpenoids (grayanotoxin). Honey made from the pollen and nectar of *R. ponticum* flowers is also toxic to humans (Koca & Koca, 2007). The first recorded case of mad honey intoxication dates as far back as the 401 BC. Xenophon of Athens, a Greek historian and soldier, gives an account of the effects upon soldiers in his magnum opus, *Anabasis* (401 BC, translated by H. G. Dakyns, 2007).

‘The effect upon the soldiers who tasted the combs was, that they all went for the nonce quite off their heads, and suffered from vomiting and diarrhoea, with a total inability to stand steady on their legs. A small dose produced a condition not unlike violent drunkenness, a large one an attack very like a fit of madness, and some dropped down, apparently at death's door.’

There were, however, no fatalities and all recovered after several days of severe medical treatment (Xenophon).

Non-commercial mad honey is still being made by amateur beekeepers in the eastern Black Sea Region of Turkey from the nectar of *R. ponticum* which grows natively there (Aliyev, *et al.*, 2009) It is one of the common food intoxications encountered for humans and livestock in Turkey (Koca & Koca, 2007). Symptoms are dose-related ranging from dizziness, weakness, excessive perspiration,

hypersalivation, nausea and vomiting; however, severe intoxication may lead to life-threatening cardiac complications (Koca & Koca, 2007, Dubey *et al.*, 2009).

Rhododendron ponticum can dominate the understorey below a tree canopy and it greatly increases the cost of timber harvesting and significantly reduces the yield and the rate of tree regeneration (Robinson, 1980; Esen & Zedaker, 2004). The impact on tourism can be positive, with some visitors coming especially to witness the great swathes of purple flowering blossoms in May and June, although the presence of large shrubby bushes has a detrimental effect on amenity and recreation by blocking paths and access to land (Jackson, 2008). However, a recent increase in public awareness regarding the environmental problems is beginning to cause a shift in attitudes.

1.4 TECHNIQUES AND COSTS OF *RHODODENDRON PONTICUM* CONTROL

Eradication of a non-indigenous species has the appeal of providing an apparently permanent solution to an invasion problem. It is sometimes feasible, particularly if the invasion is detected early and resources can be applied quickly. Usually, however, ongoing monitoring is not of sufficient frequency or intensity to detect an invasion soon after it occurs (Simberloff, 2003). If eradication is not possible, the goal often becomes ‘maintenance control’ at acceptable levels. Control is often targeted at habitats of conservation or amenity value that have been invaded in order to restore their status or value. The first step often involves initial removal of the established plants of the invasive species. Several different techniques have been developed for this initial removal of *R. ponticum* depending on various factors, such as: plant size, growth habit and density, site access, and post-clearance aesthetics. Three main approaches to control, applied singly or in various combinations, widely used are: chemical, physical, and biological. The main method used for *R. ponticum* is chemical where a herbicide, generally one such as RoundUp containing the active ingredient glyphosate, is applied to either the foliage (foliar application); directly into a cut or hole in the stem (stem treatment); or to the cut

surface of the remaining stump or foliar regrowth following cutting (stump treatment). Physical control, in the form of handpulling small seedlings, or using mechanized equipment can also be used but application of the latter method is limited to flat, easily accessible sites. Similar methods are used in the control of other shade tolerant, woody invasive species, such as *Prunus laurocerasus* and *Ailanthus altissima* (The Global Invasive Species, 2009). Several other techniques have been successfully applied to *A. altissima* including girdling – although this generally results in resprouting below the girdle in hardwoods – and biological control using livestock to graze the tops of seedlings and resprouts following cutting to gradually weaken the underground parts (The Global Invasive Species 2009). Girdling has been found to be ineffective for *R. ponticum* (Julian Miller, practitioner, Kehoe Countryside, pers. comm.) but there is no record of any formal studies. Attempts at ‘starving’ the remaining stump after cutting the stem to ground level by complete shading with a pegged covering of black plastic was also found to be unsuccessful due to the capacity of *R. ponticum* shoots to grow out a considerable distance horizontally from stumps to emerge from below the edge of the plastic (pers. obs. and Dave Smith, National Trust, pers. comm.). Physical removal of saplings and seedlings by pulling is not a viable option as *R. ponticum* has a dense mat of roots and uprooting would not only be hard to achieve but would cause a great deal of ground disturbance, which itself might create good microsite conditions for establishment of a new cohort of seedlings (section 5.1 above). There is some evidence of sheep grazing on small *R. ponticum* seedlings (< 2 cm in height) but they tend to avoid eating larger plants (Jackson, 2008) and therefore using livestock to browse regrowth would be ineffective and if it did work might well have animal health implications because of the grayanotoxin in *R. ponticum*. There are therefore no effective alternatives to using chemical control with herbicides such as glyphosate to remove *R. ponticum* from an invaded site.

1.4.1 Control techniques

Techniques for the control of *R. ponticum* are now well established and summarised below. Most of them involve the application of a herbicide to either the foliage or the stem. A full description of the techniques and the implementation of a

management plan for clearance of infested areas is given in the Forestry Commission practice guide for managing and controlling invasive *Rhododendron* (Edwards 2006). This guide states that for seedlings or small plants that are growing in loose substrate and easily uprooted, hand pulling is the cheapest, easiest and most environmentally friendly way of removing them. The most common method of treating bushes less than 1.5 m in height is foliar application of the herbicide glyphosate (e.g. RoundUp) and surfactant (Mixture B) using a knapsack sprayer. The guide warns that even with careful technique, some collateral damage to adjacent vegetation can occur from spray drift and drip, as leaves are usually sprayed to run-off. This method can also only be applied in dry conditions with at most a light breeze to be effective and to minimise spray drift and soil contamination.

Several different methods are described to treat larger bushes. The first involves initially cutting to ground level and applying glyphosate to the cut stump directly after cutting. In the second method the stump is left for 6 months and is followed by foliar spraying shoot regrowth with glyphosate. The guide states that the former method has had variable success sometimes merely causing a reduction in the amount of regrowth rather than plant death. Another method recommended in the guide is stem treatment, in which herbicide is applied under the bark at the base of otherwise uncut stems typically into a hole made with either an axe or a drill. Several advantages are listed for this method: it is economic, causes minimal damage (compared with foliar application technique) to non-target plants through spread of glyphosate, can be applied in varying weather conditions and has a very high kill rate. The final technique recommended involves using excavator mounted flails with powerful mulching heads. This may provide a cheaper alternative to manual techniques (where the scale of operation justifies the use of this machinery) but subsequent treatments to deal with regrowth are still be required. The main drawback to this method is its restriction to sites which can be accessed by excavator; the steep terrain on which *R. ponticum* is typically found is unsuitable. A more detailed history and description of control techniques is given in Chapter III.

1.4.2 Cost of invasive species control

The cost of global damage by invasive species has been estimated to be \$1.4 trillion per year (Pimentel *et al.*, 2001). In 2007, the Minister for Biodiversity stated that invasive non-native species costs to the British economy amounted to £2 billion per year (Parliamentary Office of Science and Technology, 2008). It is estimated that £2 million has been spent on control of *R. ponticum* in Snowdonia National Park alone since 1987 (Jackson, 2008). Jackson (2008) estimates that a further £9 million will be required to control *R. ponticum* on a Park-wide basis. The cost of eradicating current *R. ponticum* source populations from the entire Argyll and Bute landscape is estimated at > £9.3 million (Edwards & Taylor 2008). *Rhododendron ponticum* eradication and control are expensive operations and require full commitment to a long-term plan incorporating initial treatment, follow-up operations and monitoring. The cost can be variable depending on several factors: plant density, roughness of terrain, steepness and site access. There is also a wide variation in prices quoted by practitioners for contracts. Typical prices, based on work done in Snowdonia National Park by Jackson (2008) and clearance in Argyll by Edwards (2006), can range from £1000 per hectare for foliar spray application to bushes less than 1.5 m in height to £5000 per hectare for completely removing large bushes (cut, burn, foliar spray of regrowth) from a site. These prices are based on flat sites with easy access; steep sites where rope work may be required can be much more expensive (Jackson, 2008).

Even if an area is cleared of *R. ponticum* plants, in reality control is not likely to be 100% effective despite taking the greatest care. Despite following recommended procedures, plants can be missed and there can often be regrowth which can begin flowering within three years (Edwards, 2006). Incompletely treated stands can recover rapidly with subsequent high control costs that can exceed comparable untreated areas (Jackson, 2008). It is therefore of vital importance that follow-up surveys are conducted to assess whether further treatments are required. Due to the short-term nature of clearance grants, which are often paid in lump sums, there are little or no funds available to deal with follow-up treatments. In addition to this, disturbance associated with clearance may actually have the effect of increasing the probability of reinvasion through the creation of suitable microsites (Stephenson

et al., 2006). This is especially of great concern where there is an adjacent stand of mature flowering *R. ponticum* acting as a seed source. This situation can occur where there is not enough money available to clear the entire stand, or where land is under management by a different owner who does not intend to clear the invaded area. It is therefore important to develop a post clearance management strategy to prevent reinvasion; this could include planting native vegetation to compete with and exclude *R. ponticum* seedlings, or perhaps a grazing regime to impede establishment.

1.5 INVASIVE SPECIES LEGISLATION

Section 14 of The Wildlife and Countryside Act (1981), as amended by the Nature Conservation (Scotland) Act 2004 in Scotland and the Natural Environment and Rural Communities Act (2006) in England and Wales, is the principal legislation dealing with the release of non-native species and makes it illegal to plant or otherwise cause to grow in the wild any plant listed on Schedule 9 of the Act (DEFRA 2007). In England and Wales, Schedule 9 currently includes two terrestrial plant species (Japanese Knotweed, *Fallopia japonica*, and Giant Hogweed *Heracleum mantegazzianum*). Following the 2007 consultation more species are likely to be added to the list, including perhaps *R. ponticum* which was on the proposed species list (DEFRA, 2007).

The UK is obliged by several international agreements to prevent, control or eradicate invasive non-native species which are harmful. The UK is a contracting party to the following European conventions: The Convention of the Conservation of European Wildlife and Natural Habitats, Article 12 of which, states that each contracting party to the convention undertakes to strictly control the introduction of non-native species; and The Directive on the Conservation of natural habitats and wild fauna and flora (EU Habitats Directive, 1992), which requires members to ensure that the deliberate introduction of any species which is not native to their territory is regulated (NNSS, 2009).

EU and UK policy on invasive species is founded on the worldwide Convention on Biological Diversity (CBD), (1992/1993), which states under Article 8(h) that members shall "prevent the introduction of, control or eradicate those alien species which threaten ecosystems, habitats or species". Contracting parties to the CBD also agreed to "achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national level" (2010 Biodiversity Target) (NNS, 2009).

1.6 PROJECT OBJECTIVES

An understanding of the invasive dynamics of *R. ponticum* is key in identifying potentially vulnerable habitats and predicting future range expansion. It is also critical to develop a better understanding of the physiological mechanisms behind control techniques with the aim of improving the efficacy of practical control measures. An essential step in learning more about the underlying mechanisms of plant invasions is not only the identification of traits which make plant species invasive, but also investigating the factors that make a habitat vulnerable to invasion, and how these factors interact with the invasive plant traits. For example this interaction could be between the seed biology of the invasive plant species and microhabitat conditions. A series of experiments were carried out in the greenhouse and growth chambers to look at the effect of microhabitat on germination and seedling survival of *R. ponticum* under controlled conditions. This information would be of great value for the prevention of not only invasion or re-invasion but also for prediction of future range expansion.

The use of herbicides, particularly glyphosate is essential to the removal of *R. ponticum* in an invaded area (section 1.4 above). However little is known about the action of the herbicide in *R. ponticum*. Knowledge of the absorption, translocation and the factors that affect these processes within the plant is therefore very valuable to optimise control efficacy and potential re-establishment. A set of trials in the field in Snowdonia National Park, North Wales, UK, and in laboratory under controlled conditions were carried out to investigate this.

'CHAPTER II: The effect of substrate, watering and light on germination and seedling survival of the invasive species *Rhododendron ponticum*

2.1 ABSTRACT

Rhododendron ponticum L. constitutes an invasive species in several countries across Europe. The relative importance of microhabitat limitation is one of the critical elements in effective management strategies and for predicting future range expansion. A series of controlled experiments were conducted to explore the effect of microhabitat on *R. ponticum* germination and seedling survival. Light environment in the form of different shade treatments, watering regime and seedbed substrate were manipulated in a fully factorial design to investigate main effects and interactions.

Short-growing moss, bare soil and sown lawn grass were the best seedbed substrates for *R. ponticum* establishment, exhibiting a combination of high germination rates (20, 28 and 28%, respectively) and high seedling survival (69, 41 and 50%, respectively). Both rates were much lower in tall growing moss, leaf litter and grassland turf with survival rates as low as 0% in some treatments.

Periodic droughting had a significant negative effect (overall reduction from 21% to 14%) on germination rates especially in the more exposed substrates (bare soil and short moss) with seed sown in leaf litter and grass being less affected.

Germination rate, seedling survival and biomass were all significantly affected by light treatment, though the nature of this effect was dependent upon seedbed type. Green shade caused significant reductions in germination in the sown grass (39 to 16%), grass turf (16 to 1%) and leaf litter (20 to 11%) seedbed substrates. However, it had a positive effect on germination in bare soil which increased from 20% with no shade to 35%, and in short moss from 9% to 30%. Similar patterns were followed for seedling survival with green shade reducing survival in lawn grass, grass turf, leaf litter and tall moss, but having no effect in bare soil. Investigation into the mechanism behind light limitation revealed that a

reduction in R:FR ratio was responsible for inhibiting germination and reducing seedling survival. Seedlings grown under no shade had greater root and shoot biomass. There was some indication of an effect of population and genotypic plasticity on germination under different light treatments.

2.2 INTRODUCTION

Invasive species are now recognised as one of the key threats to biodiversity, second only to habitat destruction (Mack *et al.*, 2000; Keane & Crawley, 2002). Although many exotic plant species escape from cultivation or are directly introduced into semi-natural habitats, only a fraction (0.1%) become aggressively invasive and displace native species (Williamson & Fitter, 1996). Invasion success by new species is influenced by three factors: propagule pressure, the characteristics of the invading species, and the susceptibility of the environment to the new species (invasibility) (Lonsdale, 1999). Invasibility is the outcome of several environmental factors, including competitive ability of the resident species, local climate and disturbance regime as well as a range of intermittent stochastic events that can increase invasibility (Lonsdale, 1999). Therefore, predicting invasion success requires an understanding of the site-specific and species-specific factors that contribute to the successful emergence of the invader.

Seed germination and seedling establishment are key processes that influence plant distribution and abundance. The transition between these phases is considered one of the most critical stages in the life cycle of plants (Harper 1977; Kitajima & Fenner 2000). The likelihood of a propagule surviving each of these life stages may vary both spatially and temporally and can be affected by many abiotic and biotic factors (Baskin and Baskin, 1998). While seedling establishment in some species is largely affected by seed size (Burke & Grime 1996) and number (Jakobsson *et al.*, 2006), in many species the availability of suitable microsites or 'safe sites' limits species recruitment (Eriksson & Ehrlén, 1992; Jones & del Moral, 2005). The term 'safe site' refers to the biotic and abiotic conditions that promote emergence and survival of seedlings of a given species (Harper *et al.*, 1965). Many abiotic and biotic

factors can affect seed germination and seedling establishment such as distance from closest seed source (Stephenson *et al.*, 2006), light (Paynter *et al.*, 1998; Leishman *et al.*, 2000) and substrate type (Herrera & Littera, 2009). Ground cover (live vegetation plus leaf litter) is an important environmental factor that can influence seedling emergence as well as survival (Isselstein *et al.*, 2002; Gómez-Aparicio *et al.*, 2005) and may control the existence of safe sites for plant recruitment (Harper 1977; Xiong *et al.*, 2003).

2.2.1 Microclimate and microhabitats

Plants are dependent on a range of environmental resources for which they can potentially compete: light, water, nutrients and space, and in some cases pollinators and seed dispersers. The modification of above-ground microclimate by established vegetation as well as inter-annual climatic fluctuations can also have a major role to play in seedling establishment. They may alter the evaporative regime, temperature, wind flow, gaseous concentrations (e.g. water vapour and carbon dioxide), and soil moisture availability through their physical presence and physiological activities (Hunter & Aarssen, 1988). The presence of a vegetative canopy alters the light environment below by reducing both light quantity and causing a reduction in the ratio of red to far-red (R:FR) wavelength. A low R:FR can reduce the growth and other developmental responses of plants (Geiger, 1965; Mitchell, 1992), notably seed germination (Yirdaw & Leinonen, 2002). For plant species that are 'light-demanding' (positively photoblastic), a low R:FR typical of shady habitats can inhibit germination (Seiwa *et al.*, 2009) and reduce seedling survival (de Souza & Válio, 2001).

As well as reducing resources through competition, plants may alter environmental conditions in a way that benefits neighbours, e.g. by increasing resources through canopy leaching, microbial enhancement, mycorrhizal networks and hydraulic lift and providing physical or chemical protection from herbivores, potential competitors or extremes of climate (Brooker *et al.*, 2008). The positive influence of neighbouring plants (nurse plant syndrome) has been described for a wide range of ecosystems but is particularly common in arid and semi-arid environments (Maestre *et al.*, 2001; Munguia-Rosas & Sosa, 2008). Commonly

reported effects of the nurse canopy include: a reduction in temperature and lower rate of evaporation (Valiente-Banuet & Ezcurra, 1991; Suzan *et al.*, 1996), lower photosynthetic flux density (PPFD) (Bush & van Auke, 1990), and increased soil moisture (Belsky, 1994). A review of the facilitative interactions in plant communities carried out by Brooker *et al.* (2008), found that evidence of positive facilitative effects between plants tended to occur in severe environments, such as deserts, arctic or alpine (Callaway *et al.*, 2002; Castro *et al.*, 2004; Cavieres *et al.*, 2005; Cavieres *et al.*, 2006). In Mediterranean ecosystems, the emergence and survival of seedlings is highly microclimate dependent, being much lower in open spaces than under pre-established vegetation such as trees or shrubs (Herrera *et al.*, 1994; Castro *et al.*, 2004; Gomez-Aparicio *et al.*, 2005). Pre-existing vegetation can facilitate germination and seedling survival by causing a reduction in direct solar radiation and soil temperature and an increase in soil moisture and air humidity thereby improving the water status of the seedlings (Maestre *et al.*, 2003; Castro *et al.*, 2004). Certain plant species, either during particular life-history stages or throughout their lives, require the humid conditions beneath a canopy of other species and may not respond to full sunlight with increased productivity as do upperstorey light demanding species (Hunter & Aarssen, 1988). Leaf litter can reduce the amplitude of temperature and reduce evaporation while imposing shaded conditions (Eckstein & Donath, 2005). Whilst this can create a beneficial microclimate for seedlings vulnerable to desiccation, the overall effect on seed germination and seedling establishment is usually negative especially on smaller-seeded species with higher light requirements (Xiong and Nilsson, 1999; Jensen & Gutkunst 2003). Litter influences both light quantity and quality causing a change in the spectral composition beneath the litter layer through a reduction in the red component of light. The effects of shading, biochemical effects, and physical obstruction to the emergence of a seed's plumule and radicle by forest-floor litter decrease germination and seedling emergence in many species (Ellsworth *et al.*, 2004).

2.2.2 *Rhododendron ponticum* autoecology

The purpose of this study is to further understand the recruitment dynamics of the invasive species *R. ponticum*. *Rhododendron ponticum* was first introduced to

the British Isles in 1763 as an ornamental plant in gardens and was also extensively planted as game cover in woodland (Elton, 1958). It has since become widely established across Great Britain and Ireland where conditions were favourable. Local populations are mostly thought to have originated from *R. ponticum* L. subsp. *baeticum* in the Iberian Peninsula though there is some evidence for crossing with the more frost tolerant *R. catawbiense* and the American *R. maximum* (Milne & Abbott, 2000). Research by Erfmeier and Bruelheide (2005) found evidence for a genetic shift in invasive populations of *R. ponticum* from Ireland which exhibited faster germination and greater growth rates than their native counterparts from Georgia and Spain. They also found a great variation among populations nested within countries. The present study area is focused on Snowdonia National Park in North Wales, UK, where the core distribution of *R. ponticum* appears to be associated with planting in large estates from which it has since spread rapidly into the surrounding countryside in one of the most serious invasions in the British Isles (Jackson, 2008).

The relative invasion success of *R. ponticum* in different areas where it has been planted in the British Isles has been attributed to a combination of several factors: the climatic and edaphic suitability of the invaded habitat, the absence of natural predators, prolific seed production, widespread planting and habitat disturbance (Cross, 1981; Shaw, 1984). A major factor thought to limit the spread of *R. ponticum* is the availability of suitable safe sites for seedling germination and survival (Rotherham, 1986, 2001). *Rhododendron ponticum* can grow well under both deciduous and evergreen canopies (unless the light levels are extremely low) as well as in more exposed open areas. In a survey of *R. ponticum* distribution in the Snowdonia National Park, Gritten (1995) found that the habitat most invaded was coniferous woodland (49.8% of *R. ponticum* stands) with 14.7% of populations being in broadleaved woodland sites and 23.5% on open mountain. There is limited information on the effect of different light environments on *R. ponticum* germination and seedling survival. The only available information seems to be solely based on readings under different canopies in the field. For example, Cross (1975) (citing Cross, 1973), states that the rate of germination after 49 days under a *R. ponticum* canopy and an oak/holly canopy was 17% and 75% respectively. While this indicates that a denser shade reduces germination there is no quantitative description of the

light environment in terms of photosynthetic photon flux density (PPFD) or R:FR and there is no information on whether the seed was sown or how much. The confounding effects of varying environmental factors could also be responsible for any differences in germination rates. Cross (1975) also states that light is essential for germination and that R:FR in the ratio 1:20 (i.e. 0.05) with moss green filter was not inhibitory, but again there is no indication of experimental method or rigour. There is also no information on the effects of light or water availability on the survival of *R. ponticum* seedlings and habitat preferences are solely based on field distribution observations.

There are two studies recording occurrence of *R. ponticum* in the field, both based in woodland habitats. The first, by Cross (1981) was carried out in an oak woodland in Killarney, Co. Kerry, Ireland. A representative area of woodland was mapped and frequencies of *R. ponticum* seedlings on different ground cover recorded. Seedlings were localized to slopes on thin bryophyte carpets, such as short-growing pleurocarpous mosses, and acrocarpous mosses but not thicker moss mats. Cross found no occurrence of *R. ponticum* seedlings on bare soil or in leaf litter and suggested that this could be due to desiccation. Mejías *et al.* (2002) attributes recruitment failure of *R. ponticum* seedlings in relict populations in its native Iberia to the lack of suitable safe sites free from drought. The other study by Stephenson *et al.*, 2006 recorded the presence/absence of *R. ponticum* seedlings (<7 years old) in a single site within a mixed woodland (oak, beech, spruce) in Argyll, Scotland. They found that *R. ponticum* occurrence was highest on fallen logs or decaying tree stumps covered in moss or a thin layer of decomposing leaf litter. They recorded no seedlings in areas dominated by grass or with a mix of grass and moss.

2.3 OBJECTIVES

Four hypotheses were tested.

1. Is establishment of *R. ponticum* limited by availability of very specific safe sites ('seed bed')?
2. Is establishment of *R. ponticum* limited by drought?

3. If it is limited by drought, does the presence of a canopy mitigate this effect and contribute to a site being 'safe'?
4. To what extent can shade be a limiting factor on establishment? Does filtering of red and blue by a green canopy (which also reduces R:FR ratio) limit the rate of seedling survival, and growth. And, as a potential evolutionary consequence of this (out-weighting the potential benefits of shade, does R:FR limit germination?

In order to test these hypotheses three experiments were established:

Experiment 1 tested hypotheses 1, 2 and 3 with a factorial combination of different seed-bed substrates, light quantity and watering regimes.

Experiment 2 tested hypotheses 3 and 4 with a comparison of different levels of neutral (high R:FR) and green (low R:FR) shade, in glasshouse and in growth chamber conditions.

Experiment 3 tested hypothesis 4 by comparing the effect of light quantity and quality on germination rates of different provenances of *R. ponticum*.

2.4 METHODS

2.4.1 Seed collection

Experiment 1 & 2

Collection sites were selected to cover the altitudinal and geographical range in this area, including different habitat types, open grassland, forest edges and forest interior. Seeds were collected from five populations in North West Wales: (1) Bangor: 53°11'23 N, 4°10'56 W, roadside, woodland edge; (60 m alt.); (2) Beddgelert: 53°00'00 N, 4°06'13 W, open heath (140 – 190 m); (3) Nant Gwynant: 53°01'38 N, 4°02'06 W, open grassland (150 -170 m); (4) Penmaenuchaf Hall Hotel, 52°54'52 N, 3°55'36 W, deciduous woodland (50-60 m alt.); and (5) Sygun Copper Mine, 53°01'03 N 4°04'54 W, open mountain (70-100 m alt.). Capsules containing

seed were collected from at least 20 individuals in each population. Seeds were thoroughly mixed to minimise the effects of population and parent individuals.

Experiment 3

Seeds were collected from 6 populations – Sygun Copper Mine, Nant Gwynant, Wales (WNG) 53°01'03 N 4°04'54 W (70-100 m alt.) open mountain; Penmaenuchaf Hall Hotel, Mawddach, Wales (WDOL) 52°54'52 N, 3°55'36 W (50-60 m alt.) old estate, deciduous woodland; Kylemore Abbey, Co. Galway, Ireland (IKYL) 53°33'41 N, 9°53'21 W (40 m alt.) old estate, open; Kilbride, Co. Wicklow (IKIL) 53°12'13 N, 6°27'40 W (220-230 m) coniferous forest edge; Loch Eck, Scotland (SCLE) 56°04'35 N, 4°59'21 W (250 m alt.) open space in mixed woodland; and Drimsynie, Scotland (SCB839) 56°11'05 N, 4°55'47 W (40 m alt.) coniferous woodland/road edge - in December 2007 and January 2008. Capsules containing seed were collected from at least 20 individuals from each population. Seeds were thoroughly mixed within each population to minimise the effects of single parent individuals.

Although *R. ponticum* seeds (Fig. 2.1) are small, the mature seed is ~ 2mm in length, making it easily distinguishable from much smaller (< 0.5mm in length) undeveloped ovules. Only mature seeds were chosen, at random, for the experiments. Preliminary germination tests on filter paper in Petri dishes found germination capacity to be between 70 and 95%.

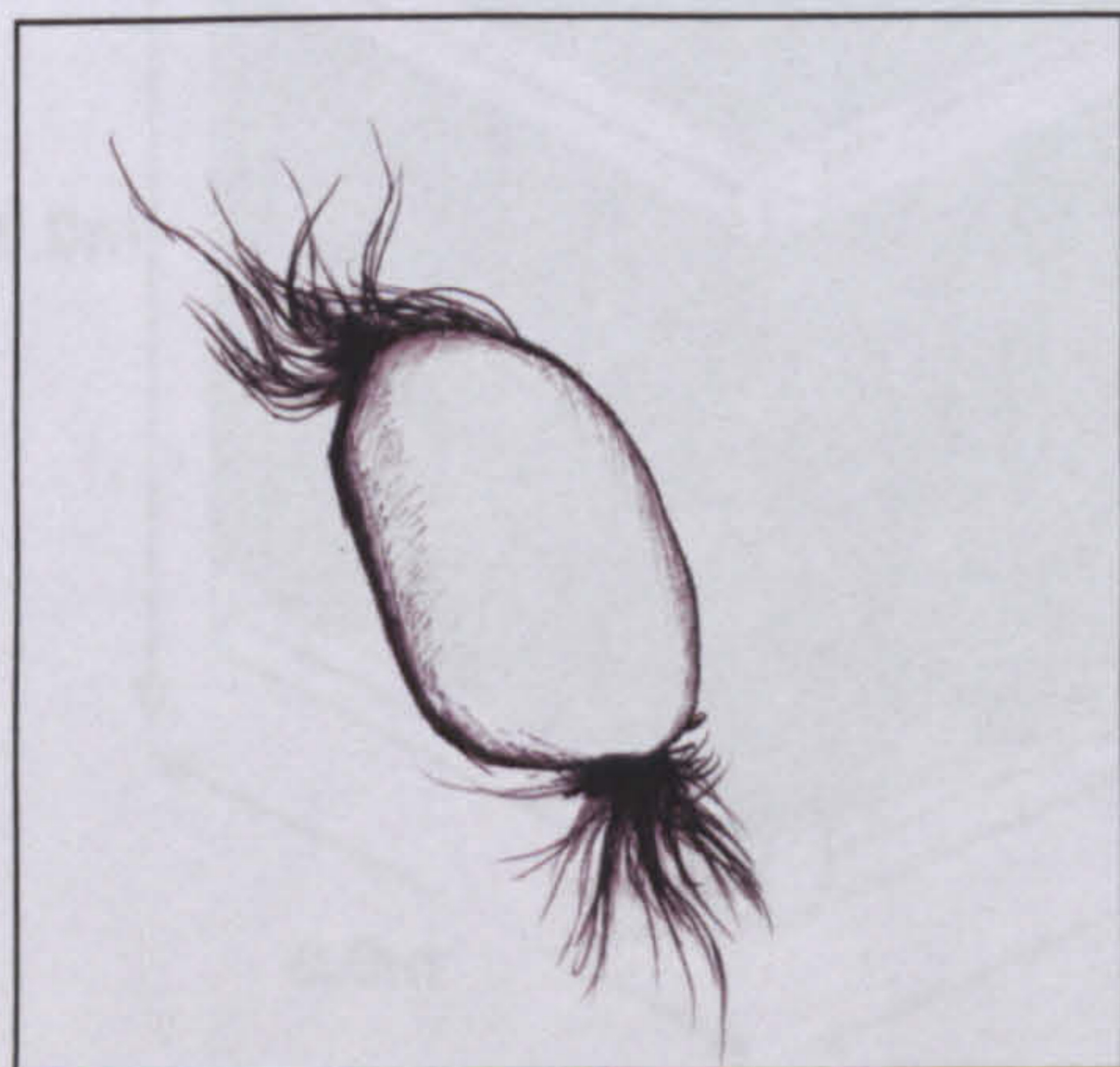


Figure 2.1 Drawing of *R. ponticum* seed approximately 2 mm in length bearing a frill of fibres, by author.

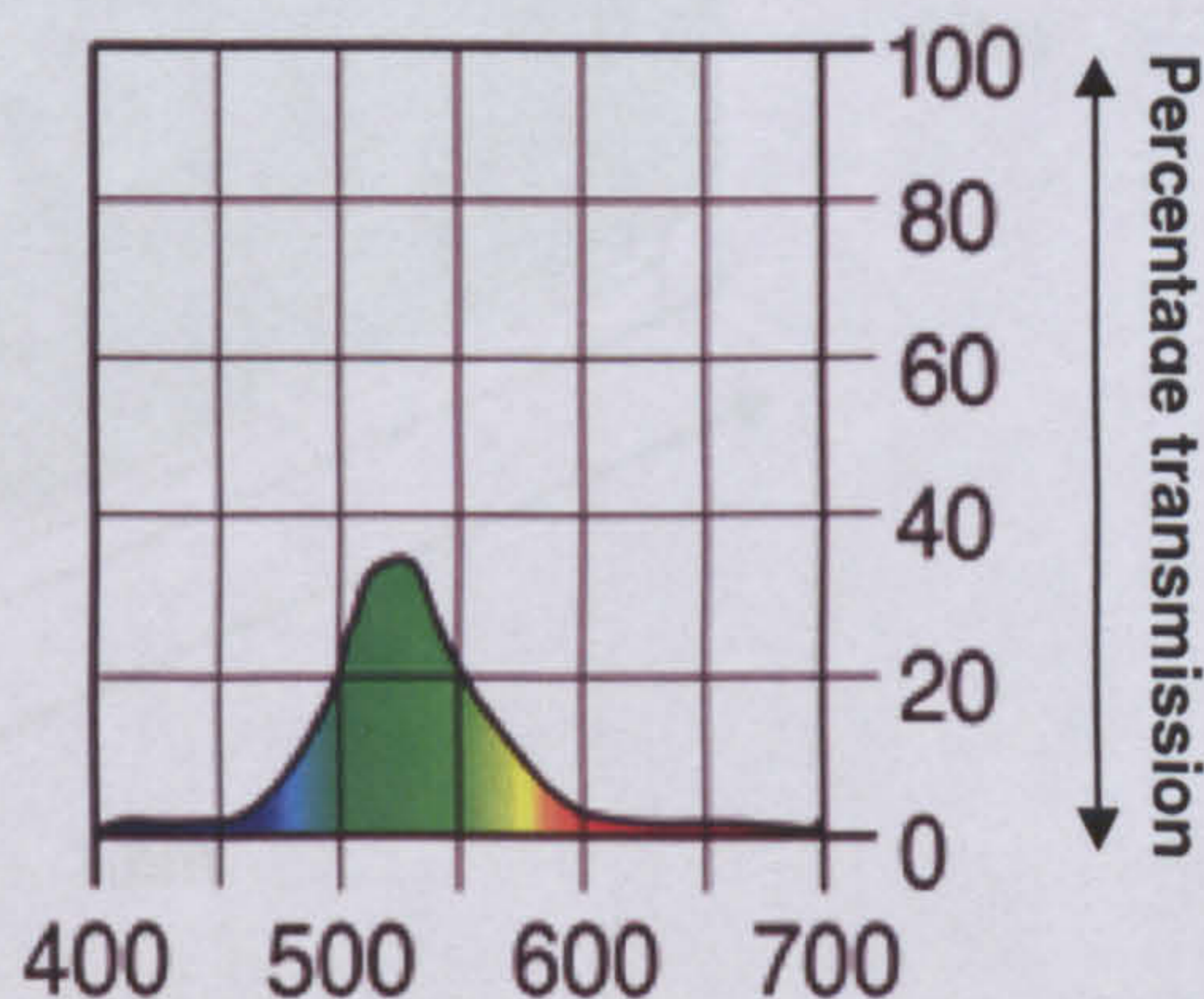


Figure 2.2 Percentage transmission of Lee 139 primary green film transmitted across the visible portion of the electromagnetic spectrum (x axis – wavelength)

2.4.2 Experimental design

Experiment 1

Two different types of light treatment chambers were constructed to examine effects of simulated forest understory light on germination and seedling survival: one using clear film the other Lee 139 primary green filter film (Stage Electrics, Brighton, UK; Fig. 2.2 gives the absorption spectrum). Chamber dimensions (Figure 2.3) were made large enough to leave a buffer of 10 cm around the seed trays to limit any variation of light quantity and quality by edge effects. A small gap of 3 cm was left at the top and bottom to allow airflow. Average values for maximum photosynthetic photon flux density (PPFD) and the red to far red light ratio (R:FR) under the different chambers were measured using a Skye PAR (SKP 215) sensor and a Skye 660/730 nm (SKT 110) sensor (Skye Instruments Ltd., Powys, UK). Calibration of the instruments was carried out to national standards by Skye Instruments, Powys, 6 months prior to use to national standards. Average PPFD was 20% (SD = 3%) and 90% (SD = 3%) of ambient light under the green and clear chambers respectively. The R:FR (0.45) was 45% (SD = 5%) of ambient light (R:FR = 1.04) under the green film and 97% (SD = 2%) of ambient light (R:FR = 1.01) under the clear chambers.

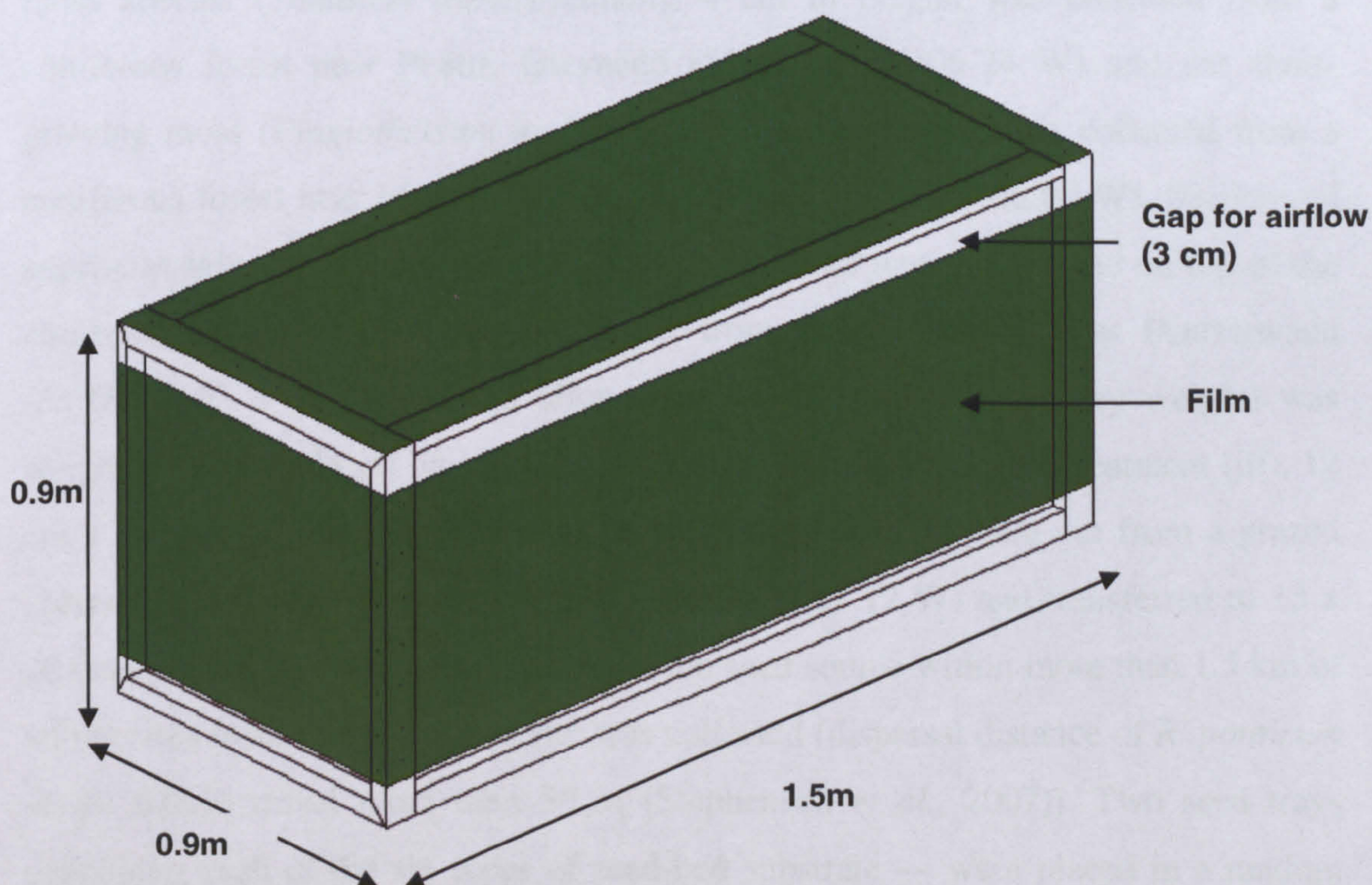


Figure 2.3 Shade chamber construction and dimensions.

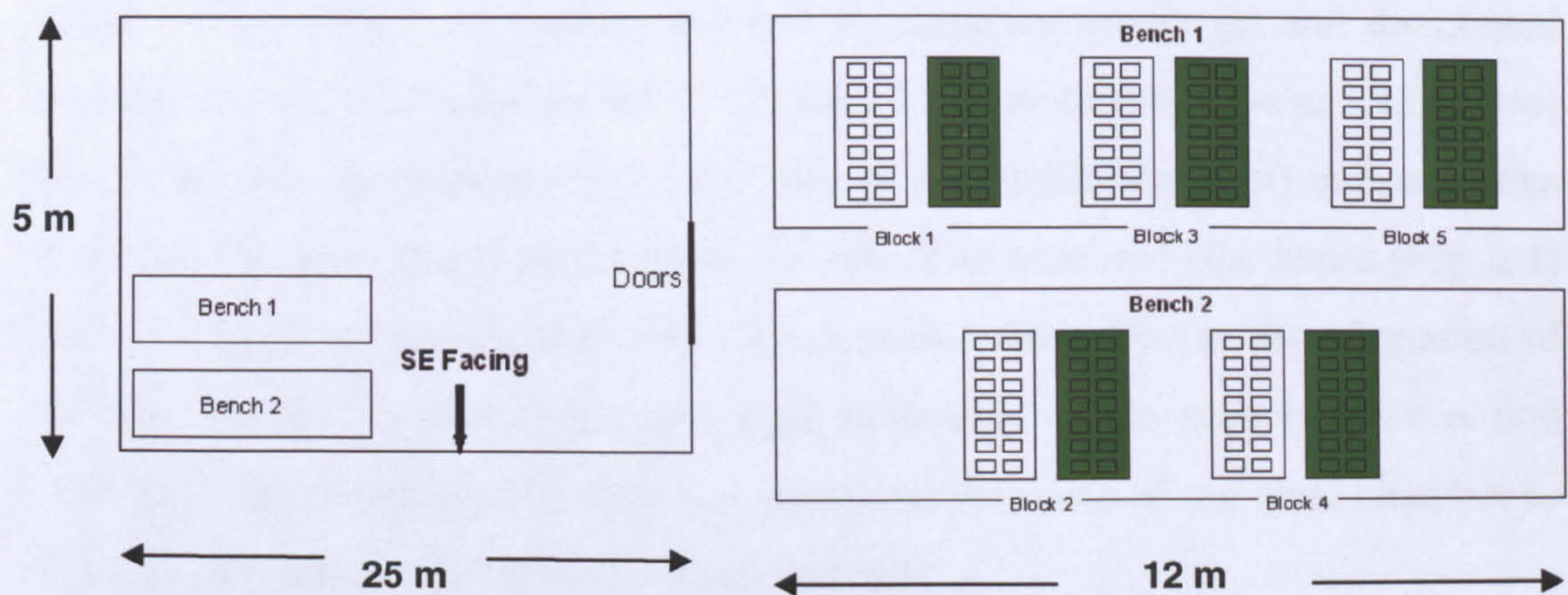


Figure 2.4 Arrangement of chambers on benches and arrangement of benches in the glasshouse.

Six seed-bed treatments were established: (i) tall moss 4 cm in height (*Thuidium tamarascinum*); (ii) short moss 0.5 cm in height (*Plagiothecium undulatum*); (iii) perennial rye grass turf (*Lolium perenne*); (iv) lawn grass seed mixture of *Lolium perenne*, *Festuca ovina* and *Festuca rubra*; (v) oak leaf litter; and (vi) bare compost. For treatments i, ii, iv, v and vi, 400 g (fresh weight, mean 200 g dry weight) of John Innes No. 3 seeding compost was placed in 15 x 20 cm seed trays. The tall growing moss species (*Thuidium tamarascinum*), 4 cm in height, was collected from a coniferous forest near Pentir, Gwynedd (53°10'45 N 4°06'24 W) and the short-growing moss (*Plagiothecium undulatum*), 0.5 cm in height, was collected from a coniferous forest near Moel y Ci, Gwynedd: (53°10'38 N, 4°06'00 W). Sections of approximately 15 x 20 cm were taken from the moss mats and placed on top of the compost. Oak leaf litter was collected from oak woodland near Penisarwaun (53°09'54 N, 4°09'55 W) and allowed to air dry and 8 g (air dry weight) was weighed out and placed on top of the compost. For the grass turf treatment (iii), 12 cm x 27 cm x 4 cm (depth) blocks of perennial rye grass were cut from a grazed upland field in Abergwyngregyn (53°13'58 N, 4°01'12 W) and transferred to 15 x 20 cm seed trays. There was no *R. ponticum* seed source within more than 1.5 km of all the sites from which the material was collected (dispersal distance of *R. ponticum* seeds: 0.02% travel more than 50 m (Stephenson *et al.*, 2007)). Two seed trays containing each of the six types of seed-bed substrate — were placed in a random position on a bench below each of ten of the light treatment chambers. Two of each

seed-bed substrates (one under each light treatment), fully watered with no seed added were included (to identify any non *R. ponticum* seedlings) and distributed between the five blocks for a total of 132 trays (120 treatments trays and 12 control trays). Chambers were arranged in pairs (one of each light treatment) in five blocks distributed between two benches on the SE side of an unheated glasshouse (Fig. 2.4) on top of capillary matting and porous black plastic sheet. Due to the orientation of the benches the position of the two light treatments within each block was non random so that the green chamber was always to the north of the clear chamber to minimise any green shading of the clear chambers.

Two watering treatments were applied to both shade treatments, one set was watered daily (WD) to field capacity, and the other was subjected to periods of drought (PD). However despite being watered daily, in summer months, some substrates such as bare soil and moss were still prone to drying out. Watering frequency for the PD treatment varied depending on environmental conditions: all PD trays were watered one day after a soil surface crust had formed in the bare soil seedbed substrate. Average weight of bare soil in a tray (based on 5 weightings) at field capacity was 400 g; at time when crust formed it was 255 g (28% soil water content); and dry weight was 200 g. Watering was carried using a watering can with a fine rose from above. Grass was trimmed when necessary to keep it at a height of no more than 5 cm.

The treatment trays were allowed two weeks to equilibrate and on 1st May 2008, 13.6 mg of *R. ponticum* seed (equal to 200 seeds based on five weightings) was scattered evenly across the substrate in each of the trays.

The trays were checked every two days for signs of emergence, and germinants were first counted 48 days after the trays were first watered. Twenty of the seedlings were marked with 3-cm plastic sticks (for monitoring of seedling survival) and the rest were gently removed (to maximise the accuracy of subsequent counts of new emergents). Measurements were taken at three-week intervals thereafter to determine subsequent germinations (all of which were removed) and seedling mortality. The most likely cause of any death was recorded, distinguishing between drought (seedlings brown and shrivelled), physical damage from water drops (seedlings

uprooted) and limitation by low light (seedling with extremely thin almost transparent wilted stem and leaves) although death by pathogens could not be ruled out. The experiment was monitored for six months when growth had ceased and no further changes in seedling mortality or germination had occurred for four weeks. To measure seedling growth, 72 surviving seedlings were harvested after six months from the bare soil and short moss substrates (combined), 36 from the clear and 36 from the green light treatments. Seedling height (cm), and root and shoot fresh weight (g) were measured. To examine the effect of competition from grass on *R. ponticum* seedling growth 36 seedlings were also harvested from the clear chamber sown grass and grass turf treatments. In the clear chamber treatment, it was not possible to compare these with the green shade treatment as there were not enough surviving seedlings to sample from. Shoot height and weight were measured but root weight was not, as it was not possible to remove the roots from the soil intact.

Experiment 2

Three different light treatments were constructed to examine the effects of simulated forest understorey light and neutral shading on *R. ponticum* seed germination and seedling survival. This was done using clear film, Lee 139 primary green filter film (Fig. 2.3) and clear film covered in a thin layer of black spray paint. Average values for maximum PPFD and R:FR of the different light treatments were measured using a Skye quantum (SKP 215) sensor and a Skye 660/730nm (SKT 110) sensor (Skye Instruments Ltd., Powys, UK). Average PPFD was 20% (SD = 3%) of ambient light under the green and neutral shade chambers and 90% under the clear chamber. The R:FR was 45% (SD = 5%) of ambient light (R:FR = 1.04) under the green film and 97% (SD = 2%) of ambient light (R:FR = 1.01) under the clear chambers.

Based on the experience from the first experiment, the following three growing media were chosen to represent the different effects of seed-bed vegetation (and its interaction with shade treatment) on seed germination and seedling survival: tall moss, sown grass and bare soil. The details of the establishment of these seed-bed treatments in the seed trays were identical to Experiment 1.

One seed tray containing each of the three types of seed-bed substrate was placed in a random position on a bench below each of 15 of the 'light treatment chambers', giving a total of 45 trays. The boxes were arranged in groups of three (one of each treatment) in five blocks distributed between two benches on the SE side of an unheated glasshouse (Fig. 2.5). Due to the orientation of the benches the position of the two light treatments within each block was non-random so that the green chamber was always to the north of the clear chamber to minimise any green shading of the clear chambers. Plants were watered daily to field capacity using a watering hose from above. The treatment trays were allowed two weeks to equilibrate and on 1st July 2008, 13.6 mg of *R. ponticum* seed (equal to 200 seeds based on five weightings) was scattered evenly across the substrate in each of the trays.

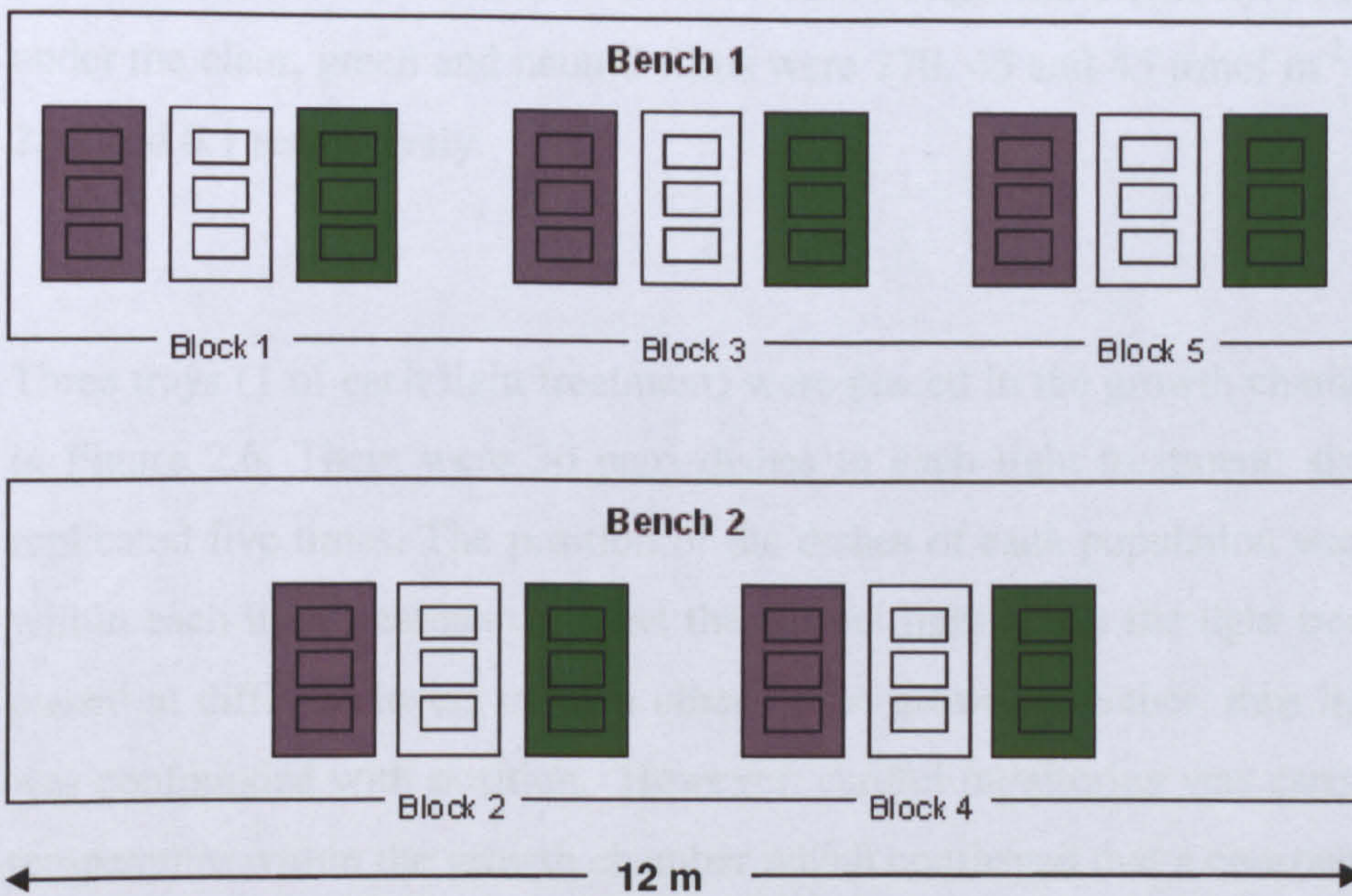


Figure 2.5 Arrangement of replicate blocks and shade boxes on the benches.

The seed trays were monitored weekly and counts were taken of new seedling emergents. Germinants emerged for the first time after 28 days. Twenty of the seedlings were marked (with plastic sticks) and the rest were gently removed (to maximise the accuracy of subsequent counts of new emergents). Measurements were taken at weekly intervals thereafter to count subsequent germinations which were removed and mortality of the marked seedlings.

Experiment 3

Germination tests were carried out in petri dishes with 20 seeds from one of the six populations placed in each petri dish. Seeds were placed on filter paper discs of 85 mm diameter in 90 mm petri dishes. The experiment was conducted in a controlled growth chamber environment with a constant temperature of 20 °C (the optimum germination temperatures for *R. ponticum* in both invasive and native populations have been found to be in the range 16 to 23 °C (Erfmeier & Bruelheide, 2005)). The seeds were subjected to three different light treatments - clear film, green plastic film and clear film covered in a thin layer of black spray paint. The light environment under each of the films was determined using a Skye PAR quantum (SKP 215) sensor to measure photosynthetic photon flux density (PPFD) and a Skye 660/730nm (SKT 110) sensor to measure the red to far red light ratio (R:FR). PPFD and R:FR under the clear, green and neutral films were 270, 45 and 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 2.42, 2.42 and 0.1 respectively.

Three trays (1 of each light treatment) were placed in the growth chamber as shown in Figure 2.6. There were 30 petri dishes in each light treatment: six populations replicated five times. The position of the dishes of each population was randomised within each light treatment. To get the correct light levels the light treatments were placed at different levels to each other in the growth chamber, thus light treatment was confounded with position. However, careful monitoring was carried out of the temperature within the growth chamber which confirmed that a constant temperature of 20 °C was maintained throughout, minimising the risk that this was a confounding error. A seed was considered to have germinated when the radicle had emerged. Dishes were checked every second day for new germinants. All the dishes were carefully watered using a dropper pipette every second day with de-ionised water and with 50% ethanol solution once a week to prevent infection by mildew.

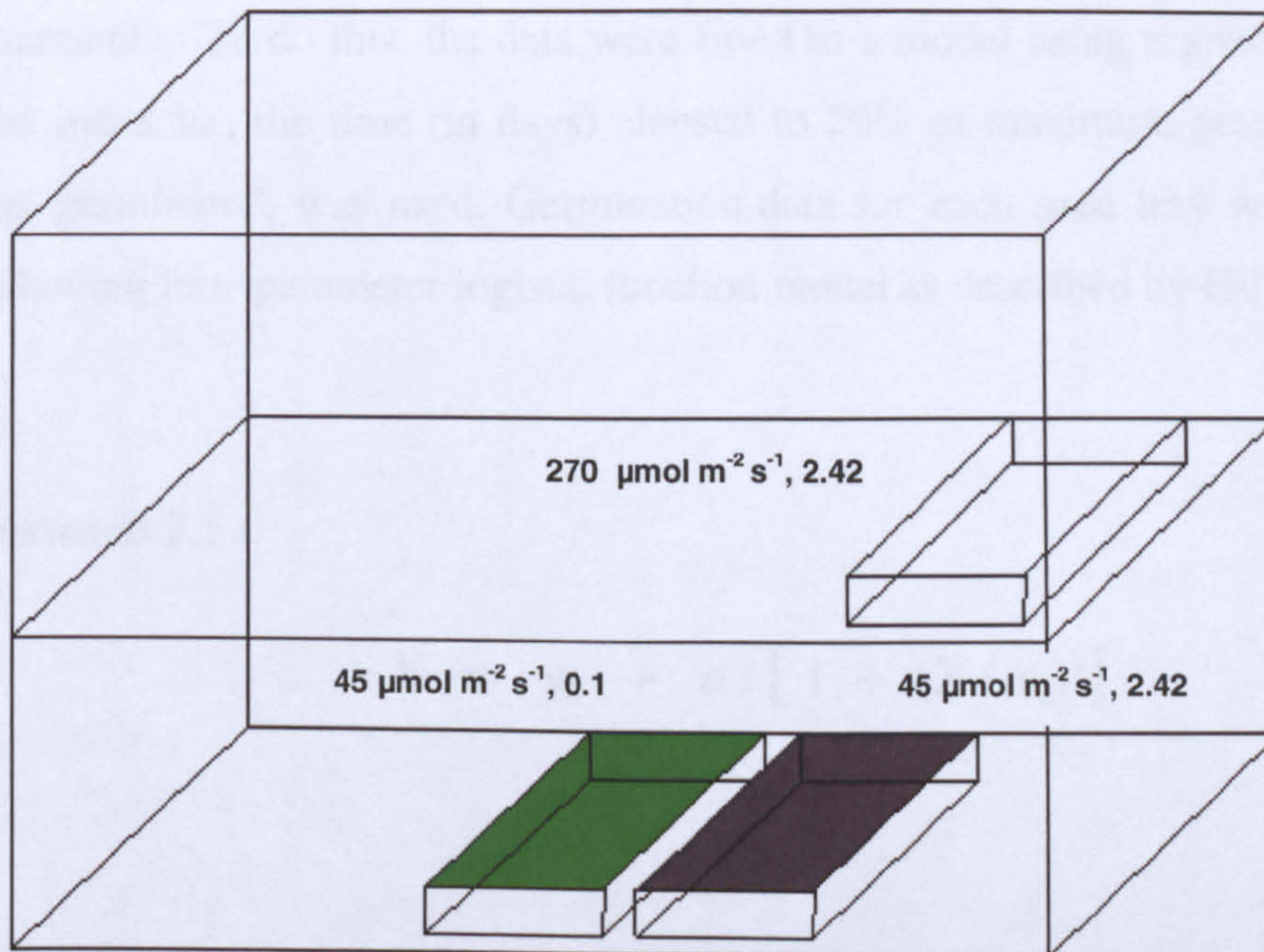


Figure 2.6 Experimental set up in growth chamber, 6 populations x 5 reps = 30 Petri dishes arranged within each light treatment. Numbers represent the levels of PAR ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and R:FR.

2.5 STATISTICAL ANALYSES

Experiments 1 & 2

Germination capacity was expressed both as a cumulative percent germination (seeds germinated/ total seeds sown) and as germination rate (velocity of germination over time). Residuals of data were plotted (SPSS V17.0, 2008) for a visual inspection of normality and the data were found to be normally distributed. Univariate analysis of variance was carried out to test for significant differences in the effects of treatment: habitat (wet vs. dry; light v shade), seed-bed substrate, and habitat \times substrate (microhabitat) on each of the two measures of germination capacity. The substrate effect directly tested the hypothesis that more seeds germinate on low-growing moss than on the other substrate types. The interaction term between substrate and watering regime was included to verify that this effect was not mediated by soil moisture.

In calculating the dependent variable of germination rate, it was important to consider the potential variation in the time course of germination in each of the

treatments. To do this, the data were fitted to a model using regression procedures. The index $t_{1/2}$, the time (in days) elapsed to 50% of maximum percentage of seeds that germinated, was used. Germination data for each seed tray were fitted to the following four-parameter logistic function model as described by Hill *et al.* (2005):

Equation 2.1 :

$$Y = y_0 + a / [1 + (X / x_0)^b]$$

where X = any given day from 0 to 147, Y = the relative germination for Day X , y_0 = the predicted y intercept; a = maximum germination, x_0 is the calculated time in days necessary to achieve one-half of a , and b = the calculated slope at $1/2 a$. Independent y_0 , x_0 , a , and b values were calculated from the logistic function for each tray. Analysis of variance was also used to examine the treatment effects on the logistical parameter, b . Seedling survival was the total number of seedlings remaining at the final recording (6 months) expressed as a percentage of the number of germinants. The data were normally distributed so ANOVA was carried out to test for significant differences in seedling survival amongst the various treatments.

Some trays experienced no germination at all and therefore rates of germination or seedling survival could not be included for them (hence reduced degrees of freedom in ANOVA error for germination rate and seedling survival).

Experiment 3

Analysis of data (germination capacity, germination rate) was carried out in the same way as for experiments 1 and 2. The light treatments were applied to different parts of the growth chamber so light treatment was confounded with position and therefore, while statistical comparison between the two is valid, the interpretation must acknowledge that any differences cannot be attributed with certainty to light treatment rather than position.

2.6 RESULTS

Experiment 1

2.6.1 The effect of substrate on seed germination and seedling survival

After 21 weeks of treatment exposure, germination and seedling survival of *R. ponticum* was strongly affected by light regime, water availability and seed-bed substrate (Fig. 2.7 – 2.9, Table 2.1). Emergence varied amongst the different substrates with the highest final percentage seed germination occurring in sown lawn grass and bare soil (Fig. 2.7a). Analysis of percentage germination data with the Tukey HSD Post Hoc test, subdivided the substrates into three groups that were significantly different from each other ($p < 0.001$) based on percentage germination: the first comprising sown grass (27.5%) and bare soil (27.5%); the second, short moss (19.8%) and leaf litter (15.3%); and the third, grass turf (8.6%) and tall moss (7.2%). There was little difference in time elapsed to 50% maximum germination in the various substrates. Time ranged from 34 to 37 days for all but one of the substrates (Fig. 2.7b; r^2 values for all logistic regression between 0.9900 and 0.9999); $t_{1/2}$ in the grass turf was significantly longer ($p < 0.001$) at 45 days though this was most likely due to the difficulty in identifying newly germinated seedlings in the thick grass.

There was a large significant ($p < 0.001$) effect of substrate on seedling survival with the highest percentage surviving in the short moss, sown grass and bare soil (Fig. 2.7a). Seedling mortality was highest in tall moss, leaf litter and grass turf (Fig. 2.7a). Many of the dead seedlings in short moss, bare soil and, to some extent, tall moss had a brown and shrivelled appearance which appeared to indicate that they had died of drought. However, this interpretation is not supported by the highly insignificant effect of the watering treatment, or the (marginally) insignificant interaction of watering and substrate. The other major cause of death in bare soil was physical uprooting, presumably by water drops. Inspection of the seedlings that died in leaf litter and tall moss indicated that physical obstruction to seedling roots reaching the soil was a major cause of mortality. The appearance of the seedlings that died in sown grass, grass turf and leaf litter indicated that low light levels were

the major cause of mortality (the dead or dying seedlings were characterised by extremely thin etiolated, almost transparent wilted stems and leaves), although death by pathogens cannot be discounted.

Table 2.1 Univariate analysis of variance on the effect of light treatment (green shade v no-shade); watering regime (daily watering v periodic droughting) and seedbed substrate (bare soil, lawn grass, short moss, grass turf, leaf litter, tall moss) on various aspects of *Rhododendron ponticum* seedling establishment: germination capacity (cumulative seeds germinated / total seeds sown (%)); germination rate ($t_{1/2}$ = time taken for half of the seeds to germinate); and seedling survival, after 21 weeks in an unheated greenhouse. See Appendix 2.1–2.3 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
<i>Germination capacity</i>			
Substrate	7900.642	5	0.000
Light	56.033	1	0.425
Water	1562.408	1	0.000
Block	1435.571	4	0.004
Substrate x Light	7876.967	5	0.000
Substrate x Water	947.892	5	0.064
Light x Water	80.033	1	0.340
Substrate x Light x Water	313.417	5	0.611
<i>Germination rate</i>			
Substrate	1699.676	5	0.000
Light	286.934	1	0.019
Water	942.995	1	0.000
Block	346.360	4	0.153
Substrate x Light	930.850	5	0.004
Substrate x Water	745.868	5	0.016
Light x Water	40.162	1	0.374
Substrate x Light x Water	621.148	5	0.039
<i>Seedling survival</i>			
Substrate	60009.510	5	0.000
Light	17461.343	1	0.000
Water	15.005	1	0.848
Block	1344.416	4	0.511
Substrate x Light	20827.970	5	0.000
Substrate x Water	4400.597	5	0.065
Light x Water	245.315	1	0.439
Substrate x Light x Water	644.483	5	0.901

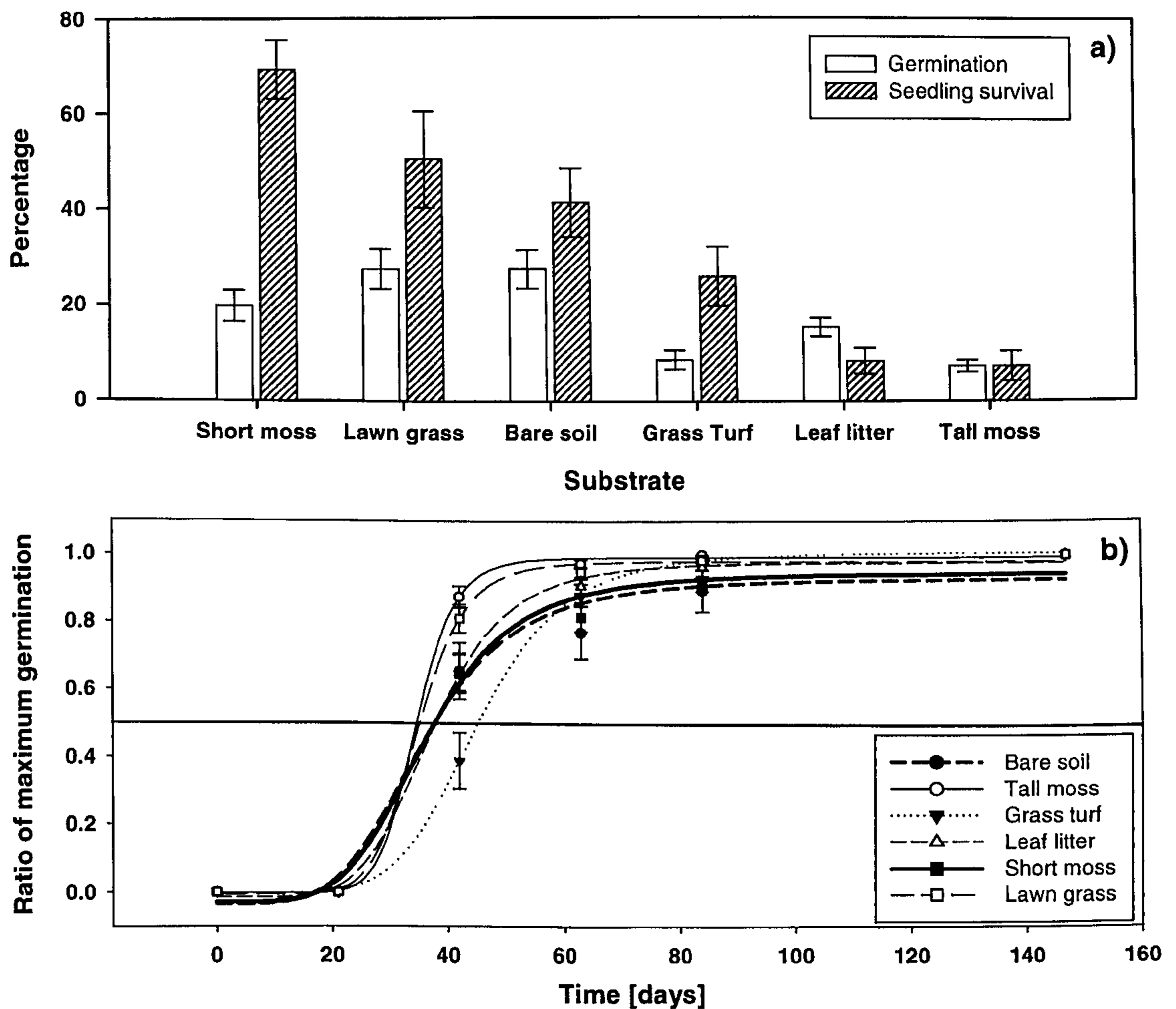


Figure 2.7 The effect of substrate on establishment of *Rhododendron ponticum* seedlings: a) the effect on final germination (expressed as a percent of total seed sown) and seedling survival at 21 weeks after sowing seed; b) the effect on germination rate (expressed as a ratio of the maximum germination). Curves were fitted using a logistic 4 parameter function model (Hill *et al.*, 2005), see Equation 2.1. Vertical bars are standard errors of the mean.

2.6.2 The effect of light on seed germination and seedling survival

Although there was no significant overall effect of green shade on final percentage germination (mean clear: 18 % (SEM = 2); green: 17 % (SEM = 2)) there was a highly significant interaction effect of light and substrate ($p < 0.001$). The response of both measures of germination to light treatment was highly dependent upon the seedbed substrate. A significantly higher final percentage germination was observed in bare soil and short moss under the simulated green canopy than under their no-shade counterparts (Fig. 2.8a). However, green shading had a significantly negative effect on germination success on other substrates: sown grass, field grass and leaf litter rates reduced from 39.4, 19.7 and 15.8% under the clear chamber to 15.6, 11

and 1.4%, respectively under green shade (Fig. 2.8a). The shape of the cumulative germination curves under the two light regimes indicates that in both the majority of seeds completed germination over a relatively short time period; however, this was significantly ($p = 0.019$) accelerated by the green shade treatment (Fig. 2.8 b). Average time ($t_{1/2}$) taken for half of the seeds to germinate with no shade was 41 days (SEM = 1 day) compared to 37 days (SEM = 1day) under the simulated green shade canopy.

Green shade significantly ($p < 0.001$) reduced seedling survival from a mean of 43.9% (SEM = 4.5%) under the clear film to chamber 21.5% (SEM = 4.3%) under the green shade). This effect showed a strong interaction ($p < 0.001$) with seedbed substrate. The deleterious effect of green shade was very evident in the grass turf, sown lawn grass, leaf litter and tall moss substrates but not on bare soil or short moss (Figure 2.8c). The lowest levels of seedling survival under green shade were in the grass turf, leaf litter, tall moss and sown grass substrates with 0, 1, 2 and 11% surviving, respectively. This increased to 44, 16, 12 and 90%, respectively for seedlings grown under no shade.

2.6.3 The effect of periodic droughting on seed germination and seedling survival

Daily watering significantly increased final percentage germination over droughting (from 14% to 21%) and reduced $t_{1/2}$ germination (both $p < 0.001$). This direction of watering effect occurred for all of the substrate types except for the grass turf (Fig. 2.9a), and was significant in bare soil, short moss and tall moss. There was a significant effect of watering treatment ($p < 0.001$) on time taken for half of the seeds to germinate ($t_{1/2}$) when they were watered daily was 36 days (SEM = 1 day) compared with 42 days (SEM = 1 day) for those that were subjected to periods of drought (Fig. 2.9b). There was no overall effect of periodic droughting on seedling survival, though there was a nearly significant ($p = 0.065$) interaction with substrate type; it was notable that survival was higher for seedlings grown in bare soil that were watered daily, whereas in grass turf and tall moss periodic droughting appeared to produce higher seedling survival than daily watering conditions (Fig. 2.9c).

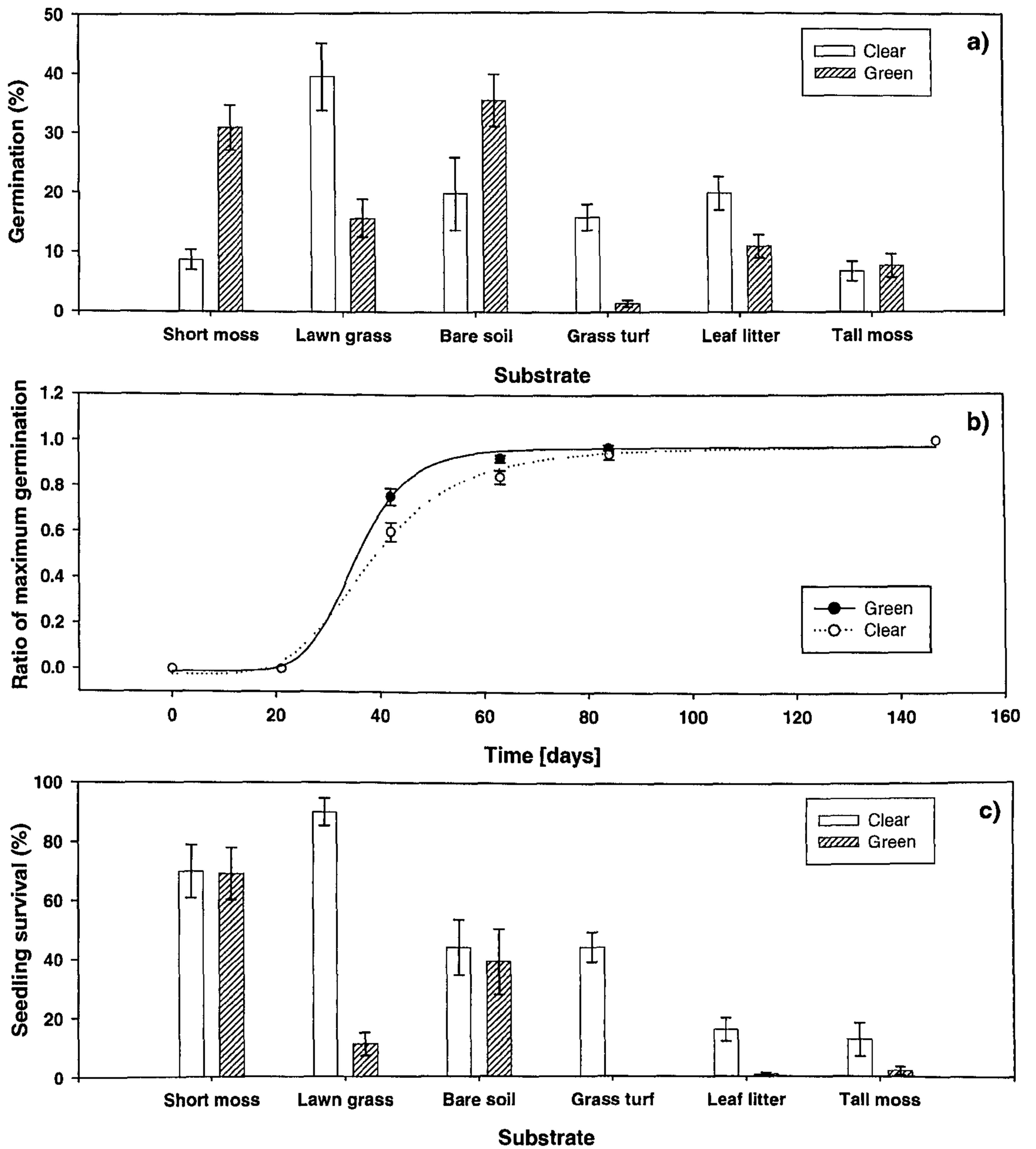


Figure 2.8 The effect of green and clear light on establishment of *Rhododendron ponticum* seedlings. a) The effect of the interaction of light and substrate on final germination (expressed as a percent of total seed sown); b) the effect of shade treatment on time taken for half the seeds to germinate ($t_{1/2}$), curves were fitted using a logistic 4 parameter function model (Hill *et al.*, 2005), see Equation 2.1; and c) the effect of the interaction of light and substrate on seedling survival percentage. Vertical bars are standard errors of the mean.

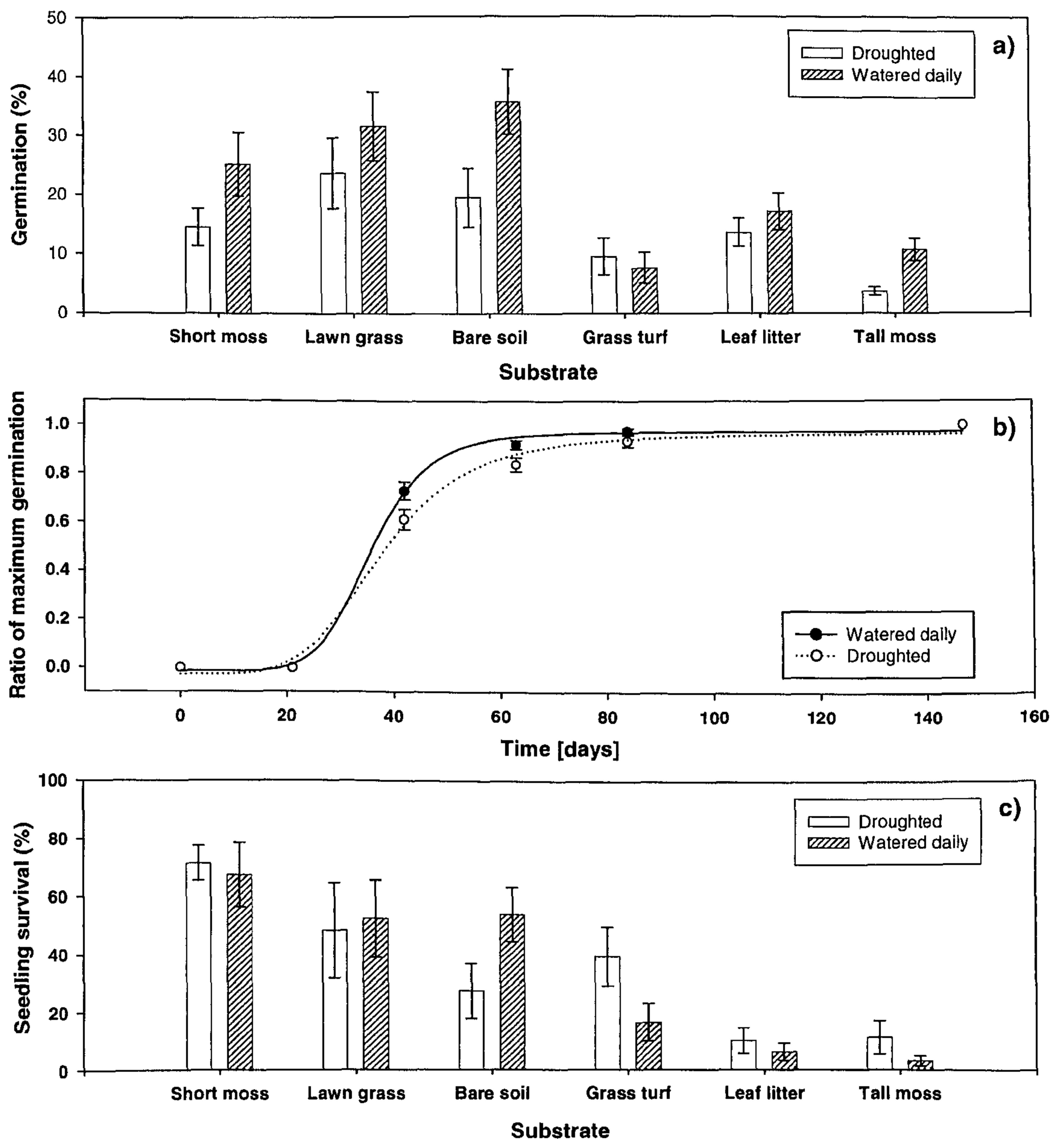


Figure 2.9 The effect of watering treatment with different substrate types on: a) final germination (expressed as a percent of total seed sown); b) time taken for half the seeds to germinate ($t_{1/2}$), curves were fitted using a logistic 4 parameter function model (Hill *et al.*, 2005), see Equation 2.1; and c) seedling survival (%) of *Rhododendron ponticum* 21 weeks after sowing seed. Vertical bars are standard errors of the mean.

2.6.4 Seedling height growth and root to shoot ratio

At harvest, after six months the height of seedlings in the bare soil and short moss substrates combined (no significant difference between those two seedbed substrates) was significantly increased ($p < 0.001$) by the green shade treatment from 1.9 cm (SEM = 0.1) to 2.8 cm (SEM = 0.1). However, the green shade significantly reduced both the shoot and root biomass of the seedlings ($p = 0.003$ and $p < 0.001$ respectively, Table 2.2). The weight of shoots and roots was 24.8 mg and 1.4 mg respectively under the green shade compared with 56.6 mg and 22.5 mg respectively under the no-shade chamber (Fig. 2.10 a). Root and shoot weight under each light regime were related by a fitted linear regression (Fig. 2.10b) defined by the following equation.

Equation 2.2:

$$f = y_0 + ax$$

Where f = root weight, x = shoot weight and a = estimated constant representing the root to shoot ratio. The root:shoot ratio for seedlings grown under green shade (0.044) was significantly lower than under the clear chamber (0.423) ($p < 0.001$, Table 2.2). However, seedling shoot biomass was much lower under clear in sown grass and grass turf than in bare soil and short moss ($p < 0.001$, Table 2.2). Average shoot weight (Fig. 2.10c) and height (Fig. 2.10d) were both significantly ($p < 0.001$) higher in bare soil and short moss than in the grass seedbeds (Table 2.3).

Table 2.2 Results of one-way analysis of variance tests on the effect of light treatment (green versus clear shade) on *Rhododendron ponticum* seedling growth during the first six months after seed sowing in bare soil/short growing moss. See Appendix 2.4 for full ANOVA.

Source of variation	Sum of Squares	df	p
Height	13.3477	1	0.000
Shoot	0.018	1	0.003
Root	0.008	1	0.000
Root:Shoot	2.596	1	0.000

Table 2.3 Results of one-way analysis of variance tests on the effect of light treatment and seedbed substrate (bare soil/short moss v lawn/turf grass) on *Rhododendron ponticum* seedling height and shoot weight during the first six months under no shade. See Appendix 2.5 & 2.6 for full ANOVA.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Seedling height	7.933	1	7.933	43.274	0.000
Seeding shoot weight	0.052	1	0.051670	31.111	0.000

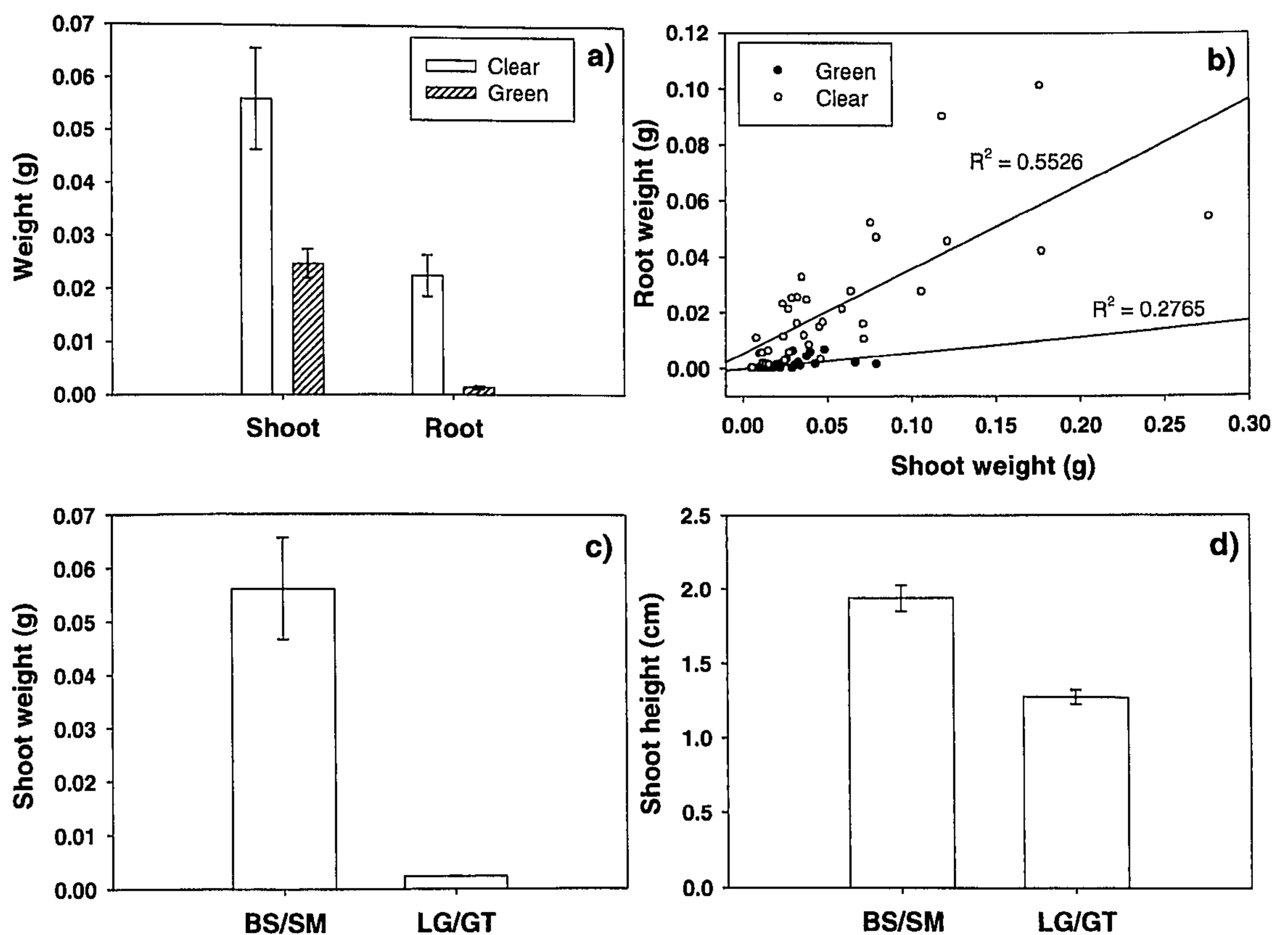


Figure 2.10 Effect of light treatment on growth of *Rhododendron ponticum* seedlings at six months after seed was sown in different seedbed substrates. The effect of green/no-shade on a) root and shoot biomass; b) linear regression of root versus shoot weight. Effect of substrate type on growth of *R. ponticum* seedlings at six months under clear chamber: c) shoot weight; d) shoot height. BS/SM = bare soil/short moss; LG/GT = lawn grass/grass turf. Vertical bars are standard errors of the mean.

Experiment 2

2.6.5 The effect of substrate on germination and seedling survival

Emergence started 28 days after the seeds were sown. The final percentage germination at 12 weeks differed significantly amongst the substrates ($p < 0.001$, Table 2.4) being highest in bare soil (36.9%) followed by sown lawn grass (26.3%) with tall moss being lowest (4.5%). These differences between substrates appeared soon after first emergence and remained consistently (Fig. 2.11a). There was a difference amongst the substrates in $t_{1/2}$ for germination: bare soil, tall moss and sown grass being 21, 23 and 31 days respectively (Fig. 2.11b; r^2 values between 0.9897 and 0.9992). Substrate had a more marginally significant effect ($p = 0.029$, Table 2.4) on seedling survival with the highest survival in sown grass (44%, SEM = 9.8), followed by bare soil (28.6%, SEM = 6.9) and tall moss (18.6%, SEM = 5.1).

Table 2.4 Univariate analysis of variance on the effect of light treatment (green, neutral and clear) and seedbed substrate (bare soil, sown lawn grass and tall moss) on: germination capacity (cumulative seeds germinated / total seeds sown (%)); germination rate ($t_{1/2}$ = time taken for half of the seeds to germinate); and seedling survival of *Rhododendron ponticum* seeds, 12 weeks after sowing in an unheated glasshouse. See Appendix 2.7-2.9 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
<i>Germination capacity</i>			
Substrate	8201.144	2	0.000
Light	1610.811	2	0.002
Block	1052.867	4	0.066
Substrate x Light	1650.622	4	0.012
<i>Germination rate</i>			
Substrate	163.808	2	0.094
Light	203.929	2	0.056
Block	480.429	4	0.014
Substrate x Light	172.458	4	0.274
<i>Seedling survival</i>			
Substrate	4685.302	2	0.029
Light	7613.274	2	0.005
Block	4030.307	4	0.171
Substrate x Light	3986.177	4	0.176

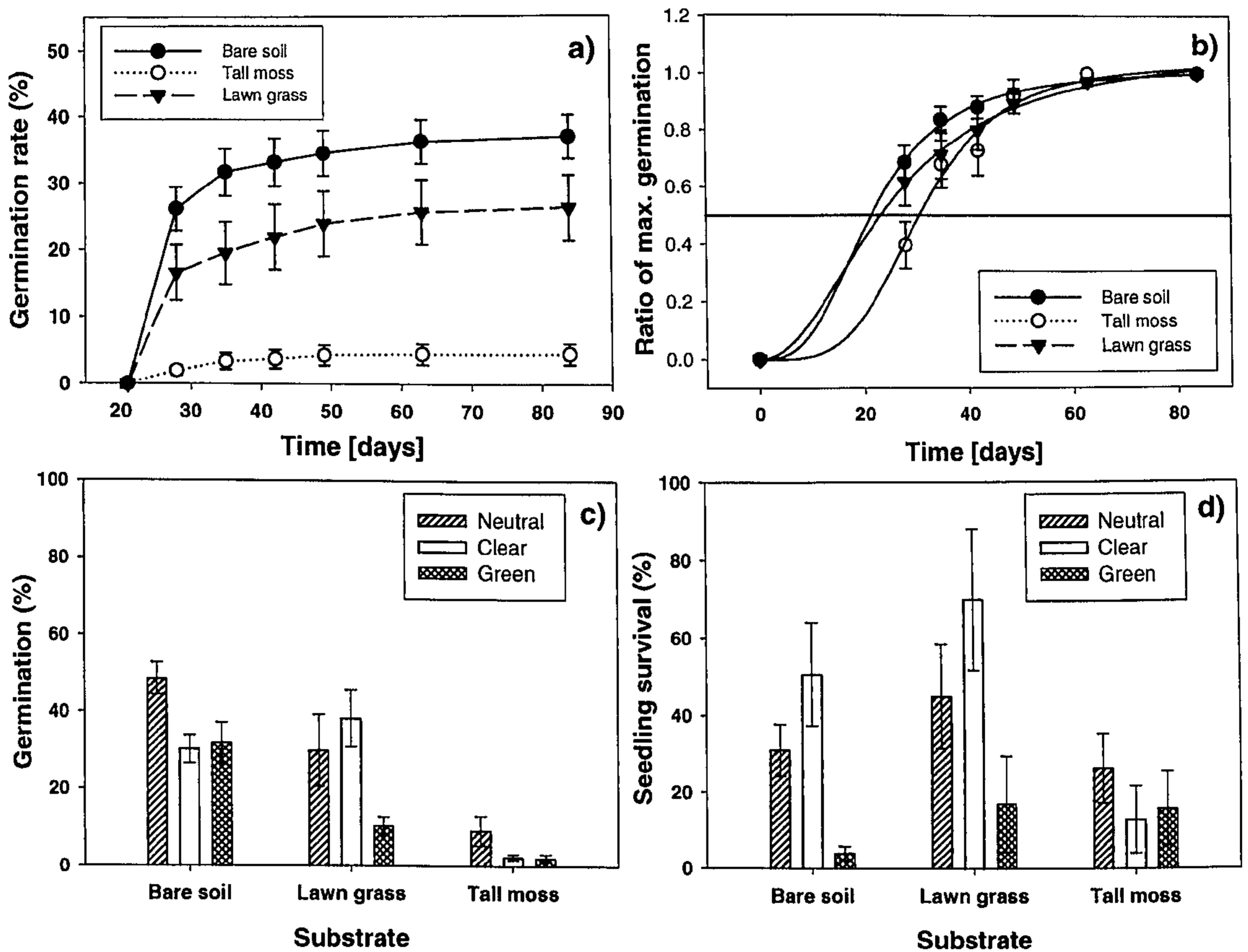


Figure 2.11 The effect of light regime and substrate on establishment of *Rhododendron ponticum* seedlings: a) main effects of substrate on cumulative germination rate over 12 weeks after sowing; b) the effect of substrate on time taken for half the seeds to germinate ($t_{1/2}$), curves were fitted using a logistic 4 parameter function model (Hill *et al.*, 2005), the r^2 values were 0.992, 0.9982 and 0.9897 for bare soil, tall moss and lawn grass, respectively; c) total mean percentage germination (expressed as a percent of total seed sown); and d) seedling survival (%) 12 weeks after sowing. Vertical bars are standard errors of the mean.

2.6.6 The effect of light on germination and seedling survival

Germination capacity was highest under neutral shade (29.3% (SEM = 4.9%)), intermediate under no shade (23.7% (SEM = 5.5%)) and lowest under green shade (14.8% (SEM = 3.8%)), the difference being highly significant ($p < 0.001$, Table 2.4). As in experiment 1, the difference in germination under the clear and green chambers was not consistent amongst all the substrate treatments; however, analysis with the Tukey HSD Post Hoc test, revealed that germination under the neutral shade was significantly ($p = 0.002$) higher than under the green shade for all three substrates. There was again a significant interaction of light and substrate on germination capacity ($p = 0.012$, Table 2.4), with the trend between light treatments being different for the sown grass substrate (where germination capacity was highest

under the clear canopy) than for the other two. The green shade treatment had a similar effect on germination rate in these substrates to the experiment 1 daily watering treatment: reducing it in sown grass, with no effect in tall moss or bare soil. There was a marginally insignificant effect ($p = 0.056$) of light on germination rate ($t_{1/2}$).

The effects of the shade treatments on seedling survival were different: the mean rates were highest under no shade (46.8%) intermediate under neutral shade (34.7%) but again lowest under green shade (11.7%). This effect of light treatment was quite significant ($p = 0.005$, Table 2.4); however, standard errors were high within substrate treatments and there was no significant interaction between light and substrate. The most common cause of death inferred from observations of the seedlings was light levels in the sown grass; the dead or dying seedlings were characterised by extremely thin etiolated, almost transparent wilted stems and leaves. The major cause of death in bare soil was observed to be physical uprooting by watering.

Experiment 3

Across the six populations the light treatment had a highly significant effect on final germination rate ($P < 0.001$, Table 5), and this was entirely attributable to the large reduction in germination rate under the green shade (from 75% to 18%, Fig. 2.12a). Green shade did not noticeably delay the time to first germination (nine days after sowing), but it did reduce the rate from this time onwards (Fig. 2.12b; r^2 values between 0.9918 and 0.9943). The germination $t_{1/2}$ under green shade (20 days) was twice as long as under both no shade and neutral shade (10 days). There was no significant difference in final germination rate between the seed from the six populations across the three light treatments (Table 2.5); the mean rates of the populations covered a narrow range from 53% to 60% (Fig. 2.12c). However, there was a strong interaction between light treatment and population ($p = 0.003$, Table 2.5). The most notable component of this was that the populations with the lowest germination rates under no shade and neutral shade (IKIL and SCB839, from Ireland and Scotland, respectively) had the highest rates under green shade (Fig. 2.12f).

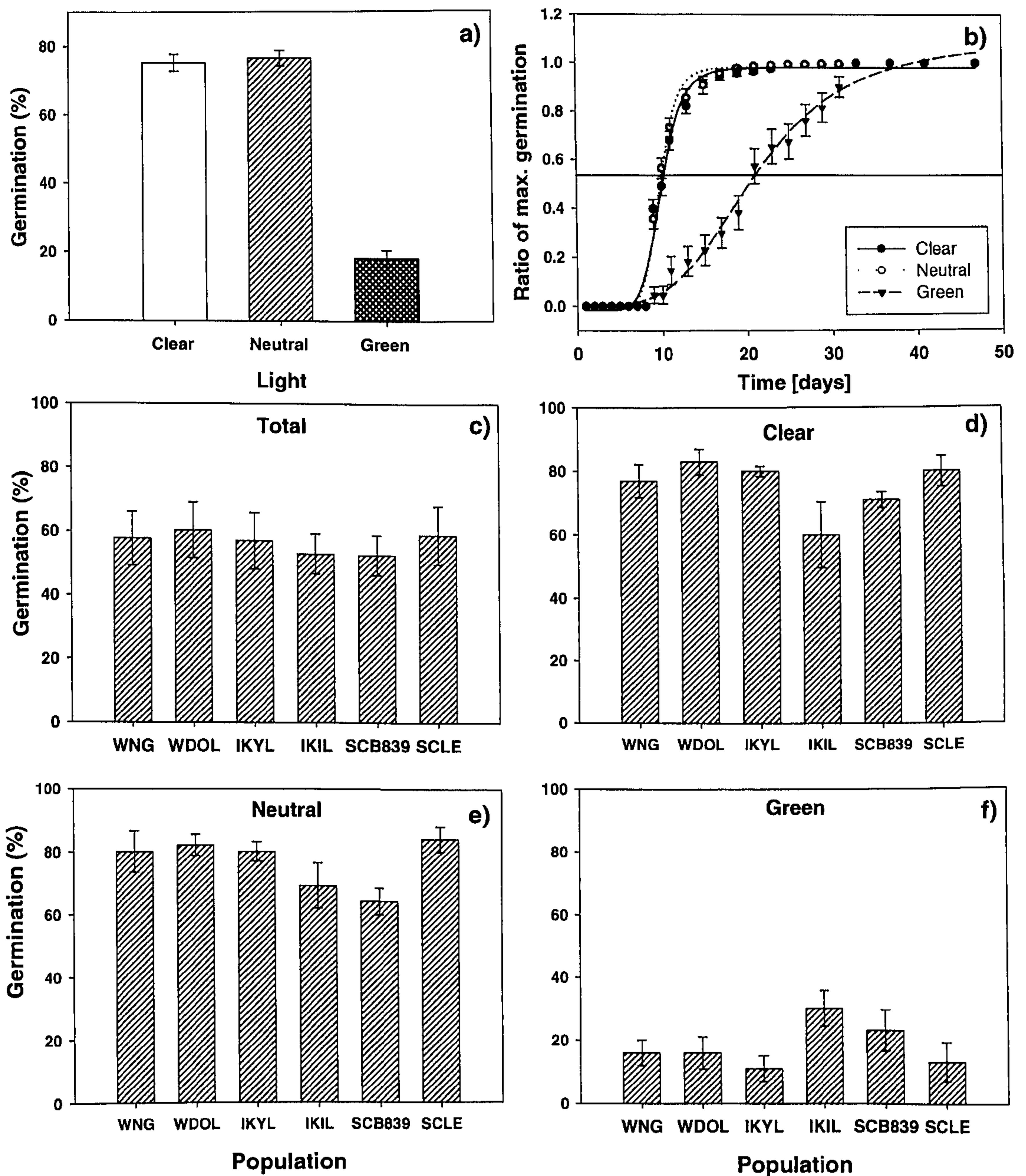


Figure 2.12 Effect of neutral and green shade on germination rate over seven weeks of seeds from six populations of *Rhododendron ponticum* sown on filter paper in petri dishes under three different light treatments in a growth chamber. a) Mean final percentage germination rate under the three light treatments (clear - high PAR, high R:FR; neutral - low PAR, high R:FR; green - low PAR, low R:FR); b) mean rate of germination over time under the three light treatments (a regression line is fitted for each using the formula of Hill *et al.* (2005), the r^2 values were 0.9920, 0.9942 and 0.9940 for clear, neutral and green, respectively); Final germination rates of seeds from the six populations - Wales (Nant Gwynant (WNG), Dolgellau (WDOL)), Ireland (Kylemore Abbey (IKYL), Kilbride (IKIL)) and Scotland (B839 near Drimsynie (SCB839), Loch Eck (SCLE)) - under light with c) a photosynthetic photon flux density (PPFD) of $270\mu\text{molm}^{-2}\text{s}^{-1}$ and red to far red ratio (R:FR) of 2.42 (no shade/clear); d) light with a PPFD of $45\mu\text{molm}^{-2}\text{s}^{-1}$ and R:FR of 2.42 (neutral shade); and e) under light with a PPFD of $45\mu\text{molm}^{-2}\text{s}^{-1}$ and R:FR of 0.1 (green shade); f) Mean final germination rates of seeds from the six populations (vertical bars are \pm SEM). Vertical bars are standard errors of the mean.

Table 2.5 Univariate analysis of variance on the effect of light (clear - high PAR, high R:FR; neutral – low PAR, high R:FR; green – low PAR, low R:FR) and provenance (six populations) on germination rate over seven weeks of seeds from of *Rhododendron ponticum* sown on filter paper in Petri dishes in a growth chamber.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Light	66535.556	2	33267.778	238.574	0.000
Population	741.389	5	148.278	1.063	0.388
Light * Population	3924.444	10	392.444	2.814	0.005
Error	10040.000	72	139.444		
Total	81241.389	89			

2.7 DISCUSSION

The main effects of the treatments tested in this quantitative experimental study are largely in agreement with previous research on *R. ponticum*. However, the striking results of the interaction between seed-bed substrate and light, and between seed provenance and light are new. This study has also provided strong evidence of the relative contribution of light level and R:FR spectral composition in mediating the effect of shade on *R. ponticum* establishment. The quantification of the relative effect of different substrate types in combination with different shade levels on *R. ponticum* germination and seedling survival is particularly useful in informing management techniques and prediction of the future range expansion of this invasive species.

All three experimental variables, light, moisture and substrate, together with some of the interactions between them, were found to have a significant effect on germination and seedling survival, both of which were highest in the short moss, bare soil and lawn grass substrates and lowest in grass turf, leaf litter and tall moss in Experiment 1. Combining both components of the process of seedling establishment, short moss was the most suitable substrate. An increasing number of studies deal with the role of bryophytes in plant communities and how their presence affects the germination, establishment or growth of vascular plants (Keizer *et al.*, 1985; Malmer *et al.*, 1994; Zackrisson *et al.*, 1997). The nature of the relationship, that is whether it is facilitative or inhibitive, depends on various factors, such as carpet depth (Zamfir,

2000; Sedia & Ehrenfeld, 2003; Ijima *et al.*, 2004), environment, and presence of a vegetative canopy, all of which effect light and moisture availability (Delach & Kimmerer, 2002; Groeneveld *et al.*, 2007; Jeschke & Kiehl, 2008). In this study, the presence of a thick moss layer (*Thuidium tamarascinum*) reduced emergence considerably: both seed germination and seedling survival were much higher on the short moss mat (*Plagiothecium undulatum*) than the tall moss mat. Although thin moss layers have been found to facilitate vascular plant establishment (Sedia & Ehrenfeld, 2003, Dovciak *et al.*, 2008), thick layers can exert negative effects on germination and establishment through physical interference and shading in thick, densely growing mats (Jeschke & Kiehl, 2008). According to Cross (1981) recruitment of *R. ponticum* seedlings in the field is dependent on the occurrence of thin moss carpets (< 1cm), which is also consistent with these results. Seedlings in the field were generally not observed in tall moss carpets with the exception of some *Sphagnum* spp. (Cross, 1981, Jackson, 2008), which could be explained by the high water retaining capacities of these species.

Despite daily watering in Experiment 1, both moss carpets were susceptible to drying out rapidly on hot days in the no-shade light treatment. *Rhododendron ponticum* germinated less in thin moss than in bare soil under the no-shade treatment, whereas in the green shade treatment germination in thin moss was not different from bare soil. The presence of a simulated canopy above the bare soil and short moss carpet seedbed substrates, however, greatly increased germination. Canopy interception of incident radiation significantly reduces soil evaporation (Lambers *et al.*, 2008). Thus, in periods of extreme drought even a thin moss cover may inhibit rather than facilitate germination and in the absence of a shade canopy, moss carpets are highly vulnerable to desiccation with the effect of reducing germination. However, light or watering treatment had no significant effect on seedling survival in a thin layer of moss whereas periods of drought did reduce survival in bare soil. Conditions affecting survival of seedlings were therefore different from those affecting seed germination. Various studies have reported higher vascular plant seed germination on bare soil than in bryophyte layers (e.g. During & van Tooren, 1990; Zamfir, 2000). For example, a study into the germination of four species of grass found that a biological soil crust dominated by short mosses had a negative effect on seed water status and significantly reduced seed germination (Serpe *et al.*, 2006). Although germination and early seedling development were

highly affected by moisture, this dependence lessened over time as seedlings became rooted in the soil beneath the thin moss layer. This was not the case in bare soil, where the negative effect of periodic droughting continued from germination to seedling survival. The results suggest that as seedlings became rooted in the soil beneath the thin moss layer, a greater amount of soil moisture becomes available to them and the bryophyte layer may have offered physical protection. The survival rates of seedlings and juvenile plants have been found to be significantly higher in moss patches than on bare soil (During & van Tooren, 1990, Kameyama *et al.*, 1999). Safe sites for seedlings of *Rhododendron metternichii* var. *hondoense* were determined to be highly dependent on ground conditions with extremely low seedling density on bare soil but high density on moss mats either in the open or under a deciduous canopy (Kameyama *et al.*, 1999). In another related species, *Rhododendron hodgsonii*, at early life stages seedlings were found on all microsites but over time there was an increase in the number of seedlings found on bryophyte mats and a decrease on all other microsites (Gratzer & Rai, 2004). This could explain the occurrence of *R. ponticum* seedlings on thin layers of moss carpets and their absence from bare soil in field studies (Cross, 1981, Rotherham, 1986). This highlights the vulnerability of seedlings to desiccation and/or seedlings to uprooting by water drops. Cross (1981) noted the absence of *R. ponticum* seedlings on bare soil, which considered to be most likely due to physical destruction by water drops, trampling, or drying out. Cross (1981) noted that *R. ponticum* seedlings on bare soil were confined to moister sites or linked with loose boulders, rocky outcrops and breaks in slope. These results help in explaining the distribution of *R. ponticum* seedlings in the field (Cross, 1981; Stephenson *et al.*, 2006) on thin moss carpets under shaded conditions and further support the overall positive effects of moss as a seedbed (McLaren & Janke, 1996; Parker *et al.*, 1997; Su *et al.*, 2009).

The literature gives a clear indication of very slow height growth rates of *R. ponticum* seedlings in the field, e.g. Cross (1981) reports average heights for 1, 2, and 3 year old seedlings as 0.8 +/- 0.2, 1.3 +/- 0.4 and 2.3 +/- 0.8 cm respectively. However, in the present study (under mesic (though unheated) glasshouse conditions), mean seedling height of 6-month-old seedlings was 2.3 +/- 0.2 cm. This indicates that very slow height growth rates are not inherent to *R. ponticum* but do very much depend on microclimate conditions (or perhaps effects of herbivores or pathogens).

Rhododendron ponticum seeds, which are typically 2 mm in length (average 0.068 mg), have previously been shown to require light for germination (Cross, 1975) but to date no quantitative studies on the effect of different types of light on germination and seedling development have been reported. The current work demonstrated that spectral quality had a profound effect on *R. ponticum* germination rates, seedling mortality and development. Germination in Experiment 1 of *R. ponticum* under the simulated green canopy was higher than in the 'open' on bare soil and short moss (but the opposite trend with shade was seen on most of the substrates with taller vegetation or leaf litter). On bare soil and short moss there was no difference in seedling survival between the green shade and no shade treatments but seedlings grown under no shade had a significantly greater root and shoot biomass. In contrast green shade greatly reduced seedling survival on all of the other substrate types. Low levels of incident light under a canopy are widely known to reduce seedling growth and development (e.g. Thompson & Harper, 1988; Holt, 1995).

The interaction between the effect of green shade and the substrate type described above is very striking. It indicates that additional canopy shade is very detrimental to *R. ponticum* seedling establishment when already shaded (or subject to other interference) by ground vegetation or litter. The second experiment allowed the mechanism of this interaction to be investigated further. Any beneficial effect of shade for *R. ponticum* establishment on bare soil was attributable to a reduction in PPFD and not a change in R:FR (germination rate was only increased by neutral shade, and reducing R:FR to 0.47 (45% of that in incident radiation) was detrimental to seedling survival). However, the detrimental effect of canopy shade on *R. ponticum* establishment in sown grass and tall moss was clearly attributable to the reduced R:FR (germination and seedling survival rate were not generally reduced by neutral shade, but were by green shade (that had the same effect on the PPFD)). This suggests that the cumulative effect of two vegetative canopies produced a R:FR low enough to be inhibitory. This conclusion is further supported by Experiment 3 in which germination rates were greatly reduced under a green filter with a more strongly reduced R:FR of 0.1, but not under neutral shade of equal PPFD but much higher R:FR (2.42). Light, through its interaction with other environmental factors, is well known to control the dormancy relief of seeds in many 'light-demanding' plant species (Gorski *et al.*, 1978). Canopies not only reduce the amount of solar radiation

but chlorophyll also absorbs incident photosynthetically active radiation, altering spectral composition, specifically the red to far-red ratio by transmitting a greater amount of far red light (720–740 nm) than red light (660–680 nm). There is a large variation in R:FR under vegetation canopies with values ranging anywhere between 1.2 and 0.1 in woodlands (Smith, 1982; Erwin *et al.*, 2006). A low red to far-red ratio (R:FR) detected by the phytochrome system of seeds inhibits germination in some species, especially in positively photoblastic seeds (Yirdaw & Leinonen, 2002) or many small-seeded species (Jensen & Gutekunst, 2003). In the present study, although *R. ponticum* germination and seedling survival in a grass seed-bed with no higher canopy were high, seedling biomass growth rate was greatly reduced (compared with bare soil/short moss substrate). Thompson and Harper (1988) found that grass canopies reduced the quality of transmitted radiation and suppressed the growth of other plant species.

Although seeds germinated under green shade in a layer of deciduous leaf litter 3 cm in depth and in tall moss in Experiment 1, their seedlings had a low probability of survival. This is likely to be due to the combined effects of a reduction in R:FR by the ‘double’ filter of ground level vegetation/litter and a canopy, drought, and physical interference. Preliminary tests showed that approximately 20% of the seed applied fell through the leaf litter onto the soil surface. Any surviving seedlings were found at the tray edges where leaf litter was less dense and light was able to penetrate to the soil surface. In previous field studies, *R. ponticum* seedlings have been found to be both negatively associated with leaf litter (Cross, 1981) and positively with thin layers (depth not specified) of mixed leaf litter on fallen logs and decomposing stumps (Stephenson *et al.* 2006). A review of 35 studies by Xiong and Nilsson (1999) found that leaf litter had an overall largely negative effect on vegetation establishment. Tree species were more affected than herbs and grasses by litter, at least at the colonisation stage and litter inhibited germination more than establishment. Forest floor litter has been found to decrease germination and seedling emergence through shading, biochemical effects, and physical obstruction to the emergence of a seed’s cotyledons and radicle (Ellsworth *et al.*, 2004; Jensen & Gutekunst, 2003). Furthermore, litter has an influence on light quantity and changes its spectral composition through causing a reduction in the red component (665 nm) of light beneath a litter layer (Silvertown, 1980, Facelli & Pickett 1991). Although some studies have shown that litter may favour vegetation establishment by causing

a decrease in maximum temperature and reduction in evaporation (Eckstein & Donath, 2005), the negative effects of plant litter generally outweigh the positive ones (Xiong & Nilsson, 1999).

The results of this study indicate that there may be some degree of adaptation by populations in Britain and Ireland to shady conditions. There was a reduction in germination capacity of seeds sampled from populations at the edge of *Picea sitchensis* (Sitka spruce) forests in Ireland and Scotland. Of the remaining populations two were sampled from old populations on estates, one in deciduous woodland, the other in an open site, and the other two were from populations growing in the open spaces. There have been many independent introductions of *R. ponticum* in different parts of the British Isles (Dehnen-Scmutz *et al.*, 2004). Whilst it is unlikely that there has been sufficient time for natural selection on these different populations to have resulted in differences in their environmental responses, very little is known about the origins or hybridisation history of the seed that were planted. Changes in phenotype in response to environmental conditions that enhances the individual performance under the prevailing conditions is common in plants (reviewed in Schmitt *et al.*, 2003, Galloway & Etterson, 2007). In an analysis of 133 invasive plant species, Ren & Zhang (2009) found that phenotypic plasticity (either in environmental tolerance or in resource allocation) was responsible for the invasiveness of about 50% of the species. Alien species often exhibit a greater plasticity in their response to changes and disturbances in the environment and may therefore have a greater capacity to shift the physiological optimum to a range that is favourable in a changing climate (Verlinden & Nijs, 2006). There is some evidence that transgenerational plasticity has evolved in response to natural variation in light and provides a flexible mechanism by plants to cope with heterogeneous environments (Galloway & Etterson, 2009). Therefore, there remains a clear possibility that differences between these populations exist as a legacy of the differences in environmental conditions where their origin populations evolved.

CHAPTER III: The effect of seasonal growth stage on the control of *Rhododendron ponticum* using the herbicide glyphosate

3.1 ABSTRACT

The first step in restoration involves the removal of invasive plants and the use of chemical control is often necessary in clearing an invaded area. The herbicide glyphosate is widely used to control the invasive evergreen plant *Rhododendron ponticum* yet little is known about its action in this particular species. The effect of season of application and hence growth stage on the translocation of the herbicide glyphosate on *R. ponticum* plants less than 1.5 m in height was investigated. The crowns of the plants were treated with a 2% glyphosate and 2% surfactant solution using a knapsack sprayer to 0, 25, 50, 75 and 100% of the plant's crown either in early February, May, August or November. Plants which were 100% sprayed were "completely controlled" (killed) for each of the four times of treatment. Damage caused to untreated parts of the treated plants was proportional to the percentage of the crown treated; treating 0, 25, 50 and 75% of the crown resulted in the death of 0, 49, 74 and 89% respectively of shoots in the untreated portions of the crown. Mean shoot growth and the lengths of new leaves on untreated parts of treated plants were significantly lower than on untreated (control) plants. Seasonal timing of the treatment also had a major impact; plants sprayed in May and August suffered most damage in the untreated parts of their crowns, whereas spraying in February had the least effect.

3.2 INTRODUCTION

Rhododendron ponticum subspecies *baeticum*, an evergreen shrub native to southwest Spain and central and south Portugal, Bulgaria, Turkey, Caucasus and Lebanon, was introduced to Britain and Ireland as an ornamental around 1763 (Elton, 1958). It has since become naturalized in a wide range of sites, including deciduous and coniferous woodland interiors and edges, coastal dunes, heaths and bogs (Cross, 1981; Fuller & Boorman, 1977; Rotherham, 1986). *R. ponticum* is estimated to currently occupy 2,075 of the 213,000 hectares of the Snowdonia National Park, Wales, an increase of approximately 20% over the last 20 years (Jackson 2008). Of this, 70% occurs within deciduous and coniferous woodland with 11% in grassland. However, 230 hectares has been effectively controlled in that time. It is estimated that £2 million has been spent on control in Snowdonia National Park since 1987 and that a further £9 million will be required to control *Rhododendron* on a park-wide basis (Jackson, 2008). The total cost of eradicating *R. ponticum* source populations from the entire Argyll and Bute landscape (3,950 ha) is estimated at > £9.3 million. However delaying control management would increase the cost of eradication by allowing expansion of the current populations by 23% after 20 years and by 58% after 50 years potentially increasing the cost to more than £19 million in 2028 and £65.4 million in 2058 (Edwards & Taylor, 2008).

The shrub is one of several understory rhododendron species that have been shown to compete with, and, in some cases, totally inhibit tree regeneration in temperate forest ecosystems throughout the northern hemisphere (Nilsen *et al.*, 1999; Peterken, 2001 and Lei *et al.*, 2002). Due to the negative effects on native flora several control methods have been attempted including pulling, clipping or cutting shrubs, foliar, and stump application or stem injection of herbicides. Few rigorous studies of these methods have been performed and there is currently no standard method of control as no single method has been proven to be the most effective (Eşen and Zedaker, 2004). Methods of application vary depending on bush size and form, stand density, site access and topography. These variables are also the factors that determine cost, with bush density probably being the single most important factor (Jackson, 2008). However, there is still a large disparity in the contract prices quoted by practitioners.

3.2.1 Herbicide application methods

The efficacy of a range of herbicides and surfactants for the control of *R. ponticum* has been tested both in pot and field trials (Clay *et al.*, 1992; Lawrie & Clay, 1993a,b; Edwards & Morgan, 1996; Edwards, 1999; Edwards *et al.*, 2000; Dixon & Clay, 2002; Esen *et al.*, 2004; Esen *et al.*, 2006). A review by Tyler and Pullin (2004) collated all accessible information and critically appraised the evidence for effective control of *R. ponticum* using current management interventions. The evidence suggested that application of the herbicides ¹Imazapyr or ²Metsulfuron-methyl to *R. ponticum* stands, and post-cut foliar application of ³Glyphosate, significantly reduced *R. ponticum* abundance. Only five studies provided data for analysis of Metsulfuron-methyl and were either performed in an unspecified habitat or in pots in glasshouses. There was also significant bias present in the post-cut glyphosate application meta-analysis and Tyler and Pullin advise treating the results with caution. Other herbicides were also included in the review but were either ineffective (such as ⁴Triclopyr) or there was insufficient evidence to test their effectiveness on *R. ponticum* control. There is therefore a lack of rigorous unbiased replicated results under different field conditions with a diverse range of interacting variables for available herbicides.

¹Imazapyr is the active ingredient in compounds that are marketed under the trade names Chopper, Arsenal, and Assault. Imazapyr is a non-selective broad-spectrum systemic herbicide, absorbed by the foliage and roots, with rapid transfer to the xylem and phloem to the meristematic regions, where it accumulates and causes disruption of protein synthesis (Tomlin, 1994).

²Metsulfuron-methyl is a residual sulfonylurea compound used as a selective pre- and postemergence herbicide for broadleaf weeds and some annual grasses. It is a systemic compound with foliar and soil activity and inhibits cell division in the shoots and roots of the plant. It is sold under the trade name Ally, Allie, Gropper, and Escort (Extension Toxicology Network, Pesticide Information Profiles).

³Glyphosate or N-(phosphonomethyl) glycine is a broad spectrum, non-selective, post-emergence systemic herbicide normally goes under the tradename RoundUp patented and sold by the company Monsanto.

⁴Triclopyr is a systemic, foliar herbicide in the pyridine group. It is used to control broadleaf weeds while leaving grasses and conifers unaffected and is sold under the trade name Garlon and Release (Triclopyr Technical fact sheet, National Pesticide Information Center, Oregon, US).

Both Imazapyr and Metsulfuronmethyl have been banned for use in the European Union from 2003 and 2009, respectively (Chemicals Regulation Directorate, UK, 2009). Glyphosate, the active ingredient in 'RoundUp', is therefore currently the recommended herbicide for *R. ponticum* control. There are three recommended glyphosate application methods for controlling *R. ponticum* plants: cut stump application for plants > 1.5 m in height, stem injection again for plants > 1.5 m and foliar spray application for plants < 1.5 m. Control can be successful if recommended removal and herbicide regimes are employed (e.g. Eşen and Zedaker, 2004; Edwards, 2006; Barron, 2008).

Foliar spray

Foliar spray is the cheapest and most commonly used method for treating bushes less than 1.5 m in height. Typical contractor costs, based on easy flat sites with good access and bushes with good all round access is £1000 per hectare (Jackson, 2008). The most widely used herbicide for foliar application is a 2% solution of glyphosate in water at a maximum rate of 10 litres of RoundUp (containing 360 g isopropylamine glyphosate salt) per hectare. The addition of a surfactant such as Monsanto's 'Mixture B' (a blend of surfactants, wetters, spreaders and penetrants), as 2% by volume, often improves the effectiveness of RoundUp, and has been shown to increase the phytotoxicity of glyphosate to *R. ponticum* (Lawrie & Clay, 1993a). The only recorded replicated studies of foliar application with glyphosate are to shoot regrowth following cutting to a stump (Edwards & Morgan 1996; Edwards, 1999; Edwards *et al.*, 2000). These were included in Tyler and Pullin's review who concluded as mentioned before that glyphosate had a significant negative effect but that the results were biased. The other recommended foliar spray application herbicide is triclopyr as a 2.5% solution in water (Barron, 2008). The herbicide is normally applied using a knapsack sprayer at low pressure. Foliar application with glyphosate can only be applied in dry conditions when no rain is expected for at least 6 hours. Spraying in windy conditions should be avoided as this will lead to spray drift resulting in collateral damage to non-target vegetation. If a surfactant has been added to the solution, spraying bushes by aquatic habitats should be avoided as surfactants are often more environmentally damaging than the

herbicides themselves (Edwards, 2006; Barron, 2008). Foliar application of herbicide by hand to bushes greater than 1.5 m is not advised as it poses a risk to the practitioner (and surrounding vegetation from drift) and the height/diameter is too dense to allow good access for spraying. There are several techniques to deal with plants greater than 1.5 m including stump and stem treatment.

Stump treatment

Cutting all of the stems rarely results in the death of *R. ponticum* bushes and generally results in the proliferation of fast growing shoots from dormant basal buds (Cross, 1975; Fuller and Boorman, 1977). There are therefore two types of post-cut-stump herbicide application practices: the first involves applying the herbicide directly to the cut stump and the second allows regrowth of shoots for a certain period followed by foliar spraying with herbicide and surfactant. Successful trials in Killarney National Park, Ireland have been carried out using the former method by applying 20% or 10% glyphosate concentration in water or triclopyr in an 8% solution in water to the stump surface directly after cutting. Treatment between June and September is recommended for optimum control (Barron, 2008). Advantages of the cut stump followed by immediate treatment is that all initial clearance work can be carried out in one visit and collateral damage to the surrounding non-target vegetation is minimal. However, this technique has had mixed success in the UK (Julian Miller, Kehoe Countryside; Peter Jackson, Snowdonia National Park pers. comm.). The second method allows time (about 18 months) for shoots to regrow on surviving cut stumps of *R. ponticum* before applying herbicide to make it easier to distinguish the surviving from the dead stumps (Edwards, 2006). No evidence has been found to date of a reduction in the health of planted trees in the vicinity of the cut stumps to which herbicide has been applied (Edwards & Morgan, 1996; Edwards, 1999), whereas off-target damage has been observed with the foliar spray technique (Esen *et al.*, 2006). Cutting followed by herbicide treatment therefore provides an effective and safe control for *R. ponticum* plants large enough to make cutting an efficient method (generally taken to be taller than 1.5 m). Cutting the plants generates a large amount of brash, and removing this from the site to be burned is a laborious and costly process, however, by leaving the brash in place,

subsequent regeneration of *R. ponticum* is greatly reduced and therefore re-invasion is unlikely to occur (Edwards *et al.*, 2000). The average costs of these methods on easy access, flat sites is £3,500 for the cut stump, foliar spray, follow-up spray technique, and £5,000 per hectare if the brash is cleared from the site by burning or chipping (Jackson, 2008).

Stem treatment

An alternative to the cutting stem technique is the stem treatment and is useful where there is no need to cut or remove the dead standing bushes. It was first tested on *R. ponticum* by Edwards *et al.* (2000) and involved making cuts of approximately 2-3 cm wide and deep into the stem using an axe and applying herbicide into the cuts. They found that stem injection of imazapyr and glyphosate are the best control of *R. ponticum* plants. Results from an unreplicated study by Edwards *et al.* (2000) suggested that only a percentage of stems might need to be treated with imazapyr for complete plant control to be achieved, whereas glyphosate would need to be applied to every stem to achieve complete plant control. It can also be applied by drilling a hole into the bark at an angle and applying 2 ml of glyphosate (25% solution in water, no surfactant required) into the well. This method is best suited to large bushes with few, but substantial, easily accessible stems and is particularly useful in difficult, sloping terrain where other methods are impractical. Other advantages of this method include low cost, application in moist conditions, minimal habitat disturbance and high kill rate. The drill-hole injection technique seems to be equally effective at all times of year for *R. ponticum* (Julian Miller, Kehoe Countryside Forestry Contractors; Colin Edwards, Forestry Commission, pers. comm.). Typical contractor costs per hectare for this technique are £2500 on an easy site including follow-up spray/weed (Jackson, 2008).

3.2.2 The mode of action of the herbicide glyphosate and the effect of season on rate of absorption and translocation

N-(phosphonomethyl) glycine (glyphosate) is a broad-spectrum, non-selective, post-emergence systemic herbicide used for control of essentially all annual and perennial vascular plants including grasses, sedges, broad-leaves weeds and woody plants. Glyphosate itself is an acid, but it is most commonly used in the isopropylamine salt and is generally distributed as water-soluble concentrates and powders. It was first patented and released by Monsanto (Missouri, USA) under the trade name 'RoundUp' in 1976 and can now also be found under a range of other trade names including Gallup, Landmaster, Pondmaster, Ranger, Rodeo, and Touchdown (Extension Toxicology Network, Chicago State University, 2009). Glyphosate exerts phytotoxicity by competitive inhibition of 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase, an enzyme of the shikimic acid pathway. The main consequences of this action is an arrest in the production of the amino acids phenylalanine (Phe), tyrosine, (Tyr) and tryptophan (Tryp) and ultimately protein synthesis (Cole, 1985). The mode of action of glyphosate is such that best efficacy results when the herbicide is translocated in the phloem to the root system and stored there over winter with retranslocation in the spring resulting in the death of new growth (Franz, 1985).

Systemic, foliage-applied herbicides are translocated with assimilates according to the 'source' to 'sink' principle (Ashton & Crafts, 1981). In herbaceous perennials, glyphosate has been found to be rapidly absorbed and translocated to the meristematic regions of shoots and roots where high rates of protein synthesis occurs (Sprankle *et al.*, 1975; Gottrup *et al.*, 1976; Fernandez & Bayer, 1977; Zandstra & Nishimoto, 1977; Smid & Hiller, 1981). The rate of glyphosate uptake in woody species is relatively slow compared with the fast rate in herbaceous plants (Lund-Høie, 1985). Factors that affect glyphosate absorption or transport include plant growth habit and consequent differences in spray coverage, cuticular permeability, plasma membrane permeability, environmental conditions, and the season of application (Neal & Skroch, 1985). Environmental conditions such as light duration and intensity, air temperature, relative humidity and dew or precipitation effect

herbicide efficacy by influencing processes such as herbicide absorption, translocation, or plant metabolism (Hammerton, 1967; Gerber *et al.*, 1983).

Although several different methods have proven effective in achieving full control of *R. ponticum* (Esen & Zedaker, 2004; Edwards, 2006; Barron, 2008), there is little knowledge on the action of glyphosate in the plant's system. The timing of treatments has been shown to be significant in determining the effectiveness of control efforts in woody plants by using different doses of glyphosate (e.g., Lynn *et al.* 1979; Pieterse & McDermott, 1994; Love & Anderson, 2009). Levels of total nonstructural carbohydrates (TNC) stored in roots fluctuate according to the plant's growth stage (i.e., dormant, bud break, flowering, fruiting, leaf senescence, and leaf abscission) which is influenced by season (Loescher *et al.* 1990). Willoughby and Dewar (1995) recommended foliar application of glyphosate to *R. ponticum* from June to September. This period was subsequently extended by Edwards (2004) who achieved complete control (plant death) of *R. ponticum* at all times of year following foliar application with 2% glyphosate, 2% Mixture B solution in water (dose of 10 lha⁻¹). Neal and Skroch (1985) applied ¹⁴C-glyphosate to eight woody ornamental plant species at different times of year. Application timing was crucial in determining tolerance with autumn applications being tolerated by all species and March treatments causing injury to all. Spring applications caused maximum injury to *Ligustrum japonicum*, *Ilex crenata* cv. *Helleri*, *Rhododendron obtusum* and *Ilex vomitoria*, while June and August treatments were most toxic to *Juniperus horizontalis*, *Ilex crenata* cv. *Compacta* and *Photinia serrulata* x *P. glabra*. In the British Isles, shoot extension of *R. ponticum* commences in early May with two main periods of shoot elongation in May-June and July-August. A third period of growth may occur in September (Cross, 1975). This would suggest that the most extensive translocation of glyphosate occurs (1) in spring when stored assimilates and nutrients are being moved from the roots to areas of new growth and for flowering and (2) in late summer/autumn when the plant is still photosynthesising but net movement of assimilates is from the leaves to the roots for winter storage. Photoassimilate translocation to roots is assumed to be more active at the latter stages of the growing season, and the herbicide, moving with the assimilate stream, would thus be more evenly distributed in the root system.

3.2.3 Collaborative projects to improve techniques

Stem treatment with Peter Jackson of Snowdonia National Park

The aim of this field trial was to test different practical glyphosate stem treatment methods on *R. ponticum*. Their relative performance would provide evidence about the translocation of glyphosate movement around the plant. The four techniques tested to prepare for the application of the glyphosate were: slicing cut with an axe (A); bark scouring with a wire brush (WB); drilling a hole 4 cm in depth (DH4); drilling a hole 10 cm in depth (DH10). Two of these application methods (Fig. 3.1a) & DH4 (Fig. 3.1b)) are tried, tested and currently in use by practitioners controlling large *R. ponticum* plants. The other two techniques were devised to investigate whether the transport of glyphosate in woody tissue would enable cheaper, simpler control methods to be used.

Glyphosate is primarily quoted as being a phloem-mobile herbicide (Caseley & Coupland, 1985). Stem sections of woody *R. ponticum* tissues (Ninaber, 2006) showed that the location of the secondary phloem (stained blue) was limited to the vascular cambium in the thin bark layer. The wire brush technique involved scratching the surface of the bark with a wire brush and applying the herbicide on the exposed area. This was intended to apply herbicide primarily to this shallowly-located phloem tissue. Two of the technique tested involved drilling a hole (using a 12 mm diameter drill bit) at an angle of ca. 30 degrees from the vertical. With the 10-cm deep hole the herbicide was applied with a dispensing pipette to the very bottom of the hole and a layer of oil was then applied to ensure that no herbicide would come in contact with the outer cambial layer; this tested whether glyphosate could be absorbed and translocated effectively in the secondary xylem. With the 4-cm deep hole the herbicide was applied with a pipette but with no oil layer on top allowing possible contact with the outer cambial layer. The axe technique involved making a cut at an angle of ca. 30 degrees from the vertical, to a depth of ca. 3 cm, and applying the herbicide into the cut that this created in the stem.

The site for the trial was an open grassland dominated by *Molinia caerulea* tussocks beside the Coederyr woodlands near Llyn Dinas in the Nant Gwynant

Valley (53°01'38 N, 4°02'06 W) which has become heavily invaded by *R. ponticum* plants. Forty large *R. ponticum* plants > 1.5 m in height and with stems > 3 cm diameter were selected, and ten were randomly chosen for each of the treatments. The treatments were applied to the base of the stem at a height of between 2 and 5 cm aboveground. Amount applied followed that recommended by the Forestry Commission Practice Guide (2006): 2 ml of glyphosate (25% RoundUp 360 in water) per 7 cm stem diameter using a dispensing pipette.



Figure 3.1 *Rhododendron ponticum* basal stem (5 -10 cm diameter) showing a) axe cut and b) drill hole technique.

The observed results were recorded 6 months after application using a health score of between 0 and 5, where: where 0 = healthy, 1 = damage, 0% defoliation, 2 = < 25% defoliation, 3 = 25 – 75 % defoliation, 4 = > 75% defoliation, 5 = dead. Results were presented in terms of mode (most frequent health score) and mean (Table 3.1) The drill hole (4-cm), axe, drill hole (10-cm) and wire scour resulted in the death of 20, 50, 40 and 0 % of the plants 6 months after application. The axe and drill hole (10-cm) techniques were the most effective and the wire brush technique was least effective resulting in only partial death and killing none of the plants. The success of the deep drill-hole (10-cm, with a layer of oil) technique provides good evidence that glyphosate can effectively be taken up and transported in the secondary xylem. This is now a hypothesis worth testing in subsequent more highly controlled experiments.

Table 3.1 Health (scored between 0 and 5, where 0 = healthy, 1 = damage, 0% defoliation, 2 = < 25% defoliation, 3 = > 25% defoliation, 4 = > 75% defoliation, 5 = dead) of large *Rhododendron ponticum* plants (> 1.5 m) treated with glyphosate (25% RoundUp 360 in water) using 4 different techniques to the base of the stem.

<i>Technique</i>	Health of plant	
	Mean	Mode
Drill hole (4 cm)	4.0	4
Axe cut	4.4	5
Wire scour	3.1	4
Drill hole (10 cm)	4.2	5

Foliar application using the controlled droplet application technique

This field trial was carried out in collaboration with Johannes Yuan Pakatul, who reported on it in his Bangor University MSc dissertation (Pakatul, 2007). It was designed to look at the environmental advantages and efficacy of using the controlled droplet application (CDA) technique for controlling *R. ponticum* plants less than 1.5 m in height with glyphosate. The location of this experiment was in the same site in Nant Gwynant where the main experiment in this chapter was carried out (details below). Glyphosate was applied at three different application rates: low (Hilite ® at 5 l per hectare), recommended (Hilite ® at 10 l ha⁻¹), and high (Hilite ® at 20 l ha⁻¹) with Hilite, a readymix pigmented oil emulsion pack containing 144 g l⁻¹ isopropylamine glyphosate salt. The mixture was applied using a CDA unit (Weed Warrior, Nomix Enviro, Hampshire, UK) on 8 June, 2007. Unlike knapsack sprayers, the speed and flow (in ml minute⁻¹) can be controlled using this CDA unit so that application is stopped when the specified dose has been applied to each plant, rather than continuing until complete leaf surface coverage ‘until runoff’ has occurred. However, none of the CDA treatments achieved complete control, i.e. none killed the entire plant after 84 days. The severity of observed damage increased with application rate, the 20 l ha⁻¹ treatment causing the greatest amount of leaf chlorosis and defoliation. There was a significant decrease (of 46%) in the rate of photosynthesis of plants treated with herbicide (at 20 l ha⁻¹) compared with unsprayed controls. I carried out further observations of these plants after another

year, and found that there was some new growth from stem buds even on the plants that had been treated at the highest rate. Although the CDA causes less collateral environmental damage from spray drift and runoff of glyphosate solution than does application with a conventional knapsack sprayer, it achieved less successful control. Further research could investigate the application of higher doses per plant using CDA. However, this will be costly: the CDA can only be used with the 5 L premixed Nomix glyphosate pack which is considerably more expensive than the unmixed knapsack sprayer glyphosate solution.

3.3 OBJECTIVES

The following two hypotheses were tested:

1. When using glyphosate to control woody shrubs, do you need to 100% spray the crowns (cost and environmental damage) or is the vascular anatomy of the plants sufficiently well internally connected to translocate glyphosate from treated to untreated parts?
2. If so, does it make a difference to this translocation in which season plants in a temperate climate are sprayed (physiologically active or not)?

These hypotheses were tested in a field experiment with small *R. ponticum* plants. A single concentration of glyphosate solution was applied to five different proportions (0, 25, 50, 75 and 100%) of the crown of individual plants at one of four different times (and hence seasonal growth stage) during the year. The extent of the damage caused to untreated parts of the crown of the partially sprayed plants (25, 50 and 75%) was taken as an indicator of the degree of translocation of the herbicide.

3.4 METHODS

3.3.1 Site

The study site is located near Llyn Dinas and the Coederyr Woodlands in the Nant Gwynant valley of Snowdonia, North Wales ($53^{\circ}01'38''$ N, $4^{\circ}02'06''$ W) (Fig. 3.2). It is at an elevation of between 150 m and 170 m with a westerly slope. *Rhododendron ponticum* forms tall dense thickets close to the woodland edge in the north part of the site and up the slope from the woodland in the north east part of the site in the area known as Hen Goed (Fig. 3.3). Clumps of mature and young *R. ponticum* are growing in land dominated by *Molinia caerulea* tussocks and moderately grazed (by sheep and cattle) grasslands over much of the rest of the site. The *R. ponticum* invasion is also spreading west from the site downhill along the Afon Llynedno River and east over the hill towards the valley of the Afon Lledyr. Many *R. ponticum* seedlings can also be found in the neighbouring *Picea sitchensis* woodland to the north and north-west the site, though the abundance is generally low below the forest canopy. Parts of the site are very wet, and the clumps of *R. ponticum* tend to be found on higher drier ground.

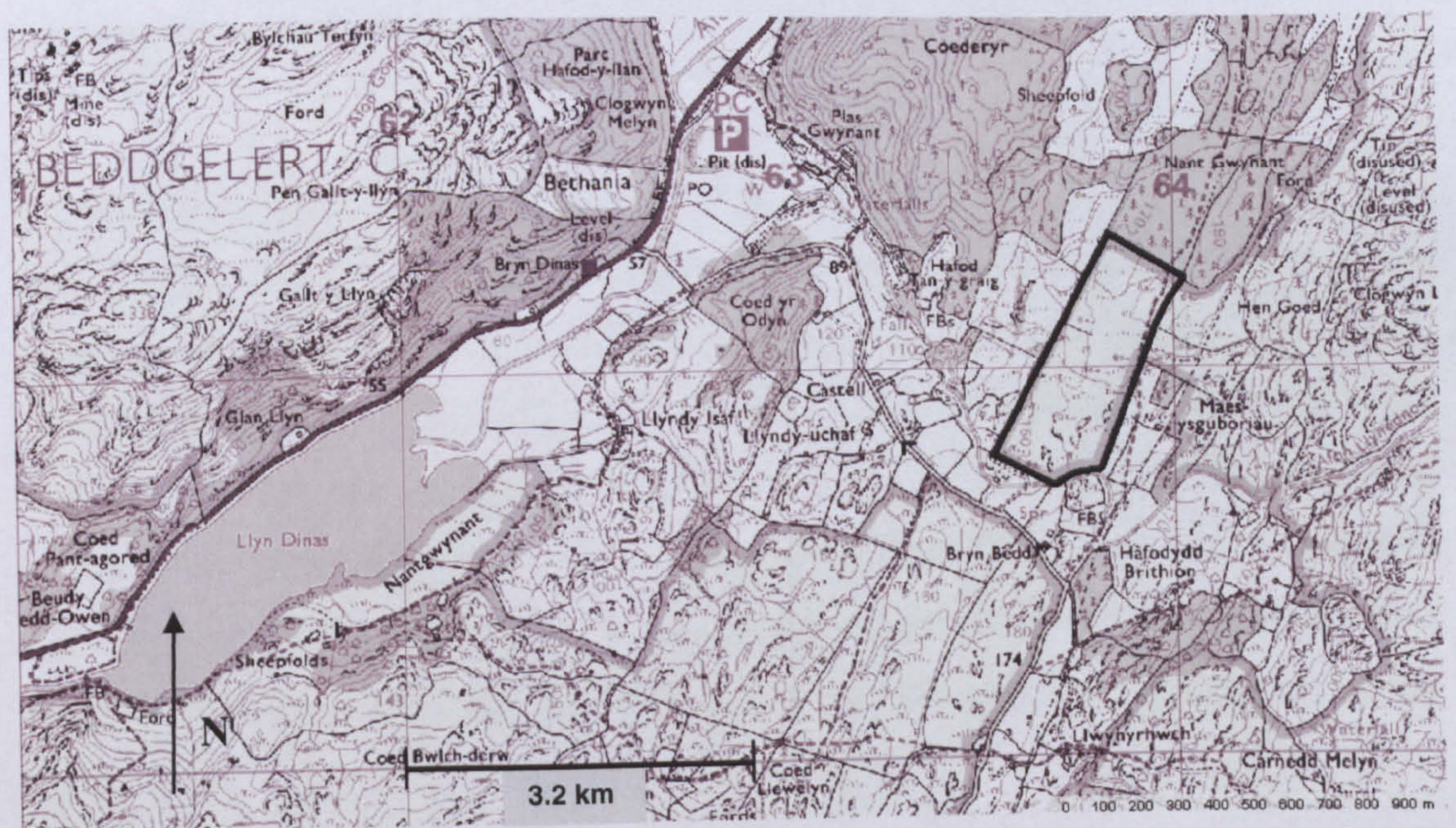


Figure 3.2 Site location in Nant Gwynant, Gwynedd, North Wales ($53^{\circ}01'38''$ N, $4^{\circ}02'06''$ W). Digimap Ordnance Survey Collection.



Figure 3.3 NE aspect of *Rhododendron ponticum* invasion from site into Hen Goed.

3.3.2 Experimental design

A randomised-block design was used. The site was surveyed and eight 0.04 ha blocks, that had i) at least 20 healthy *R. ponticum* plants between 0.9 m and 1.5 m in height of comparable growth form and ii) was at a distance of more than 20 m from another block were chosen (Fig. 3.4) . Twenty plants within each block that were most closely matched in size, growth form and health were selected and allocated randomly to 20 combinations of two treatments: five glyphosate treatments (0% (control), 25%, 50%, 75% and 100% of crown sprayed) and four times of application (August, November, February and May). In total 160 plants were included in the experiment.



Figure 3.4 The position of the experimental blocks within the site. The outline of the site is shown as a thick black line.

3.3.3 Treatments

The crown of each plant was sectioned into quadrants (northeast (NE), southeast (SE), southwest (SW) and northwest (NW)). For the 25% treatment the NE quadrant was treated; 50% the NE and SE; and 75% the NW, SW and SE quadrants (Fig. 3.5). The quadrants not being sprayed were covered with plastic dustsheets to prevent contamination via spray drift. Glyphosate solution – RoundUp 360 ProBiaoactive at 2% solution with Mixture B at 2% solution in water (Monstano, Oregon, USA), the amount recommended by Forestry Commission (Edwards, 2006) – was applied using a 5-L knapsack sprayer at low pressure and medium–high volume (as would deliver at a rate of 500–750 l ha⁻¹ under normal use). The glyphosate solution was applied as a directed spray to the selected uncovered portion of the crown until the instant just before run-off occurred, i.e. when full foliar wetness was achieved. The plastic dust

sheet was kept in place over the rest of the crown until the sprayed leaf surfaces were dry. Application of the herbicide was carried out in frost-free, low-wind, rain-free conditions and when rain was not forecast for at least the following 12 hours. Plants were treated at times of year that reflected different seasonal growth stages: 1 August 2007, 1 November 2007, 3 February 2008 and 5 May 2008.

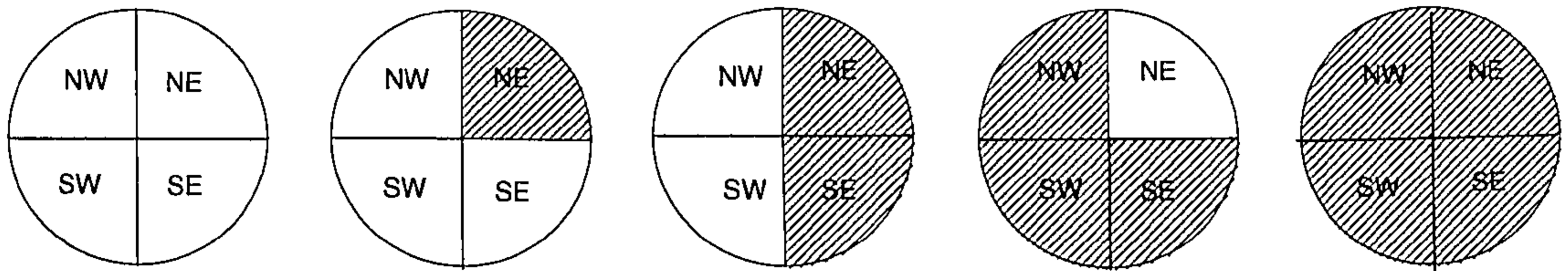


Figure 3.5 Pattern of application of the five doses of glyphosate to the crowns of *R. ponticum* plants: from left to right 0%, 25%, 50%, 75% and 100% of crown sprayed, white = untreated; hatched = treated.

3.3.4 Measurements

The health of each quadrant of the plant's crowns was assessed at the following intervals after treatment: 2, 3, 5, 8, 11, 13, 15, 17, 27 and 42 weeks using a score of 0-5 where:

0 = Live and healthy, foliage green and damage free.

1 = Slight herbicide damage to leaves, but no defoliation, all buds live.

2 = < 25% leaf defoliation, remaining leaves slightly damaged or healthy, at least 50% of buds live.

3 = 25 - 75% leaf defoliation, remaining leaves damaged, chlorotic, at least 50% of buds dead.

4 = > 75% leaf defoliation, remaining leaves severely chlorotic, > 90% of buds dead.

5 = Complete crown death, full leaf defoliation and death of all buds.

Measurements were taken toward the end of the growing season after the application of the treatments on 12 July 2008 at which time the effects of glyphosate had been seen in all seasonal treatments. For all untreated parts of the crown of each plant, the

total number of live shoots remaining was measured on 12 July 2008, 2 December 2008 and finally on 31 May 2009 (Table 3.2). This meant that at least 12 months had passed since the application of all the treatments. The number of shoots exhibiting new growth and the number of flowering shoots were counted in the growing season of 2008 (12 July) and 2009 (31 May); average leaf size and shoot extension growth were also measured on those two dates.

Table 3.2 Dates on which measurements of *R. ponticum* crowns were made and the length of time elapsed after the different months of treatment application.

<i>Month of application</i>	Time (months) to date of measurements		
	12 July 2008 (1 st growing season)	2 Dec 2008	31 May 2009 (2 nd growing season)
August 2007	11	16	22
November 2007	8	13	19
February 2008	5	9	16
May 2008	2	6	13

Photosynthetic rate of healthy leaves on control plants and leaves on untreated parts of treated plants were measured using an Infra-Red Gas Analyser (IRGA) (CIRAS-2 Portable Photosynthesis System, Boston, Massachusetts, USA) and PLC 6 Automatic Universal Leaf Cuvette (PP Systems, Massachusetts, USA). Measurements were taken on 24 October 2008 with the following settings: CO₂ = 380 ppm; temperature = 20 °C; an LED light unit (PP Systems, Massachusetts, USA) was used to maintain a constant photosynthetic photon flux density (PPFD) with light levels ranging between 0 and 1000 $\mu\text{molm}^{-2}\text{s}^{-1}$.

3.5 STATISTICAL ANALYSES

The health scores per crown quadrant were initially divided into two categories: live and dead, live representing quadrants that showed any sign of being alive i.e. that scored between 0 and 4, and dead having a score of 5. Quadrants were then divided into two subcategories, treated and untreated. The frequencies of dead treated quadrants, expressed as a percentage of the total number of treated quadrants, were determined over time. Also, the frequencies of dead untreated quadrants, expressed as a percentage of the total number of untreated quadrants, were determined over time.

Various aspects of plant health – living shoots remaining, shoots exhibiting new growth and flowering shoots for the whole crown (treated and untreated), all expressed as a percentage of the total number of shoots on the plant – had been recorded on three different dates. For the second and third measurements, they were expressed as a percentage of the total number of shoots counted in the first measurement. These data were analysed in several different ways to deal with the effect of not only treatment but also time of measurement.

The data were normally distributed so univariate analysis of variance, with a randomised block design, was used to test for significant differences in the effects of treatment. Firstly, data were subjected to ANOVA for the final recording made, 22 months after the first seasonal glyphosate application treatment (August 2007) and 13 months after the last application (May 2008). The unsprayed control plants and those with 100% of their crown sprayed were also included in the analysis (giving a total of five doses). In the two-way ANOVA, season of treatment application (four levels) was the second factor and the interaction between season and dose was also tested. A p-value less than 0.05 was taken to indicate a significant result. Secondly, to investigate the effect of time of measurement on plant health, ANOVA were carried out (same method as previous) on the other measurements taken on the earlier dates. Finally, to test treatment effects after a fixed time elapsed since treatment, measurements made closest to one year after each application time were used in an analysis of variance for differences between the treatments. Shoot growth and leaf length were also used as indices for new growth. Two-way ANOVA was

used to test for significant differences in the effects of dose and season. The number of replicates for each treatment was not equal as for some of the doses (especially the 75% dose) there were no leaves or too few leaves to measure.

Light response curves for healthy (control) and unhealthy (untreated crown of treated plants) leaves were fitted using a 3-parameter exponential rise to a maximum function after Peek *et al.*, (2002):

Equation 3.1

$$f = y_0 + A_{\max} (1 - e^{(-bx)})$$

Where y_0 denotes the y intercept, A_{\max} represents the maximum rate of photosynthesis and b corresponds to the steepest slope of the curve.

3.6 RESULTS

Treating 100% of the crown of plants with glyphosate spray led to complete plant mortality for all four seasonal spraying times. Irrespective of the proportion of the crown that was treated, the treated part eventually died, though the time taken for this to occur differed between treatment times (Fig. 3.6a, c, e). Plants sprayed in August and May, were most quickly affected by the herbicide, with the first symptoms being observed after two and three weeks respectively. Plants that were treated in February did not show signs until 11 weeks after treatment and November was the slowest at 15 weeks. Initial signs of glyphosate damage were leaf chlorosis – a yellowing of the leaf due to lack of chlorophyll – followed by defoliation. Virtually all of the treated plants showed completed mortality of the treated parts of their crowns by 27 weeks after spraying (the only exception was one 50% sprayed plant in which one quadrant which showed signs of recovery 30 weeks after treatment (Fig. 3.6c).

Glyphosate treatment also caused damage to the untreated parts of the crowns, though it showed a more complex interaction between the time of treatment and proportion of the crown sprayed. Spraying in May did always lead to the fastest occurrence of damage, but the sequence amongst the other months varied (Fig. 3.6b, d, f). By the end of the observation period, the frequency of dead quadrants in the unsprayed crown portions of plants treated in May was consistently higher than those treated in November and February for all treatment doses (Fig. 3.6b, d, f). However, the August treatment led to inconsistent results; it caused the highest mortality rate (100%) of the untreated crown quadrants, when 75% of the crown was sprayed (Fig. 3.6f) equal highest when 50% was sprayed (Fig. 3.4d), but lowest (0%) when 25% was sprayed (Fig. 3.6b). For the November, February and May treatments there was no notable increase in the mortality rate of the untreated crown quadrants between the plants 25% and 50% sprayed. However, for all four seasonal treatment times there was a marked increase in this mortality rate between the plants 50% and 75% sprayed.

Percentage of live shoots remaining in the crown, as an index of plant health, showed a clear dose-response: for all four treatment season times this percentage was greatest with 25% of the crown sprayed, intermediate with 50% and least with 75% (Fig. 3.7). The differences amongst the spraying times showed a similar pattern to that of the frequency of dead quadrants. With the 25% crown treatment, spraying in May and November resulted in the lowest percentage of live shoots remaining, and February and August the highest (Fig. 3.7a) with August showing signs of recovery with an increase in percentage live shoots remaining from 16 to 22 months. With both the 50% and 75% crown treatment May and August spraying led to the lowest percentage (Fig. 3.7b, c). Spraying 75% of the crown in August resulted in no live shoots observed on any plants.

The percentage of crown sprayed (dose) had a highly significant effect ($p < 0.001$) on all three measured indices of health of the untreated crown quadrants, two growing seasons after all applications of the treatments (Table 3.3). Season of treatment had a significant effect only on the percentage of shoots flowering ($p = 0.025$). There were no significant interactions between dose and season (though this

was close to significant for percentage of shoots flowering). These effects are now examined in more detail in the following sections.

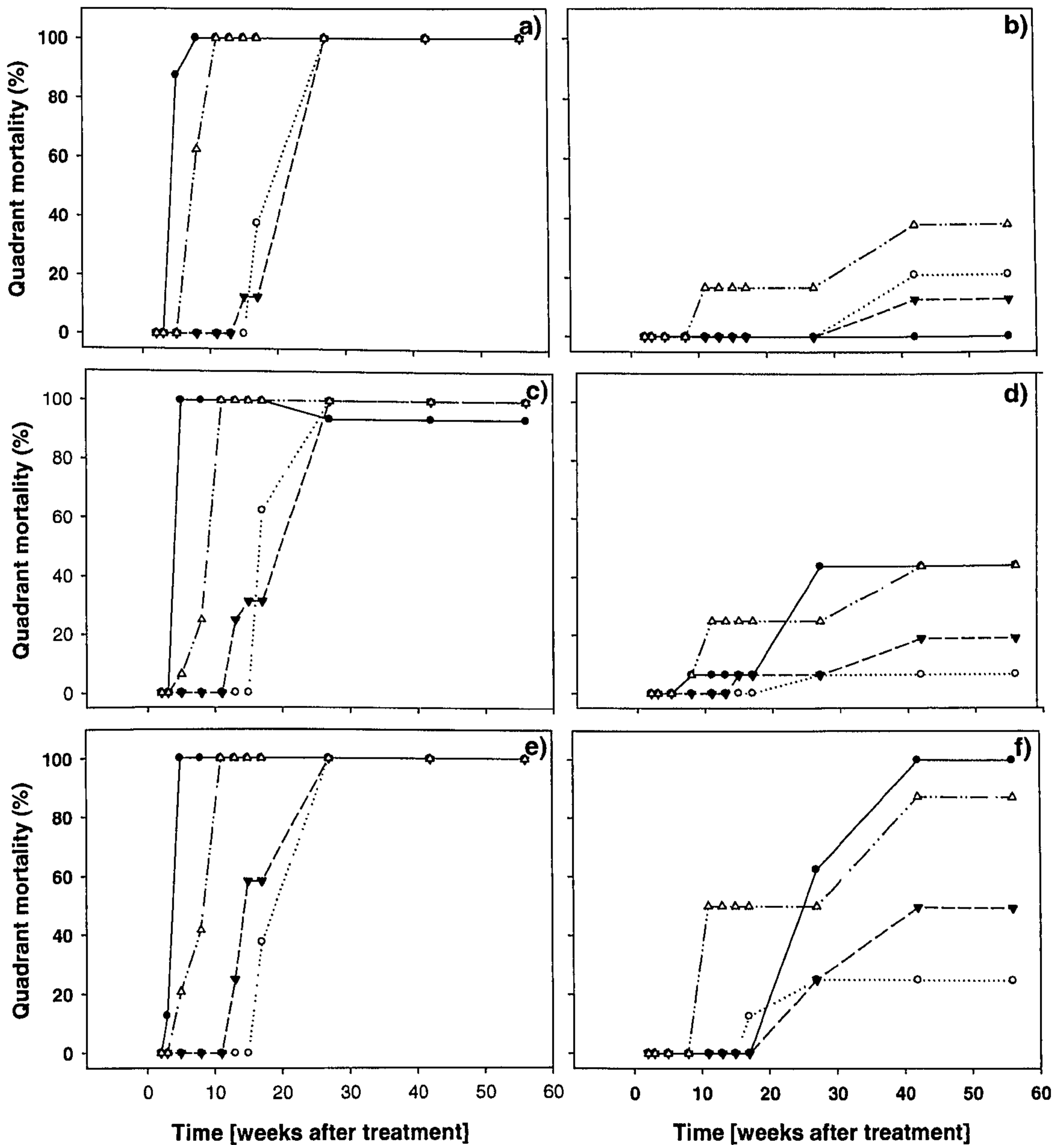


Figure 3.6 The effect of seasonal time of foliar spray (2% glyphosate, 2% Mixture B) application to *Rhododendron ponticum* plants < 1.5 m in height. The effect on treated crown quadrants (% total treated quadrants) over time after treatment where: a) 25%; c) 50% and e) 75% of the crown was treated; The effect on untreated crown quadrants (% total untreated quadrants) over time after treatment where: b) 25%; d) 50% and f) 75% of the crown was sprayed.

—●— August ◇..... November ---▼--- February -·-·-△-·-· May

Figure 3.6 The effect of seasonal time of foliar spray (2% glyphosate, 2% Mixture B) application to *Rhododendron ponticum* plants < 1.5 m in height. The effect on treated crown quadrants (% total treated quadrants) over time after treatment where: a) 25%; b) 50% and c) 75% of the crown was treated; The effect on untreated crown quadrants (% total untreated quadrants) over time after treatment where: d) 25%; e) 50% and f) 75% of the crown was sprayed.

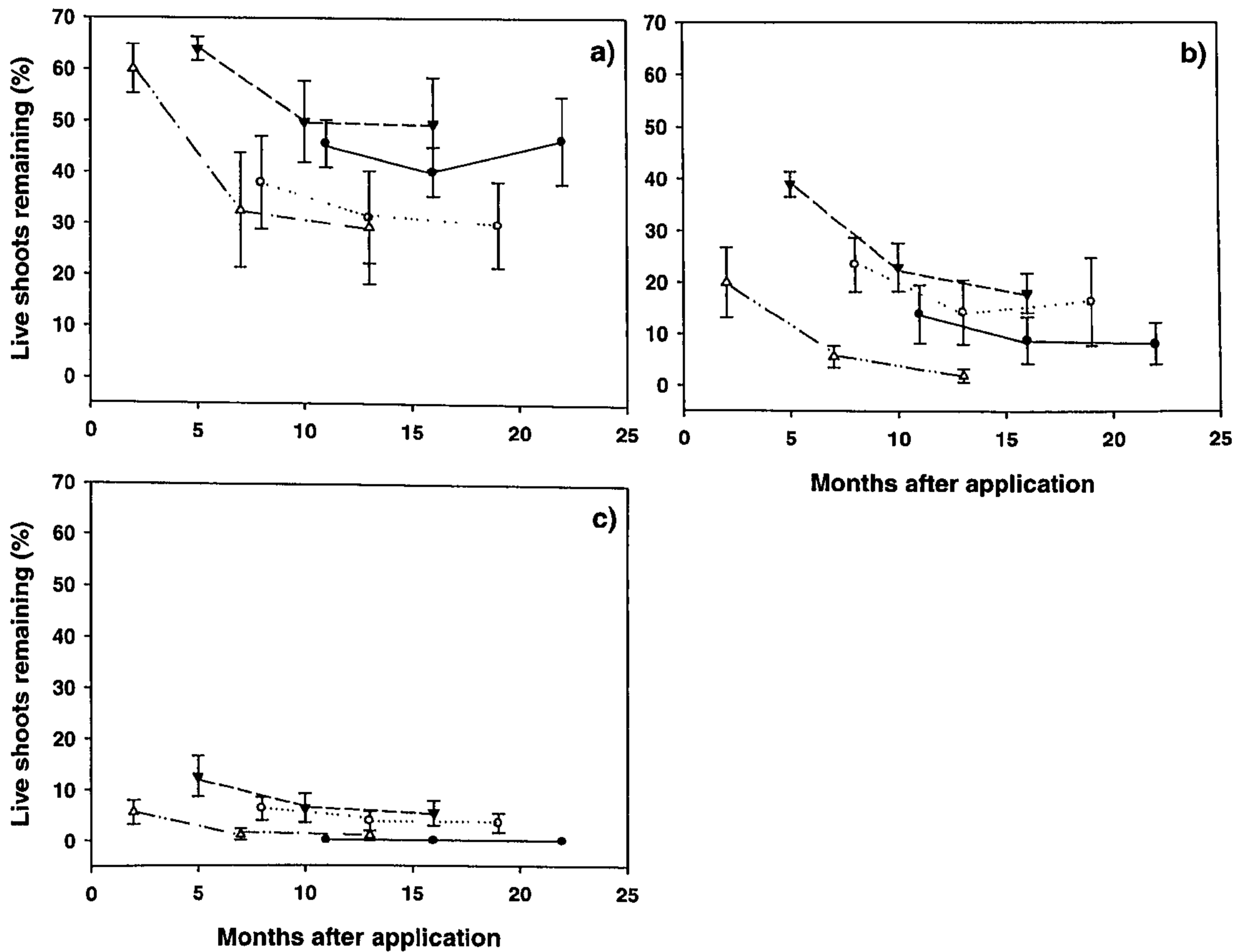


Figure 3.7 The effect of seasonal timing of foliar spray (2% glyphosate, 2% Mixture B in water) application to a) 25%, b) 50% and c) 75% of the crown of *Rhododendron ponticum* plants < 1.5 m in height on the percentage of live shoots remaining in the crown over a period of 22 months. Vertical bars are equal to the standard error of the mean.

—●— August ○..... November - - - ▼ - - - February - - - △ - - - May

3.6.1 The effect of dose on plant health

There was a significant ($p < 0.001$) difference in the total mean percentage live shoots remaining in the untreated crown quadrants after treating 25, 50 and 75% of the crown with glyphosate with 52, 25 and 6% (respectively) remaining after the first season of growth (July 2008, Table 3.3, Fig. 3.8a). After a further five months (December 2008) the effect of dose remained highly significant ($p < 0.001$) whilst

the percentages dropped to 38, 13 and 3% respectively. Six months later in May 2009 (during the second growing season) the effect of dose remained highly significant ($p < 0.001$); although there had been no subsequent reduction in live shoots remaining for all doses, there was also no increase (as there had been in the unsprayed control plants, up to 124% of the number counted at the first recording). Similar patterns were observed for new growth with a highly significant dose effect ($p < 0.001$, Table 3.3) — 31, 11 and 1% of shoots in the untreated crown quadrants exhibiting new growth in July 2008 after treating 25, 50 and 75% of the crown with glyphosate, respectively (Fig. 3.8b). The effect of dose remained highly significant ($p < 0.001$), whilst the percentages for these treatments were very similar in May 2009, whereas there was a noted increase in shoots showing new growth in the untreated control plants from 95 to 159% over the same period.

Table 3.3 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on three indices of the health of the crown of *R. ponticum* plants < 1.5 m in height at three times during the first two growing seasons after treatment. See Appendix 3.1-3.3 for full ANOVA.

Source of variation	Time of measurement		
<i>Time of observation</i>	July 2008	Dec 2008	May 2009
<i>Percentage of live shoots remaining</i>			
Block	0.661	0.409	0.422
Dose	0.000	0.000	0.000
Season of application	0.000	0.041	0.117
Dose x Season	0.004	0.536	0.215
<i>Percentage of shoots with new growth</i>			
Block	0.299	na	0.345
Dose	0.000	na	0.000
Season of application	0.235	na	0.482
Dose x Season	0.529	na	0.184
<i>Percentage of shoots flowering</i>			
Block	0.027	na	0.455
Dose	0.000	na	0.000
Season of application	0.723	na	0.025
Dose x Season	0.006	na	0.076

In both 2008 and 2009 there was a significant ($p < 0.001$) and clear dose response of reduced flowering with increased glyphosate dose (Table 3.3, Fig. 3.8c). The percentage of shoots in the crown producing flowers, increased from 2008 to 2009 for the untreated control plants (from 15% to 24%), in the plants with 25% of the crown treated (from 5% to 14%) and in the plants with 50% of the crown treated (from 2% to 3%) (Fig. 3.8c). However, in the plants with 75% of the crown treated there was no increase with $< 1\%$ of shoots producing flowers in both years.

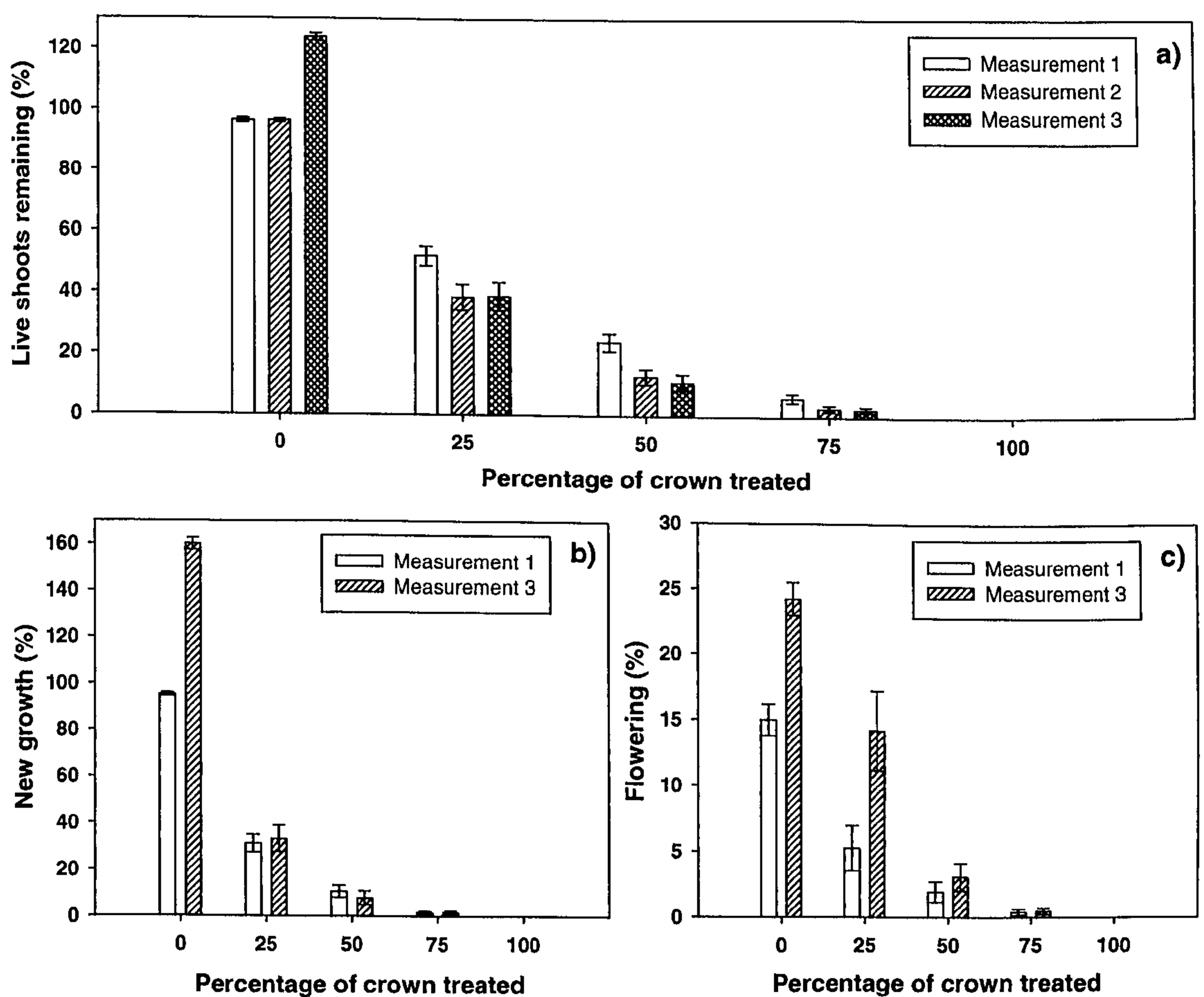


Figure 3.8 The effect of percentage of crown (0, 25, 50, 75, 100%) treated with glyphosate (2% glyphosate, 2% Mixture B) foliar spraying on the percentage of: a) live shoots; b) new growth; and c) flowering shoots remaining in the crowns of *Rhododendron ponticum* plants < 1.5 m in height at different times after treatment. The values above 100% in all panels are explained by its calculation as the percentage of the total number of shoots counted in the first recording. Measurement 1, 2 and 3 were made in July 2008 (1st growing season after application), December 2008, and May 2009 (2nd growing season after application), respectively. Vertical bars are the standard error of the mean.

3.6.2 The effect of season on plant health

Although there was no significant effect of season of treatment on the percentage of live shoots at the end of two growing seasons after glyphosate foliar treatment, this effect had been significant at intermediate times ($p < 0.001$ in July 2008 but just $p = 0.041$ in December 2008, Table 3.3). The percentage of shoots remaining alive remained highest for the February 2008 treatment throughout. However, for the May 2008 treatment this percentage, while high soon after treatment in July 2008, then fell greatly (to the lowest level for any of the seasonal treatments, 13% in December 2008 and 11% in May 2009) (Fig. 3.9a). There was also a strong dose x season interaction in July 2008 ($p = 0.004$, Table 3.3), which is attributable to the high effectiveness in killing shoots of the August 2007 75% treatment, but the low effectiveness of the August 2007 25% treatment.

Because of the effect of the variable time elapsed between the treatment times and the dates of observation, an additional analysis was carried out on the observations closest to one year after the time of each seasonal treatment (Fig. 3.9b). Both dose ($p < 0.001$) and season of treatment ($p = 0.016$) had highly significant effects on the percentage of live shoots remaining (Table 3.4).

Season of treatment had no significant effect on the percentage of shoots producing new growth in either the first or second growing season after treatment (Table 3.3, Fig. 3.9c). However, while there was no effect of season of treatment on flowering in the first growing season (July 2008), by the second growing season, the percentage of shoots producing flowers on the plants treated in May (1%) was significantly less than for the other seasons of treatment (6%-10%) (Fig. 3.9d, $p = 0.025$, Table 3.3). Although there were still some flowering shoots presents on the plants subject to all treatments, $\geq 90\%$ of these were stunted unable to reproduce.

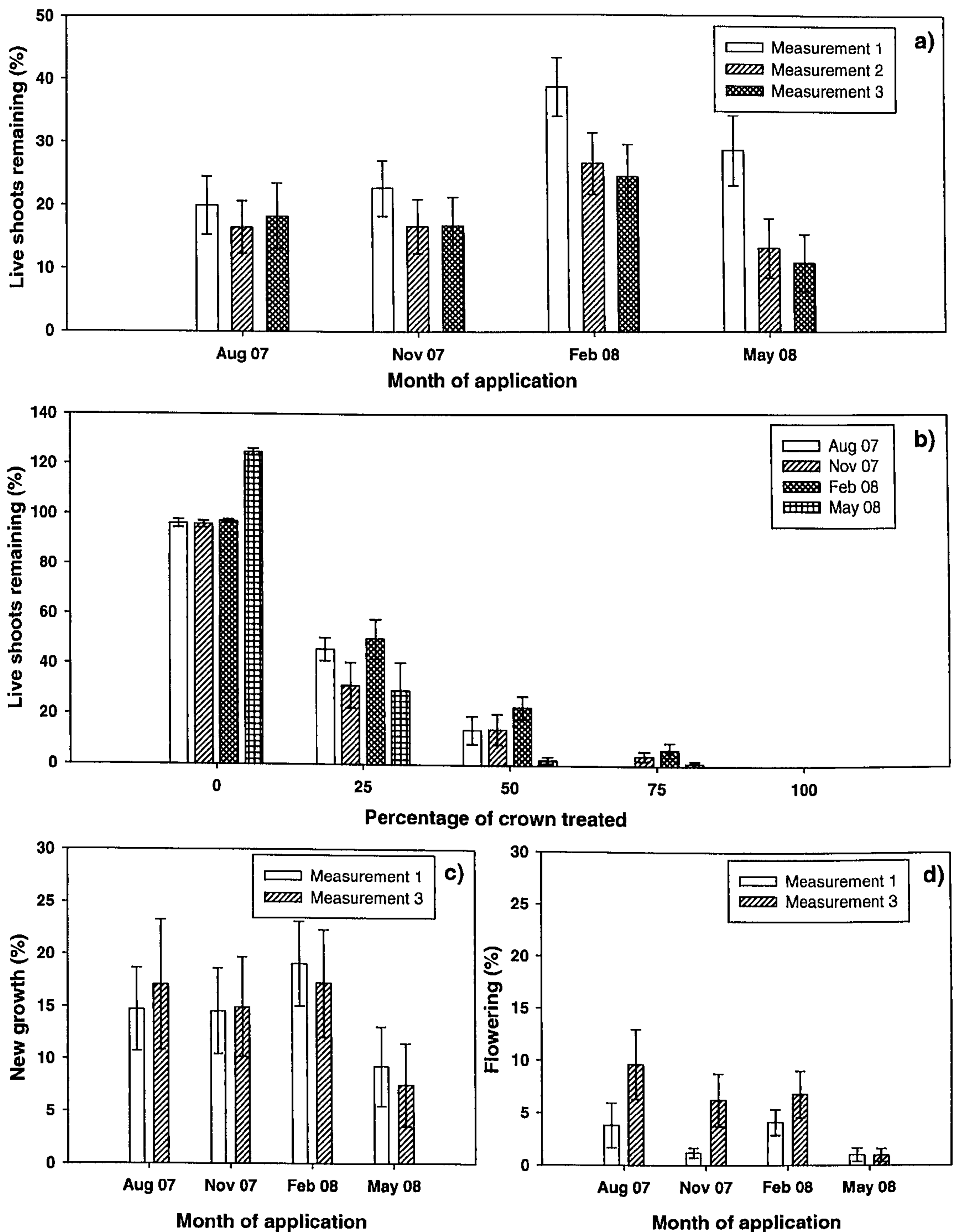


Figure 3.9 The effect of seasonal timing of treatment - glyphosate (2% glyphosate, 2% Mixture B) foliar spraying - on the percentage of a) live shoots remaining in the crowns of *Rhododendron ponticum* plants < 1.5 m in height at different times after treatment; b) The interaction of dose (% crown treated) and season of application on the percentage live shoots remaining 1 yr. (closest) after the treatments were applied; the effect of seasonal timing of treatment - glyphosate (2% glyphosate, 2% Mixture B) foliar spraying - on the percentage of: c) new growth and; d) flowering shoots remaining in the crowns of *Rhododendron ponticum* plants < 1.5 m in height at different times after treatment. Measurement 1, 2 and 3 were made in July 2008 (1st growing season after application), December 2008, and May 2009 (2nd growing season after application), respectively. Vertical bars are the standard error of the mean.

Table 3.4 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on the percentage of live shoots remaining approximately one year after treatment. See Appendix 3.4 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
Block	2206.372	7	0.328
Dose	22507.547	2	0.000
Season of application	3066.060	3	0.016
Dose x Season	1419.044	6	0.508

Table 3.5 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on the growth of new shoot growth (shoot length, new leaf length) after one growing season. See Appendix 3.5 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
<i>New shoot growth</i>			
Block	437.721	7	0.000
Season	132.281	3	0.000
Dose	1271.288	3	0.000
<i>New leaf growth</i>			
Block	203.469	7	0.173
Season	91.019	3	0.203
Dose	1314.058	3	0.000

The percentage of the crown treated had significant effects on the extension growth of new shoots and the size of new leaves in the untreated parts of the crowns after the first growing following treatment ($p < 0.001$ for both, Table 3.5). There was clear evidence of a dose-response relationship, average new shoot lengths for the 0, 25, 50 and 75% treatments were 6.0, 2.9, 2.0 and 1.4 cm, respectively (Fig. 3.10a), and average new leaf lengths were 8.0, 5.3, 4.2 and 3.0 cm, respectively (Fig. 3.10b). Shoot length for the May treatment was significantly higher ($p < 0.001$) than the other months, although this could probably be attributed to the proximity of treatment time to measurement time (2 months). There was also some variation in shoot length between blocks for new shoot growth but not for new leaf growth. The glyphosate treatment also reduced the photosynthetic rate of leaves on the untreated

parts of the crown (P_{max} being reduced from 38.6 to 16.1 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) (Fig. 3.10 c,d). A revisit to the *R. ponticum* bushes treated with the control droplet application technique (Pakatul, 2007) found that even bushes sprayed at the highest dose (20 l (Hillite) per hectare) showed some signs of recovery with new vegetative buds growing on the stems. The 20 l ha^{-1} dose is equal to 2880 g of glyphosate per hectare (144 g l^{-1}). The dose at which the 100% treatment was applied in this trial was 10 l ha^{-1} (360 g glyphosate per litre) which is equal to 3600 g of glyphosate per hectare. This is equivalent to 25 l ha^{-1} of the Hillite CDA method; similarly, the 75, 50 and 25% doses would be equivalent to 19, 13 and 7 l ha^{-1} .

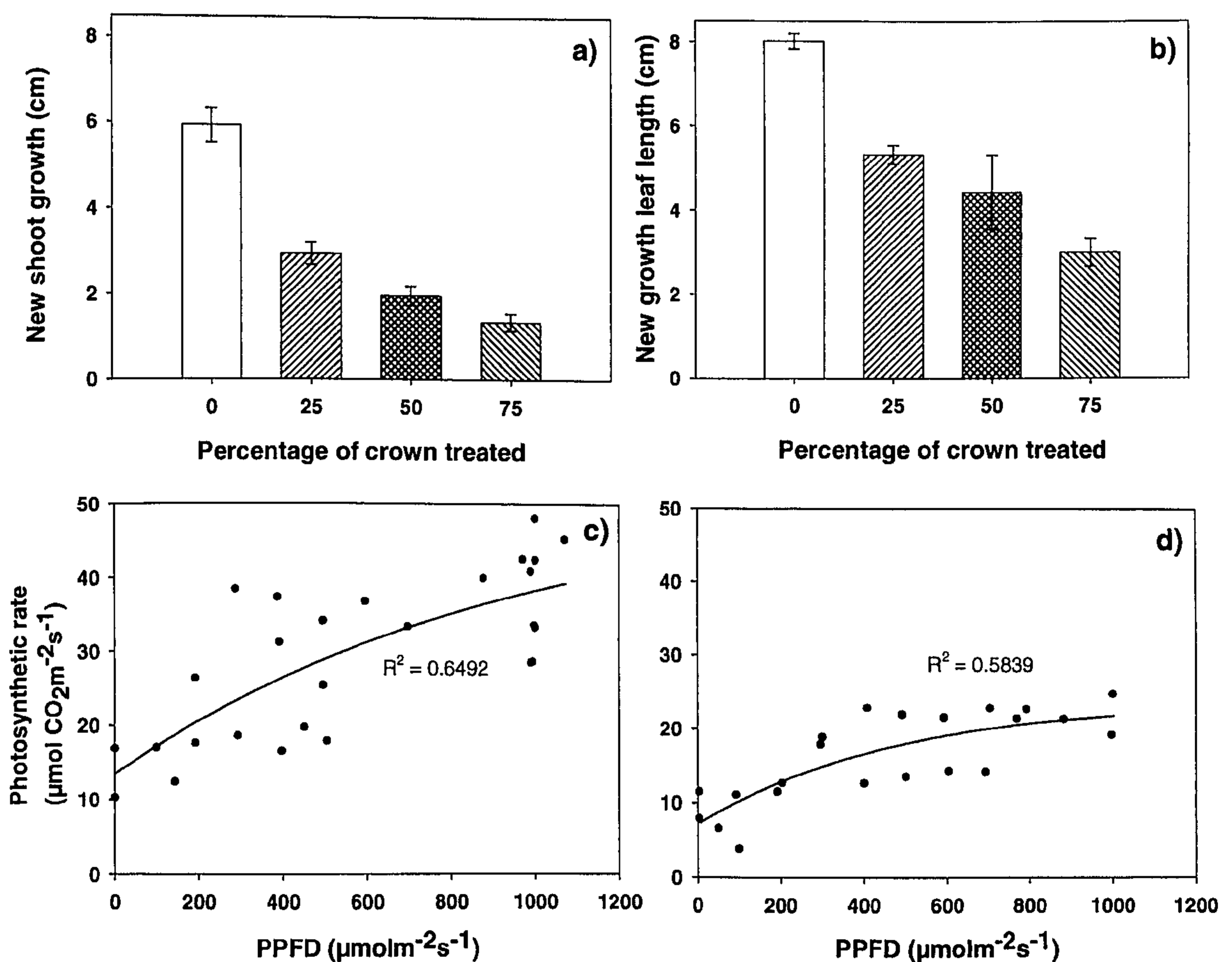


Figure 3.10 The effect of percentage of the crown treated with foliar spraying of glyphosate (2% glyphosate and 2% Mixture B) on shoot growth and physiology of the untreated crown quadrants of *R. ponticum* plants < 1.5 m in height after one growing seasons: a) length of shoot extension growth; b) length of new leaves; c) photosynthetic light response curve of untreated (healthy) control plants; d) treated (unhealthy) plants. Data was fitted with a 3-parameter exponential rise to maximum function (Peek *et al.*, 2002). Vertical bars are standard error of the mean.

3.7 DISCUSSION

Season of application had a significant effect on plant health. The different indices of damage, recorded at various times after treatment showed variation in the relative amounts of damage to untreated crown quadrants, by spraying at different seasonal times. While none of the four times proved wholly ineffective, spraying in May was the most consistently effective and February the least. Spraying in August was also effective, providing that at least 50% of the crown was treated (but not with treatment of only 25%); spraying 75% of the crown then resulted in the death of all the plants. Incomplete spraying of the crowns, especially in November and February did not cause entire plant death. Complete foliar treatment (to leaf surface saturation) with glyphosate spray of the entire crown of *R. ponticum* plants < 1.5 m in height, caused complete mortality of the plant, whatever time of the year the treatment was applied. Although Edwards (2006) showed that foliar application with herbicides, including glyphosate, is effective at any time of year, he recommends foliar application March to October for best results. Lawrie and Clay (1993b) came to a similar conclusion with the herbicide Imazapyr. While they recommended avoiding foliar spray treatment during the winter months, they found that October applications were the most effective and suggested that this may have been due to higher levels of basipetal sap flow at that time. In the present study, the higher rates of damage with August and especially May treatments correspond to times of the year when the plant is metabolically most active. Similar results have been found in other studies where application of herbicide was most successful in late or early summer (Lynn *et al.*, 1979; Nyboer, 1992). Foliar treatments applied in the summer and autumn to five Sierran shrub species were found to be less effective than spring applications, corresponding to times of higher moisture stress and rates of photosynthesis (Lanini & Radosevich, 1982). For control of Canada thistle, treatment with glyphosate is often recommended at the late-bud to midflower growth stage, or in late fall. Photoassimilate translocation to roots is assumed to be more active at these growth stages and the herbicide, moving with the assimilate stream, would thus be more evenly distributed. (Kirkland, 1977)

Damage caused to untreated crown quadrants of treated plants increased with the percentage of the crown sprayed and, in a more complex way, with the season of application. The positive relationship between percentage crown sprayed and damage can be attributed to a simple dose-response: the greater the number of sprayed leaves, the greater the total glyphosate dose received by the plant. However, it could also be attributed to the broader distribution of the glyphosate around the leaf-phloem/xylem-root components of the plants vascular system. Conforming to the pipe model theory, that a given unit of leaves is serviced by a continuation of conducting tissue of constant cross-sectional area, analogous to a pipe system (Shinozaki *et al.*, 1964), the current practice guide for managing and controlling rhododendron in the UK, suggests that there is no translocation of herbicides in rhododendron plants (Edwards, 2006). The present study found this not to be the case, with all treatments causing some levels of damage to untreated parts of the crown, showing that some translocation of glyphosate must have occurred. Leaves from untreated parts of the crown suffered from a decrease in rate of photosynthesis further supporting evidence from Pakatul (2007) who found a reduction in photosynthesis 19 days after applying glyphosate to potted *R. ponticum* plants.

Informal observations of the *R. ponticum* bushes treated with the control droplet application technique (Pakatul, 2007) found that bushes sprayed at the highest dose (20 l (Hillite) per hectare) some of which had appeared dead three months after application, showed signs of recovery two years later. This treatment dose was equivalent to treating 75% of the bush in this study which also did not achieve complete control. It is therefore imperative that the plant's entire crown is sprayed as missing even a small section may result in partial recovery. Applying the herbicide in summer or autumn reduces the likelihood of this happening in the event of partial treatment. Therefore, the completeness of the treatment applied by practitioners, especially if they are operating in late autumn or winter, will have a large impact on the effectiveness of control and the likelihood of expensive follow-up treatments being required.

CHAPTER IV: The absorption and translocation of glyphosate in *Rhododendron ponticum* under different light levels

4.1 ABSTRACT

Assumptions on the mode of action of herbicides in *Rhododendron ponticum* have been largely inferred from non-physiological trials. In order to optimize the efficacy of herbicides in particular glyphosate, knowledge about the physiological properties of the compound, its behaviour in *R. ponticum* regarding uptake and translocation, and how these processes are affected by environmental factors is essential. The effects of light on the rate of foliar absorption and translocation of glyphosate in *R. ponticum* were investigated in a glasshouse experiment. ^{14}C -labelled glyphosate was applied to the abaxial leaf surface of *R. ponticum* plants and the radiolabel was measured quantitatively by combustion and scintillation counting in plants harvested 0.25, 3, 7, 14 and 30 days after treatment. There was an initial rapid absorption of foliar applied ^{14}C glyphosate over the first 6 hours (55%) reaching 88% after 3 days, and followed by a more gradual increase to 93% after 30 days. Supplemental lighting did not increase the absorption of the ^{14}C but did increase the amount translocated to other parts of the plant. The greatest translocation was to the stem on which the treated leaf was located, followed by accumulation in the roots and the leaves on the same stem above the treated leaves.

4.2 INTRODUCTION

Rhododendron ponticum is an invasive weed that is causing severe damage to semi-natural habitats in many parts of the British Isles (Rotherham, 2001). A major component of the current control strategy is treating *R. ponticum* plants with foliar spray of glyphosate, considered to be the most effective herbicide currently licensed for this use in the European Union (Edwards, 2006). There is much concern about the cost and incomplete effectiveness of much of this control practice, with *R. ponticum* plants not being killed if complete saturation of all the leaf surfaces with glyphosate spray is not achieved or if rainfall occurs too soon after the spraying. Current knowledge of the importance of spray coverage of every stem or leaf, dose required per plant and the relative effectiveness of spraying at different times of the year is hindered by a lack of knowledge of the rate of absorbance of glyphosate into *R. ponticum* leaves, and then its rate and pattern of translocation throughout the plant. To date work on herbicide impacts on *R. ponticum* has largely been based on field and pot trials of different control practices. Other studies have concentrated on field testing the efficacy of various herbicides, adjuvants, herbicide concentrations and application rates. Assumptions on the mode of action of herbicides in *R. ponticum* have therefore been largely inferred from non-physiological trials in which only the effectiveness of killing treated plants has been monitored.

N-(phosphonomethyl) glycine (glyphosate) is a broad spectrum, non-selective, post-emergence systemic herbicide effective on essentially all annual and perennial plants (Knuutila & Knuutila, 1985). Glyphosate works by being absorbed into the plant primarily through its leaves but also through soft stem tissue (Fig. 4.1). It is then rapidly transported throughout the plant where it acts on various enzyme systems inhibiting amino acid metabolism in the shikimic acid pathway. Glyphosate is a competitive inhibitor of 5-enolpyruvylshikimic acid-3-phosphate (EPSP) synthase, an enzyme of the shikimic acid pathway (Steinrucken & Amrhein, 1984). Major end-products of the pathway are the aromatic amino acids such as phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Tryp) which are used in protein synthesis in addition to phenolic end-products, e.g. lignin precursors, flavonoids and tannins (Cole, 1985). This pathway exists in higher plants and microorganisms but

not in animals. As the primary site of action of glyphosate is associated with inhibition of meristematic activity, translocation from the intercepting leaves to both above- and below-ground meristems is an essential stage in successful glyphosate treatments.

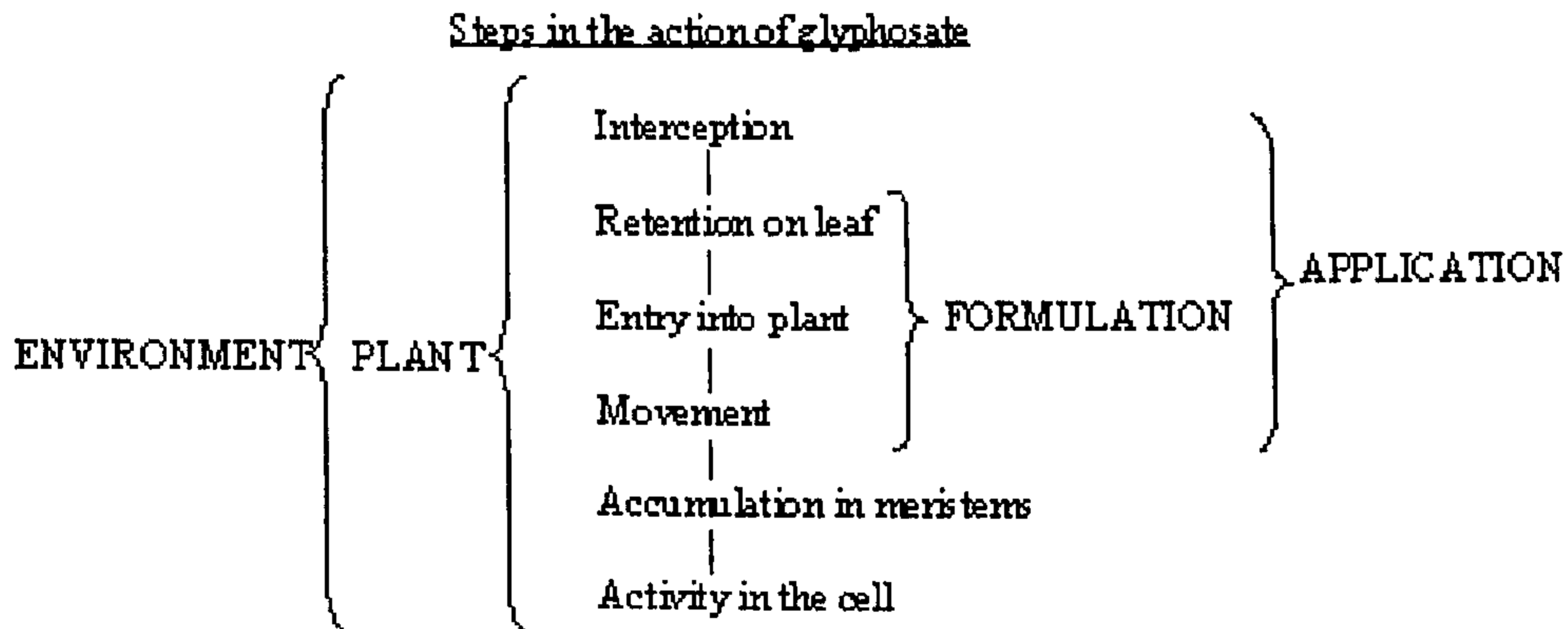


Figure 4.1 Factors affecting the steps involved in glyphosate activity (Caseley & Coupland, 1985).

A polar, water-soluble compound such as glyphosate rapidly penetrates the leaves under favourable conditions and probably enters the cuticle via the hydrophilic pathway, followed by a longer phase of slower penetration (Coupland & Caseley, 1985). Thick cuticles have been found to be the main barrier to foliar absorption of glyphosate (Bukovac, 1976). The translocation of glyphosate in plants involves cell-to-cell movement as well as longer-distance transport in the vascular tissues. Transfer across cell membranes is necessary for glyphosate to enter the symplast and to move within the phloem system. Autoradiographs have shown that ^{14}C -glyphosate is phloem-loaded and is therefore termed a phloem-mobile herbicide (Baker & Millburn, 1989; Brudenell *et al.*, 1995). However, glyphosate also exhibits apoplastic movement in between cells and in the xylem tissues (Marquis *et al.*, 1979; Bingham *et al.*, 1980; Lolas & Coble, 1980). Its distribution in plants is therefore also determined by those factors that influence plant transpiration (Devine *et al.*, 1990; Grangeot *et al.*, 2006). Little is known, however, about those factors, which determine the relative amounts of glyphosate translocated in the symplast or apoplast. Similarly, when and where this interchange between xylem and phloem occurs is little understood (Caseley & Coupland, 1985).

The mode of action of glyphosate in perennials is such that best efficacy results when the herbicide is translocated to the root system and stored over winter, retranslocation in the spring then results in the death of new growth (Franz, 1985). Glyphosate has been shown to follow photoassimilate distribution (Sprankle *et al.*, 1975a; Sandberg *et al.*, 1980; Gougler & Geiger, 1981; McAllister & Haderlie, 1985; Feng, 2003) accumulating predominantly in areas of high protein synthesis such as plant meristems in the shoots, roots and rhizomes (Sprankle *et al.*, 1975a; Wyrill & Burnside, 1976; Sandberg *et al.*, 1980; Lundhoie, 1985; Feng *et al.*, 2003; Grangeot *et al.*, 2006). The variation observed in susceptibility to glyphosate may be due to differences in plant growth stage (Rioux *et al.*, 1974; Ahmadi *et al.*, 1980; Hunter, 1995; Bariuan *et al.*, 1999), leaf cuticle and epicuticular wax (Bukovac, 1976; Green *et al.*, 1992) or environmental conditions (Hammerton, 1967; Jordan, 1977; McWhorter, 1978; Gerber *et al.*, 1983), including light photoperiod and intensity (Caseley & Coupland, 1985), that influence processes such as herbicide uptake, transpiration or plant metabolism.

A literature search revealed no references on the physiological mechanism of the action of glyphosate in *R. ponticum* or closely related species. Predictions of uptake and translocation of glyphosate (and the factors that may affect them) in *R. ponticum* is therefore largely based on extrapolation from research into glyphosate effects on other species (especially herbaceous ones). In this case it would be predicted that glyphosate flow from *R. ponticum* leaves would initially occur only in the direction of phloem flow, through the stem tissue below the leaf into the root system. It would then only enter other parts of the shoot system after it is translocated back up from the roots with xylem flow. In order to optimize the efficacy of herbicides in particular glyphosate, it is essential to gain a better understanding of the physiological properties of the compound, its behaviour in *R. ponticum* regarding absorption and distribution, and how these processes are affected by environmental factors.

4.3 OBJECTIVES

The aim of the present study was to test the following hypotheses concerning the movement of glyphosate applied to the leaves of *R. ponticum*:

1. The rate of absorption into the leaf to which it is applied is rapid, the majority occurring within the first 24 hours.
2. Rate of absorbance is increased by higher light levels.
3. Translocation of glyphosate from the leaf it is absorbed into is initially mostly basipetal, with higher accumulation in the root than the shoot system (excluding treated leaf).
4. The rate of translocation into the root system and then into other parts of the shoot system is increased by higher light levels.

Translocation of glyphosate has been studied directly, using the ^{14}C -labelled herbicide, and also indirectly by measuring certain physiological responses caused by the presence of the herbicide (Caseley & Coupland, 1985). Examples of the former include qualitative autoradiographic techniques and quantitative measurements of the amounts of radioactivity in various parts of the plant. ^{14}C -labelled glyphosate was used here to monitor glyphosate absorbance into leaves and translocation to different parts of *R. ponticum* plants in an experiment designed to test the above hypotheses..

4.4 DEVELOPMENT OF METHODOLOGY

4.4.1 Preliminary experiment I

Prior to carrying out the main experiment in this chapter, a number of questions regarding methodology (application method, design, and sample analysis) needed to be developed and tested. The first in this series of experiments was designed: (1) to test a widely-used method for determining the absorption and translocation of ^{14}C -

glyphosate; (2) to get an approximate quantification of the degree and rate of foliar absorption and translocation of ^{14}C -glyphosate to different parts of *R. ponticum* plants grown in pots in greenhouse conditions; and (3) to investigate if light level had an effect on absorption or translocation.

Methods

The plants used in the experiment were propagated from 20-cm cut stems of local source material from Dingle Nursery, Welshpool, Wales, UK and grown in peat medium in two-litre plastic pots in full light. Thirty six plants of similar growth form and size (height 30 to 50 cm), were selected and placed under two different light treatments in an unheated glasshouse. The light treatments were: (i) ambient light levels for August; (ii) supplemental lighting with a 14-hour photoperiod (Photosynthetic photon flux density (PPFD) of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$). Plants were allowed to acclimatise for two weeks before foliar application on 12 August 2007. Nine harvest times for each of the two light treatments were assigned randomly to the 36 plants giving two replications of each. Five adjacent leaves approximately mid way along the main stem were chosen on each plant and sprayed with 2% glyphosate, 2% Mixture B (in water). When the leaves had dried, a single $0.5 \mu\text{l}$ droplet containing approximately $0.2 \mu\text{Ci}$ of ^{14}C -glyphosate* was applied to the centre of the adaxial surface of each leaf using a dispensing pipette.

The plants were harvested 2, 4, 8, 12 and 24 hours and 3, 7, 14 and 30 days after treatment. These times were selected based on previous absorption and translocation studies with glyphosate; times over the first 24 hours were chosen to look at foliar uptake which has been recorded as being rapid; the longer time intervals were chosen to look at the translocation (Green *et al.*, 1992; Grangeot *et al.*, 2006).

* ^{14}C -glyphosate (glycine-2- ^{14}C) in aqueous solution (sterile water) specific activity $0.37 - 1.11 \text{ GBq mmol}^{-1}$, 95% purity, concentration 0.1 mCi/ml (American Radiolabeled Chemicals Inc., town, Missouri, USA).

The treated leaves were excised and washed three times for 15 seconds each with a 20 ml volume of distilled water to ensure complete removal of unabsorbed glyphosate and to quantify the efficacy of the washes. Radioactivity in 1 ml of the leaf wash was determined using 4 ml of Optiphase HiSafe 3 scintillation cocktail solvent (PerkinElmer, Massachusetts, USA). At each harvest time treated plants were sectioned into six different parts: the five treated leaves (each sampled separately), the section of stem on which the treated leaves were located (stem was cut 1 cm below the lowest treated leaf and 1 cm above the highest treated leaf), a section of the same stem above the treated leaves, all the leaves on the same stem above the treated leaves, all of the leaves on the same stem below the treated leaves, all the leaves on a separate adjacent stem.

The washed treated leaves and other plant parts were put into separate brown paper bags and placed in an oven at 70 °C for 48 hours to dry. The samples were removed from the oven, weighed, and the dried plant parts were ground to powder in a Retsch MM200 Ball Mill (Retsch GmbH, Haan, Germany) for 30 - 60 seconds per sample. In between sample grinds, the sample containers were washed with methanol and distilled water to prevent cross-contamination. The powder samples were then transferred into 5 ml plastic containers. Fifteen millilitres of Oxosol ¹⁴C-scintillant (National Diagnostic, Atlanta, Georgia, USA) were measured into a glass CO₂ trap and attached to a Harvey OX500 Biological Oxidizer (R.J. Harvey Instruments Corp., Hillsdale, New Jersey, USA).

A 100-mg sub-sample was weighed into a ceramic boat, which was subsequently placed on a quartz ladle and combusted in the biological oxidiser for four minutes at 900 °C. The glass trap was removed and the contents were emptied into a clean glass 25 ml scintillation vial. The trap was washed out with methanol between samples to remove any remaining radiation. ¹⁴CO₂ in the samples was determined by using a MicroBeta® TriLux scintillation counter (Perkin Elmer Life and Analytical Sciences Inc., Waltham, MA, USA) to count the disintegrations per minute (dpm).

The amount of ^{14}C -glyphosate in each plant component part was calculated by multiplying the activity per 100 mg subsample (for each plant part) times 10 to get dpm/g dry weight and multiplying this by the dry weight (g) for each different plant component where possible (leaves) – total absorbed radioactivity was the sum of the radioactivity in the different plant components. This was then expressed as a percentage of the total amount applied: the sum of unabsorbed radioactivity from leaf washes plus total recovered from the plant to give total absorbed radioactivity (%).

Translocation from the treated leaves to the different harvested plant component parts was radioactivity in different plant parts (dpm in 100 mg subsample) expressed as a percentage of the sum of radioactivity in all plant component subsamples (dpm per 100 mg).

Results and implications for methodology

Due to the low level of replication there was a high degree of variance around the mean values per treatment. Nonetheless, there was some indication of an increase in absorbance of ^{14}C -glyphosate over the 30 days. There was no evidence of a trend in absorbance between the five measurements made during the first 24 hours in either light treatment (Fig. 4.2a,b). There was also no evidence of a marked difference between the plants under supplemental light (Fig. 4.2c) and ambient light (Fig. 4.2d) up to seven days, however at 14 and then 30 days the mean absorbance increased markedly in the plants under supplemental light, while falling in those under ambient light. The distribution of the translocated ^{14}C to different plant parts was highly uneven (Fig. 24e). It was substantially highest in the part of the stem on which the treated leaves were located (58%) followed by the treated leaves themselves (21%). Activity in the four other parts ranged from 3 to 7%.

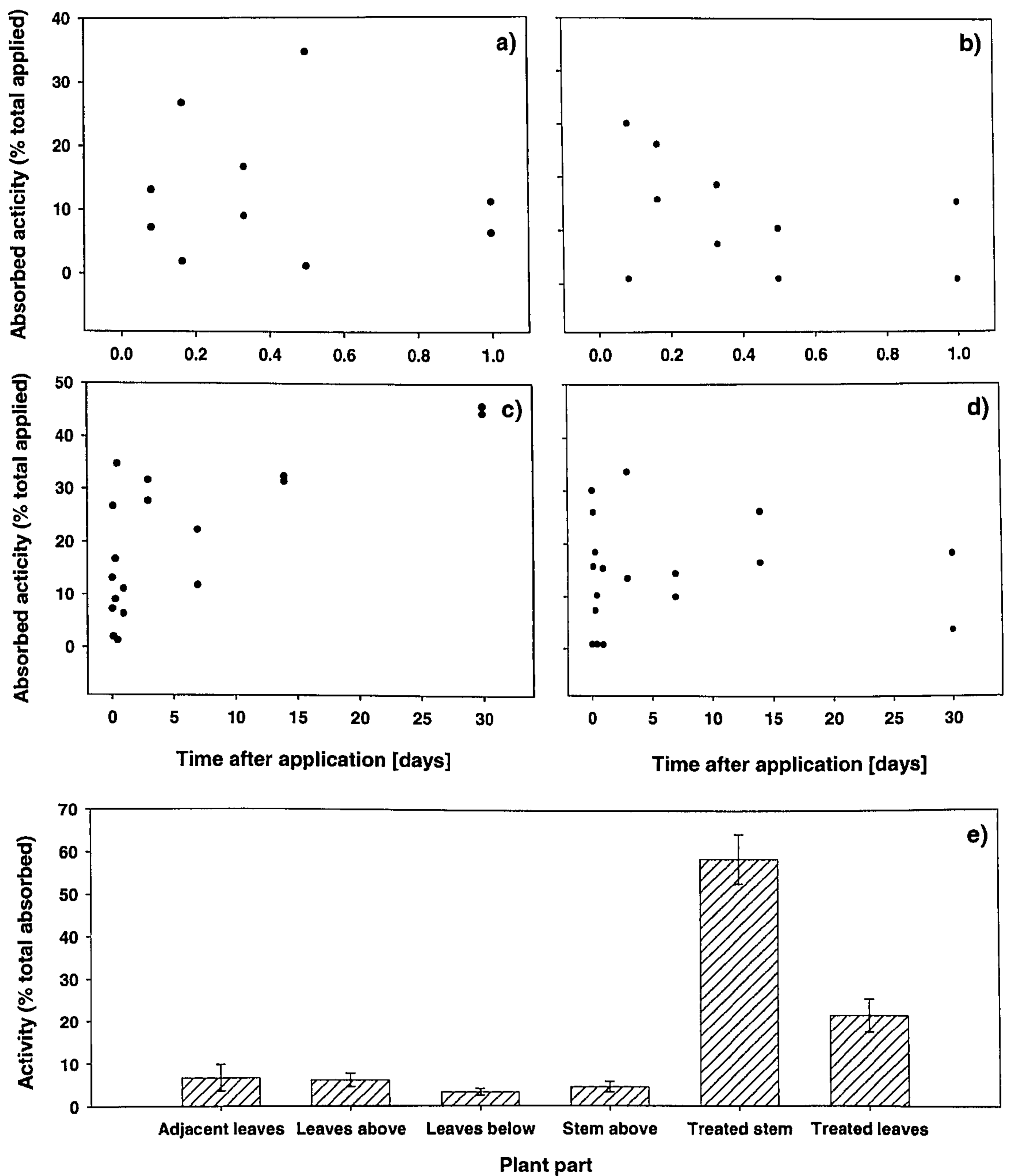


Figure 4.2 Absorption and partitioning of the activity of ^{14}C -glyphosate applied to 30 – 50 cm height *Rhododendron ponticum* plants over 30 days. Absorption (as a percentage of the total applied) over 1 day in plants kept under a) supplemental light and b) ambient light. Absorption (as a percentage of the total applied) over 30 days in plants kept under c) supplemental light and d) ambient light. e) Partitioning of this absorbed activity amongst six parts of the plants (average of plants kept under both light regimes harvested at 30 days). Vertical bars are standard errors of the means.

Based on the experience of conducting this preliminary trial, the following modifications were made for the main trial.

To reduce the amount of variability amongst replications and eliminate unnecessary measurements:

- The replication was increased to six plants per harvest per treatment.
- The number of harvest times was reduced to include only one time within the first 24 hours but maintaining the longer harvest times.
- ^{14}C -glyphosate was applied to only three leaves and time could be saved by mixing the treated leaves for sampling, with little cost to capacity to test the hypotheses (as the individual leaves are 'pseudo-replicates' within the plant).
- It was decided to continue to harvest leaves above, leaves below, treated stem, adjacent leaves, and to drop stem above but add roots.

During the application of ^{14}C -glyphosate, the droplets became unstable and dripped down onto the stem, and this was likely to have been responsible for the high levels of activity found in the treated stem. Therefore, in the main trial:

- The size of the droplet (5 μl) was reduced to 2.5 μl ; one of these smaller droplets was applied to either side of the leaf midrib to minimise spreading.
- The ^{14}C -glyphosate droplets were applied to the inside of a ring of Vaseline to minimise spreading and drip.
- Any leaf where the droplet became unstable and dripped was immediately excised and discarded and a droplet was applied to a new leaf.
- The unlabelled glyphosate (2% glyphosate with 2% Mixture B in water) was applied with a brush to untreated areas to minimise spray drift, drip and to allow the labelled glyphosate to be applied effectively at the same time (as opposed to having to wait for the surface to dry).

4.4.2 Preliminary experiment II

Use of ^{14}C -radiolabelled glyphosate to quantify its absorbance and translocations imposes many constraints on experimental design, e.g. for reasons of cost and safety. In addition, monitoring the distribution of ^{14}C activity does not necessarily correspond with the distribution of effects of glyphosate on the plant. Glyphosate competes with the substrate phosphoenolpyruvate (PEP) for a binding site on the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Cole, 1985). Upon the inhibition of EPSPS by glyphosate, levels of shikimic acid, the metabolic precursor of shikimate 3-phosphate, have been reported to increase in sensitive plants (Pline *et al.*, 2002, Bresnahan *et al.*, 2003, Mueller *et al.*, 2003). Therefore, the concentration of shikimic acid is a good candidate as an indirect assay of the disruption to plant metabolism by glyphosate. The objectives of this research were (1) to evaluate if shikimic acid accumulates in *R. ponticum* in response to glyphosate application, and (2) to correlate shikimic acid accumulation to ^{14}C -glyphosate activity and assess the possibility of using shikimic acid concentration as an assay for future trials.

Methods

The plants used in the experiment were propagated from 20-cm cut stems of local source material from Dingle Nursery, Welshpool, Wales, UK and grown in a peat medium in two-litre plastic pots in full light. Twelve plants of similar growth form and size (height 30 - 50 cm) were selected and placed in an unheated glasshouse. Plants were allowed to acclimatise for two weeks before foliar application on 2 December 2007. The plants were divided into six matched replicate pairs, within which one plant was selected randomly for treatment and one as a control. Five leaves were selected in the mid section of different shoots of each plant (leaf position matched on plants); each leaf (and the section of stem on which it was located) was considered as a separate sample. Two rings of Vaseline were smeared on the adaxial (upper) leaf surface either side of the leaf midrib. One millilitre of glyphosate solution at normal field use rate (commercial RoundUp 360 ProBioactive at 2% and

Mixture B at 2% in water) was applied to each selected leaf using a brush to minimise drip and avoid the area inside the rings. Directly after foliar application of glyphosate (outside rings), two 2.5 µl droplets, each containing approximately 0.1 µCi of ¹⁴C-glyphosate in aqueous solution were applied to each leaf inside the Vaseline rings using a dispensing pipette. After application, the drops were carefully watched to ensure that they remained where placed. If a droplet seemed unstable, the treated leaf was immediately removed and discarded and a replacement leaf was selected.

After seven days, all the plants were harvested and the treated leaves and 2-cm stem sections (1 cm either side of petiole) were excised. The treated leaves were excised and washed three times for 15 seconds each with a 20 ml volume of distilled water to ensure complete removal of unabsorbed glyphosate. Each leaf and stem section was cut in half with a razor blade and weighed. One half of each was then put in a separate brown paper bag and placed in an oven at 70 °C for 48 hours to dry. These samples were then weighed again and ground in a Retsch MM200 Ball Mill (Retsch GmbH, Haan, Germany) for 30 seconds. One hundred milligramme subsamples were weighed out and combusted in a biological oxidiser (Harvey OX500 Biological Oxidizer, R.J. Harvey Instruments Corp., Hillsdale, New Jersey, USA). Radioactive CO₂ was trapped in 15 ml of Oxosol ¹⁴C scintillant (National Diagnostic, Atlanta, Georgia, USA) which was subsequently emptied into a 25 ml glass vial and measured in a MicroBeta® TriLux scintillation counter (Perkin Elmer Life and Analytical Sciences Inc., Waltham, MA, USA). Radioactivity per 100-mg sample was displayed as disintegrations per minute.

The amount of ¹⁴C-glyphosate in the leaves and stem section was calculated by multiplying the activity per 100 mg (dpm/100mg) subsample (for each plant part) times 10 to get dpm/g dry weight and multiplying this by the dry weight (g) for each different plant component half. This was then multiplied by two to get total activity (dpm) per leaf or stem. This was subsequently expressed as a percentage of the total applied activity (total absorbed radioactivity (sum of the radioactivity in the treated leaf and stem) plus unabsorbed radioactivity from leaf washes).

The other half of each fresh sample was homogenised in liquid nitrogen with a mortar and pestle and extracted with 4 ml of 0.01 M H₂SO₄ g⁻¹ of tissue for 1 hour on a shaker. One millilitre of 0.4-M NaHCO₃ was added, and the samples were centrifuged at 10,000g for 10 minutes at 4 °C. The supernatant was filtered and stored at -20 °C until analysis. HPLC (High Pressure Liquid Chromatography) analysis was carried out according to the methods of Lydon and Duke (1988). Working standard solutions spanning the concentration range from 50 g to 800 g L⁻¹ for HPLC analysis were prepared by appropriate dilution in purified water.

The determination of shikimic acid was accomplished within 8 minutes, with the mean peak occurring at 4.5 minutes. Plate counts at this peak were determined and converted into mg of shikimic acid using a predetermined (using known amounts of shikimic acid) standard calibration curve. These data were then converted to mg shikimic acid per gramme dry weight by using the fresh to dry weight ratio data of the matching sample halves analysed for ¹⁴C-glyphosate content.

Activity in leaves and stems was converted to Bq per gramme dry weight by multiplying dpm times 10 to get dpm per gramme and dividing this number by 60 to get Bq per gramme dry weight. This was then plotted against shikimic acid (mg) per gramme dry weight.

Results and implications for methodology

The amount of ¹⁴C-glyphosate absorbed by the treated leaves was on average 4% of the total applied activity (Fig. 4.3a). Activity in the stem section was significantly ($p < 0.001$, Table 4.1) lower (0.1% of the total applied activity) despite having approximately the same average dry weight (0.16 g) as the treated leaves (0.18 g). There was a significant difference between concentration of shikimic acid in treated and untreated plant parts ($p = 0.001$). The concentration of shikimic acid was 2.3 and 5.9 times higher in the treated than the untreated leaves and stem respectively (Fig. 4.3b).

There was a notable difference between the ^{14}C activity and shikimic acid concentration in their distribution between the treated leaves and their stem sections (Fig 4.3a, b). Whereas for ^{14}C activity it was markedly higher in the leaves (ratio of 32 : 1), for the shikimic acid concentration it was slightly higher in the stem sections (ratio of 1 : 1.27).

There was a significant positive correlation between ^{14}C activity and shikimic acid concentration in both the leaves ($p = 0.038$) and stem ($p = 0.046$) sections (Fig. 3.4 c, d; r^2 values for linear regression were 0.1032 and 0.1059, respectively).

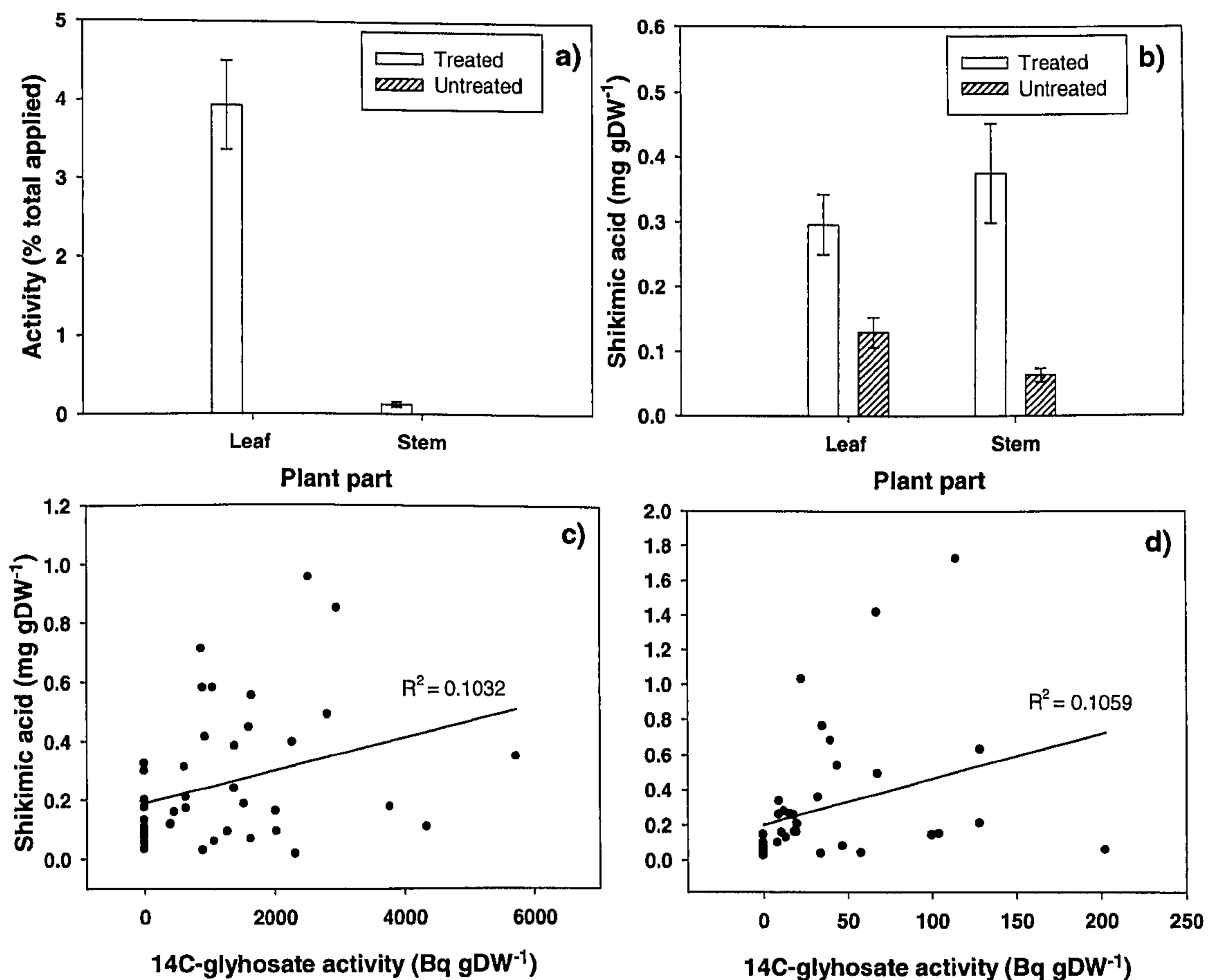


Figure 4.3 Assays of uptake and metabolic impact of ^{14}C -labelled glyphosate applied to the leaves of 30-50 cm height plants of *Rhododendron ponticum* in the leaves to which the glyphosate was applied and the section of stem these leaves were located on, seven days after application: a) ^{14}C activity (as a percentage of the total applied activity) ; b) concentration of shikimic acid (mg) per gramme of dry weight in treated and untreated plants; c) linear regression of response of shikimic acid concentration to concentration of radioactivity in treated leaves; d) linear regression of response of shikimic acid concentration to concentration of radioactivity in stem section; Vertical bars are standard errors of the mean.

The results of the shikimic acid technique were promising, although there was variability in the results between the treated leaves and stems, this was comparable to that of the ^{14}C activity assay: standard errors were 16 and 21% of the mean for the treated leaves and the stem sections they were located on (respectively) for the shikimic acid concentration assay compared with 14 and 25% (respectively) for the ^{14}C activity assay. Glyphosate treatment increased shikimic acid concentration in the treated leaves and stem by 128 and 427% which was highly significant ($p = 0.001$) nonetheless, the shikimic acid method was found to be more time consuming, and there were concerns about the functional interpretation of its results, therefore it was decided to use the radiolabelling technique to measure glyphosate absorption and translocation in the main experiment.

Table 4.1 Univariate analysis of variance on the effect of foliar application of glyphosate to levels of ^{14}C glyphosate and shikimic acid in the leaves and related stem section of *Rhododendron ponticum* woody shrubs 30-50 cm in height. See Appendix 4.1 & 4.2 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
<i>^{14}C-glyphosate</i>			
Treatment	82.366	1	0.000
Plant part	72.698	1	0.000
Treatment x Plant part	72.698	1	0.000
<i>Shikimic Acid</i>			
Treatment	1.000	1	0.001
Plant part	.001	1	0.921
Treatment x Plant part	.093	1	0.297

The method of applying two smaller 2.5 μl droplets of glyphosate solution within Vaseline rings was successful; they were much more stable than the single 5 μl droplet applied in Preliminary Experiment I, therefore this method was adopted for the main experiment. Similarly the approach of applying unlabelled glyphosate to the remaining surface of the treated leaves using a brush was more successful - there was no drip, no possible contamination of untreated leaves by spray drift and radiolabelled glyphosate was able to be applied at the same time - and was therefore adopted in the main experiment.

4.4.3 Preliminary experiment III

Previous studies have found that the leaf cuticle, which is composed of a lipophilic waxy layer, is the primary barrier to glyphosate absorption (Wyrill & Burnside, 1976; Franz, 1985). While there has been considerable research into methods to increase glyphosate penetration of the leaf cuticle these have predominantly been carried out on herbaceous plants. Surfactants improve the efficacy and rainfastness of glyphosate thus improving retention and absorption (Lawrie & Clay, 1993; de Ruiter, *et al.*, 1996; Franz *et al.*, 1997; Sharma & Singh, 2007). Leaf abaxial (lower) cuticles are normally thinner and less waxy than the adaxial (upper) leaf cuticle. The most widely used formulation for foliar spray application to *R. ponticum* is 2% glyphosate and 2% Mixture B (a blend of surfactants, wetters, spreaders and penetrants) in water. The objectives of this experiment were (1) to test whether there was greater glyphosate absorbance when applied to the upper or lower leaf surface of *R. ponticum*, (2) to test the absorbance of glyphosate mixed with three different surfactants and (3) to test the effect of surfactant concentration on foliar absorption of glyphosate.

Methods

The 12 *R. ponticum* test plants and the growing conditions were the same as for preliminary experiments I and II. The present experiment was started on 18 November 2008 (plants were allowed 2 weeks to acclimatise). There were a total of 12 treatment combinations - 3 surfactants (Mixture B, MON 0818 and MON 5911 (all supplied by Monsanto, Cambridge, UK); 2 leaf surfaces (upper and lower) and 2 surfactant concentrations (2% and 10%); glyphosate was kept at 2% concentration for all treatments. Each was applied to the surface of a single leaf to randomly selected *R. ponticum* plants. The leaves were pegged back to the stem so that the abaxial (lower) surface was facing upwards and glyphosate one millilitre of glyphosate solution at normal field use rate (commercial RoundUp 360 ProBioactive at 2% and Mixture B at 2% in water) was applied to each selected leaf using a brush. After foliar application of glyphosate (outside rings), two 2.5 µl droplets, each containing approximately 0.1 µCi of ¹⁴C-glyphosate in aqueous solution were

applied to each leaf inside the Vaseline rings using a dispensing pipette. After application, the drops were carefully watched to ensure that they remained where placed. If a droplet seemed to become unstable, the treated leaf was immediately removed and discarded and a replacement leaf was selected. The treated leaves of the plants were harvested seven days after application. The preparation of samples and measurement of ^{14}C activity were carried out in the same way as for Preliminary Experiments I and II.

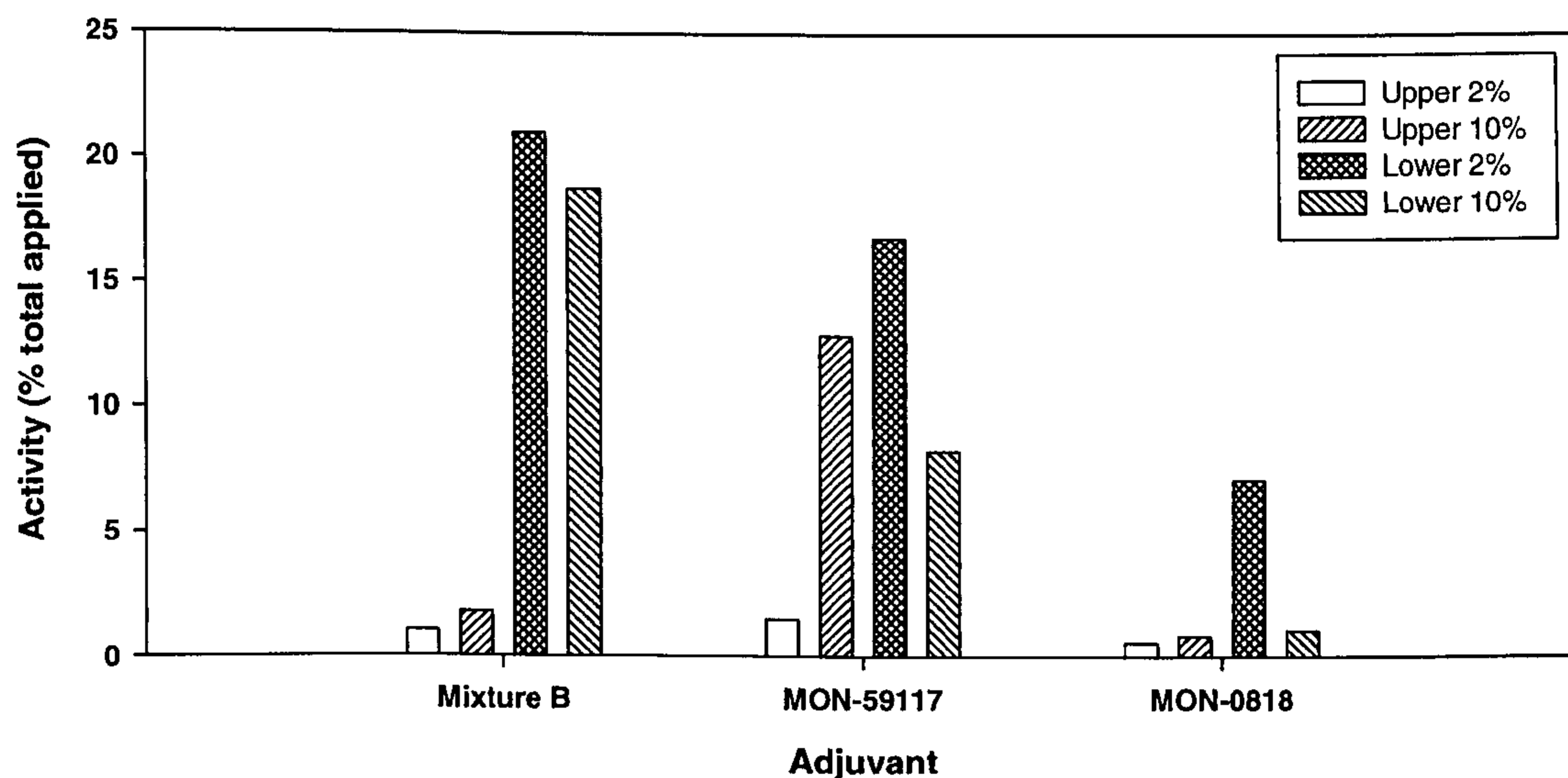


Figure 4.4 The effect of surfactant type and concentration, and the leaf surface of application (upper versus lower) on the absorption after seven days of glyphosate (2% in water) for all treatments) applied to leaves of *Rhododendron ponticum* plants.

Results and implications for methodology

Although the trial was unreplicated, clear trends were observed in glyphosate absorption amongst the different treatments (Fig. 4.4). Absorption by the lower leaf surface was consistently higher than by the upper surface. Mixture B appeared to be the most effective surfactant at both concentrations on the lower leaf surface with an average absorption rate of around 19%. In contrast MON 59117 appeared to be the most effective surfactant at both concentrations on the upper leaf surface, and MON0818 the least effective all round. Apparent difference between the 2 and 10% concentrations showed a consistent (though generally small) effect across all three

surfactants: on the upper leaf surface greater absorption was obtained with the 10% surfactant concentration, whereas on the lower surface greater absorption was obtained with the 2% surfactant concentration.

Therefore, in the main experiment it was decided to:

- Apply glyphosate to the abaxial (lower) leaf surface.
- Continue to use Mixture B as the surfactant at 2% concentration.

4.5 METHODS

4.5.1 Experimental design

The plants used in the experiment were propagated from 20-cm cut stems of local source material from Dingle Nursery, Welshpool, Wales, UK and grown in a peat medium in two-litre plastic pots in full light. Sixty plants of similar growth form and size (height 30 - 50 cm) were selected and placed under two different light treatments in an unheated glasshouse with average daily temperatures of 8.1/2.6 °C (average ambient max./min. temperatures for December 2008). The light treatments were: (i) ambient light levels for December (8 hours photoperiod, average PPFD of between 100 (cloudy day) to 250 (clear day) $\mu\text{mol m}^{-2} \text{s}^{-1}$); (ii) supplemental lighting with a 14-hour photoperiod (PPFD = 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

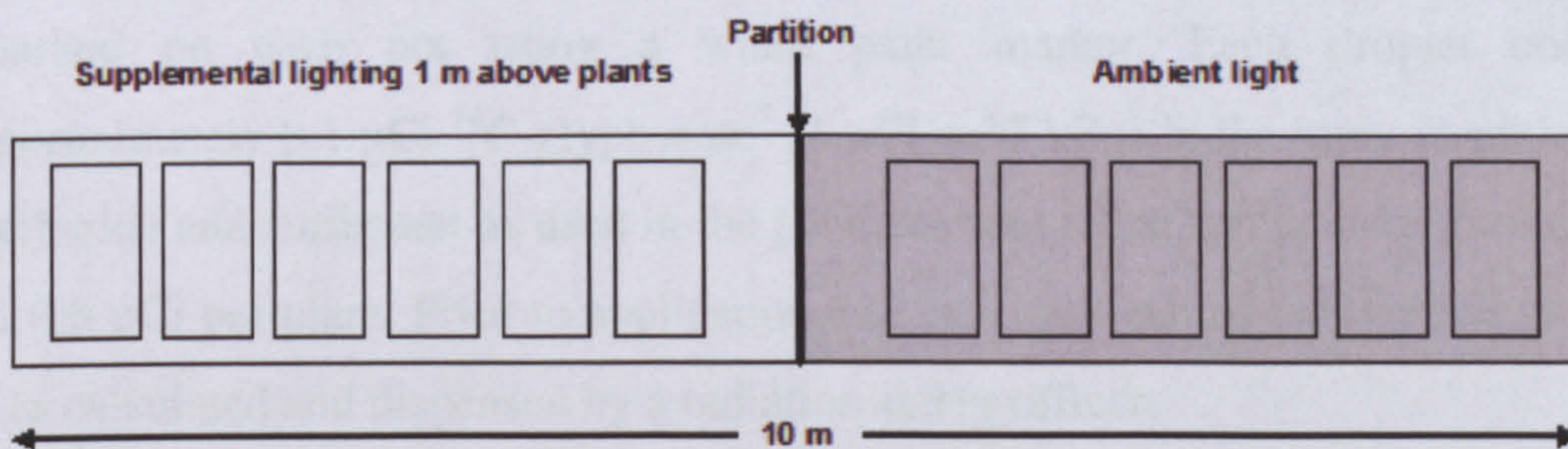


Figure 4.5 Arrangement of plants (5 plants within each 'block' for 5 harvest times) on a SE facing bench in an unheated glasshouse.

Plants were allowed to acclimatise for two weeks before foliar application of ^{14}C -glyphosate on 12 December 2008. Five harvest times for each of the two light treatments were assigned to the 60 plants giving six replications of each. Due to some variation in growth form a stratified-random allocation of plants to treatments was used, with the different plant morphologies (single-stemmed, multi-stemmed, small, tall) each distributed as evenly as possible amongst the replications.

4.5.2 Application of ^{14}C glyphosate

Three leaves adjacent to each other approximately mid-way along the main stem were chosen on each plant. Preliminary trials found that glyphosate absorption on the abaxial leaf surface was much greater than the adaxial surface. Studies have found that the leaf cuticle, composed of a lipophilic waxy layer, is the primary barrier to glyphosate absorption (Wyrill & Burnside, 1976; Franz, 1985). Two rings of Vaseline were smeared on the lower (abaxial) leaf surface either side of the leaf midrib (Fig. 4.6a). The Vaseline acted as a barrier to prevent the droplet from spreading all over the leaf surface. Leaves were pinned back using pegs so that the abaxial surface was facing up to prevent dripping. A background dose of Glyphosate at normal field use rate (commercial RoundUp 360 ProBioactive at 2% and Mixture B at 2% in water) was applied to the selected leaves using a brush (1 ml applied to each leaf) to minimise drip and to avoid the area inside the rings. Two 2.5 μl droplets of ^{14}C -labelled glyphosate were applied to each of the three selected leaves using a 2-20 μl dispensing pipette (giving a total of 15 μl applied to each plant). Treatments were applied one plant at a time making sure that the exact time of application was marked on each pot using a white paint marker. Each droplet contained approximately 0.1 μCi ^{14}C -glyphosate* (1 μCi = 37 kBq) in the same formulation of herbicide and surfactant as used in the pre-treatment foliar application, giving a total of 0.6 μCi per plant. Prior to application, the exact amount of radioactivity required was calculated and dispensed by a radiation safety officer.

* ^{14}C -glyphosate (glycine-2- ^{14}C) in aqueous solution (sterile water) specific activity 0.37 – 1.11 GBq mmol^{-1} , 95% purity, concentration 0.1 mCi/ml (American Radiolabeled Chemicals Inc., town/city, Missouri, USA).

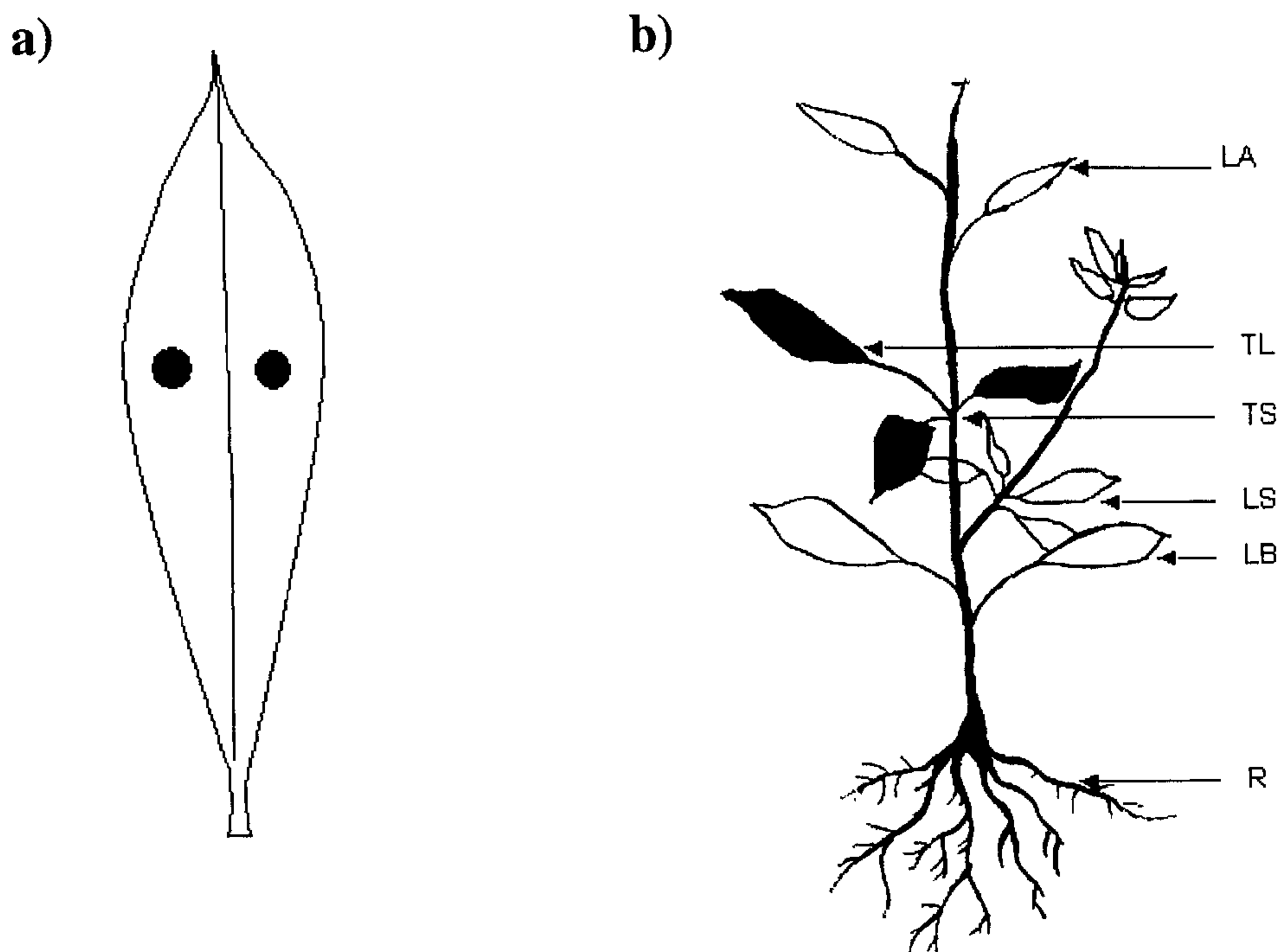


Figure 4.6 Position of glyphosate treatment and subsequent harvesting of organs on *Rhododendron ponticum* plants of 30-50 cm height: a) abaxial leaf surface where 2.5 µl ^{14}C -labelled glyphosate droplets were applied; b) organs harvested: treated leaves (TL); treated stem (TS); leaves above (LA); leaves below (LB); leaves on other shoot (LS); roots (R).

There were a total of 60 plants each treated with 0.6 µCi giving a total of 36 µl of ^{14}C -glyphosate made up to 600 µl of solution with the pre-treatment formulation. After application, the drops were carefully observed to ensure that they remained where placed. If a droplet seemed unstable, the treated leaf was immediately removed and discarded and a replacement leaf was selected. Pegs were removed once the leaf surface was dry.

4.5.3 Plant harvest and sample preparation

Plants were harvested 6 hours and 1, 3, 7, 14 and 30 days after treatment. Treated leaves were excised and washed three times each for 15 s with separate 20 ml volumes of distilled water to ensure complete removal of unabsorbed material and to quantify the efficacy of the washes. Radioactivity in 1 ml of the leaf wash was

determined using 4 ml of Optiphase HiSafe 3 scintillation cocktail solvent (PerkinElmer, town, Massachusetts, USA) – to get the total unabsorbed radioactivity in the 20 ml volumes dpm/ ml was multiplied by 20 and total unabsorbed radioactivity per leaf was the sum of the three 20 ml volumes (dpm). At each harvest time treated plants were sectioned into six different parts (Fig. 4.6b): the three treated leaves (TL); the section of stem (stem was cut 1 cm below the lowest treated leaf and 1 cm above the highest treated leaf) on which the treated leaves were located (treated stem, TS); all of the leaves above the treated leaves on the same stem (leaves above, LA); all of the leaves below the treated leaf on the same stem (leaves below, LB); all the leaves on a separate shoot adjacent to the treated shoot (leaves separate, LS); and a sample (0.2 – 0.6 g dry weight) of the roots (R). The preparation of samples and measurement of ^{14}C activity were carried out in the same way as for the Preliminary Experiments.

4.5 DATA PRESENTATION AND ANALYSES

Certain ideal procedures were not followed due to logistical or resource reasons. Ideally the entire plant should be separated into different components to dry, weigh, and grind, followed by multiple sub-sampling. However, with *R. ponticum* there were two main problems in following this procedure: (1) the stem is woody with up to a 1 cm diameter in places; the ball mill could only sample small sections - 3 cm sections of stem at most – and grind 2 samples at a time (5 minutes). To sample the entire stem or even multiple sections was not possible in the scope of this study. (2) The root system of *R. ponticum* is very complex and matted and separation from soil in the pots was too difficult to be worthwhile. Therefore, whilst complete sampling of stem and root material was not possible, it was possible to harvest all the leaves on the same stem above and below the treated leaves and all the leaves on one adjacent untreated shoot. Approximate estimates based on the reference mass: of 100 mg of the sampled stem section represented on average 2.5% (average weight of 0.5 g dry weight of 3 cm stem section from treated stem around 25 cm in length) – although this was likely an overestimate as it is highly likely there would have been a concentration gradient of ^{14}C along the stem (Brook, pers. comm. & based on

evidence from Preliminary experiment I.); of 100 mg of the sampled root system represented on average approximately 2.5% (average weight of 0.4 g dry weight root section which was approximately 10% total root mass) of the total mass of the whole root system; and that the sampled adjacent untreated shoot was on average 30% (on average 1 of 3 other untreated shoots) of the mass of the untreated shoots of the plants.

Radioactivity, in the various plant parts was first expressed as disintegrations per minute (dpm). Activity in each plant component part was expressed as activity (dpm) per 1 gramme dry weight of the plant part. ^{14}C -glyphosate activity in the treated stem, leaves above, leaves below, other leaves and roots was plotted against the activity (dpm/g dry weight) of the corresponding set of treated leaves.

The following calculation for determining absorbed radioactivity has been used in several studies (Green *et al.*, 1992; Grangeot *et al.*, 2006) and was chosen over estimating absorption as a percentage of what was thought to be applied per droplet (3.7 kBq/222000 dpm) as droplet size and ^{14}C -glyphosate concentration varied greatly between applications. The plants that were treated first had considerably higher amounts of ^{14}C (up to 500% times more) in the unabsorbed washes and in treated leaf samples. The ^{14}C glyphosate in translocated from the treated leaves to other parts of the plant was proportional to the activity in the treated leaves. To compare data between plants, activity was therefore expressed as percentages.

For the main analyses the amount of ^{14}C -glyphosate absorbed into the treated leaves was calculated by multiplying the activity per 100 mg subsample (of set of treated leaves) times 10 to get dpm/g dry weight and multiplying this by the total dry weight (g) of the harvested sets of treated leaves. This was then expressed as a percentage of the total amount applied. The total amount applied was the sum of unabsorbed radioactivity from leaf washes plus total recovered from the plant (total absorbed radioactivity). The majority of the ^{14}C was shown to remain in the treated leaves and therefore this method of determining total ^{14}C absorption was determined to be the most accurate approximation. It was assumed that essentially all ^{14}C recovered from the ^{14}C glyphosate-treated plants was in the form of intact glyphosate, based on the

results of previous glyphosate metabolism studies (Gottrup *et al.*, 1976; Schultz & Burnside, 1980).

The most reliable calculations of translocation of the applied ^{14}C -glyphosate to other plant parts were based on its concentrations within the sampled material (dpm in 100 mg subsample) and were expressed as a percentage of the amount absorbed in the treated leaves (dpm in 100 mg subsample).

The total quantity translocated to different plant parts could only be determined for the three completely sampled sets of leaves (not the stem or root tissue for the reasons described in the first paragraph in section 4.5 above). This was done by multiplying the activity per 100 mg subsample (of each set of leaves) times 10 to get dpm/g dry weight and multiplying this by the total dry weight (g) of the harvested sets of leaves – the total amount of radioactivity translocated to these sets of leaves was expressed as a percent of the total absorbed radioactivity.

All statistic analysis was done using SPSS 16.0 and graphs were plotted using SigmaPlot 10.0. The data from each plant were treated as independent replicates of each combination of harvest time and treatment (six replicates of each). The light treatments were applied to different parts of the glasshouse so light treatment is confounded with position and therefore, while statistical comparison between the two is valid, the interpretation must acknowledge that any differences cannot be attributed with certainty to light treatment rather than position. The data were normally distributed so analysis of variance was used to test for significance of light treatment, harvest time, and plant component part on levels of ^{14}C -glyphosate activity in different parts of the plant. A p-value less than 0.05 was taken to indicate a significant difference.

4.6 RESULTS

Activity, expressed as total dpm in the treated leaves of plants that were kept under supplemental and ambient light was 75,770 and 175,767 and activity in corresponding unabsorbed washes was 49,163 and 239,841 dpm, respectively. There was a positive correlation between activity in treated leaves and translocation to other plant parts especially for plants kept under supplemental lighting (Fig. 4.7). As the amount of activity in treated leaves increased so did activity in treated stem section (4.7a), leaves up the stem above the treated leaves (4.7b), leaves down the stem below the treated leaves (4.7c), leaves on untreated shoots (4.7d) and roots (4.7e). This relationship was less pronounced in leaves below and other shoots, especially under ambient light.

Foliar absorption of applied ^{14}C -glyphosate had reached a mean of 55% after six hours and 88% after 3 days, followed by a more gradual increase to 93% after 30 days (Fig. 4.8a, Table 4.2). Supplementary light had no effect on foliar absorption of applied ^{14}C glyphosate after 30 days (Fig. 4.8a, Table 4.2). The rate of absorption at the intermediate harvests (6 hours – 14 days) showed fluctuations for both the supplementary and ambient light treatments, but no evidence of significant differences in trajectory (both the overall effect of light treatment and the interaction between harvest time and light treatment were not significant).

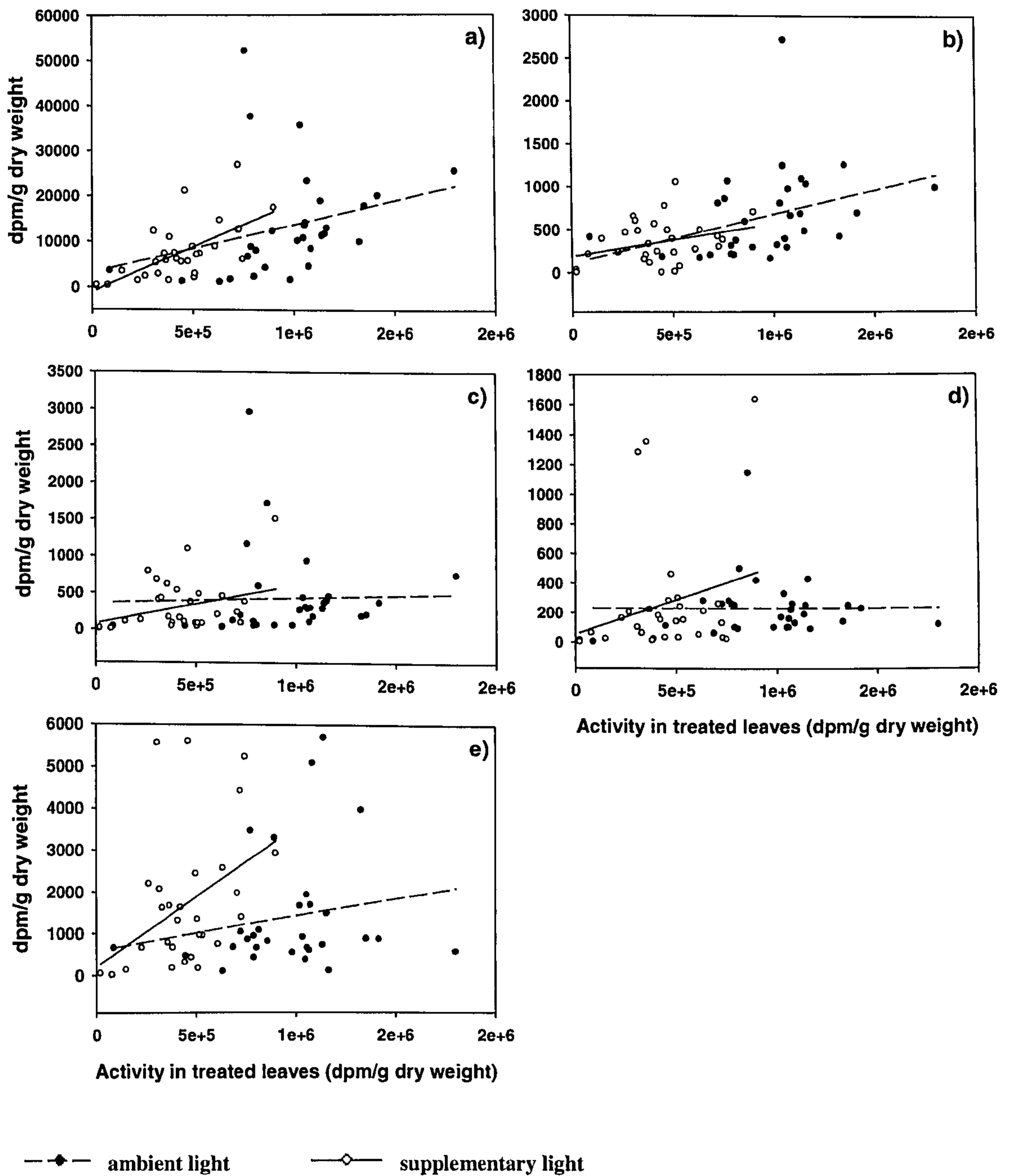


Figure 4.7 Translocation of foliar applied ^{14}C -glyphosate from treated leaves of *Rhododendron ponticum* plants 30-50cm under ambient and supplemental light to other plant parts, expressed as activity (dpm) per gramme dry weight of: a) treated stem section (r^2 linear regression values of 0.4256 and 0.049 under supplemental and ambient lighting respectively); b) leaves on stem above treated leaves (r^2 linear regression values of 0.0102 and 0.1185 under supplemental and ambient lighting respectively); c) leaves on stem below treated leaves (r^2 linear regression values of 0.1100 and 0.00164 under supplemental and ambient lighting respectively); d) leaves on untreated shoot (r^2 linear regression values of 0.0613 and 0.00007 under supplemental and ambient lighting respectively); and e) roots section (r^2 linear regression values of 0.1915 and 0.0332 under supplemental and ambient lighting respectively) against activity (dpm) gramme dry weight of corresponding treated leaves.

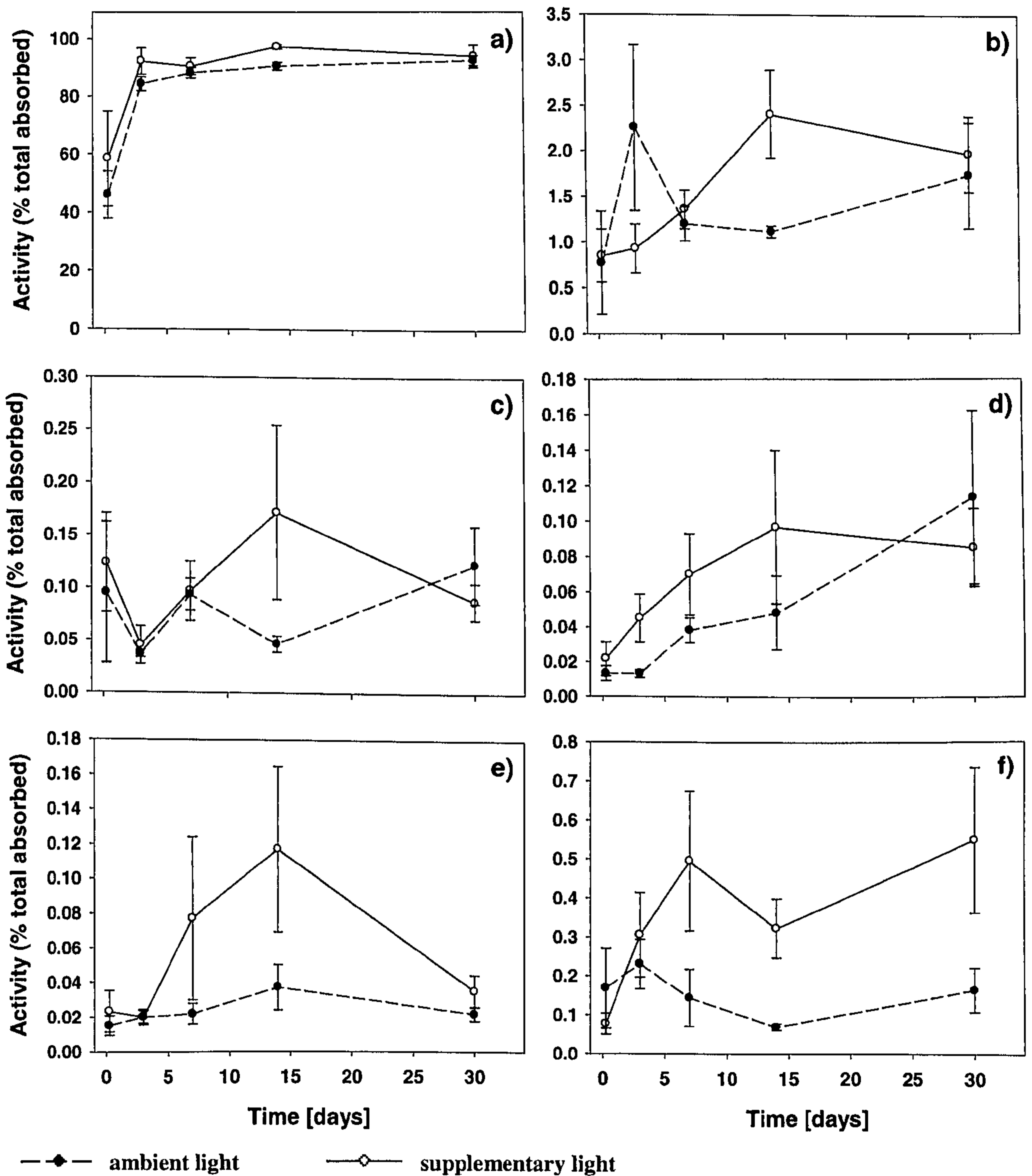


Figure 4.8 Absorption and translocation of ^{14}C glyphosate applied to leaves of *Rhododendron ponticum* plants 30-50 cm in height over 30 days (a) absorption into these leaves (as a percentage of total recovered radioactivity); Translocation of ^{14}C glyphosate to other plant parts based on the concentrations within the sampled material (dpm in 100 mg subsample): b) stem sections on which treated leaves are located; c) leaves on the same stem above the treated leaves; d) leaves on the same stem below the treated leaves; e) leaves on an adjacent untreated stem; f) roots. Vertical bars are standard errors of the mean. Note the variation between the panels in y-axis scale.

Translocation of ^{14}C to the other plant parts occurred more slowly and the trajectories differed greatly between the supplementary and ambient light treatments (Fig. 4.8b-f) such that, in contrast to foliar absorbance, the supplementary lighting did have a marginally insignificant effect ($p = 0.054$) on the rate of ^{14}C translocation out of the treated leaves to different parts of the plant after 30 days (Table 4.3) and a significant ($p = 0.011$) interaction effect of light treatment and harvest time on translocation. The difference in the amount of ^{14}C glyphosate translocated from treated leaves to different parts of the plant was also highly significant ($p < 0.001$) and post-hoc analysis with Tukey (Appendix 4.5) showed the difference between the treated stem section and the other plant parts to be the only significant difference (i.e. the differences amongst all other plant parts were not significant). The highly significant interactions between harvest time and light treatment ($p = 0.011$) and between harvest time and plant part ($p < 0.001$) correspond with the large variation between the light treatments and the plant parts in their rate of ^{14}C translocation (Fig. 4.8b-f).

In the plants kept under ambient light, 2.3% of the ^{14}C activity had been translocated to the stem section on which the treated leaves were located by the three day harvest (Fig. 4.8b). There was no consistent trend in ^{14}C activity in the stem section during the subsequent harvests; a decrease to 1.1% after 14 days may have been followed by a slight increase to 1.7% after 30 days. The rate of translocation from treated leaves to the other plant parts was low; 0.1% had reached the leaves up the stem above the treated leaves by 30 days; it rose steadily up to just above 0.1% in the leaves down the stem below the treated leaves and remained below 0.1% (with no particular trend over time) in the leaves on a separate stem; activity in the roots rose above 0.2% by 3 days but then fell.

Translocation of ^{14}C activity from the leaves to which it was applied was marginally greater in the plants under supplementary light (a total of 2.6% to all other sampled plant parts) than under ambient light (2.0%) by 30 days (Fig. 4.8). In these supplementary-lit plants the sequence of translocation appears to be as follows. The first major translocation was into the stem sections on which the treated leaves were located; it increased steadily into the stem section until 14 days (2.4%) and then fell

(this is a markedly different trend over time than in the plants under ambient light). There was a rapid translocation into the roots between 6 hours and 7 days (to 0.5%). After seven days the ^{14}C activity in the roots fell slightly and then levelled (at above 0.5%). It is notable that exactly the same trends occurred on the same time scale in the plants under ambient light, though the magnitude of translocation to the roots was much less. After 3 days there was a rise in translocation to the leaves on the same stem above the treated leaves up to day 14 (0.17%) then, as in the stem sections, the activity fell. There was a slightly slower level of translocation into the leaves lower down the stem of the treated leaves (below 0.1% up to 14 days and levelling off but with high variation amongst the replicate plants) and also a low transfer into leaves on a separate stem (never exceeding 0.12%).

Table 4.2 Univariate analysis of variance on the effect of light treatment on foliar absorption of ^{14}C -glyphosate applied to *Rhododendron ponticum* plants 30 – 50 cm in height over 30 days. See Appendix 4.3 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
Light	567.940	1	0.128
Harvest time	14786.252	4	0.000
Light x Harvest time	238.384	4	0.908

Table 4.3 Univariate analysis of variance on the effect of light treatment on the translocation to different plant parts of concentration of ^{14}C -glyphosate applied to the leaves of *Rhododendron ponticum* plants 30 – 50 cm in height over 30 days. Appendix 4.4 for full ANOVA.

Source of variation	Sum of Squares	df	<i>p</i>
Harvest time	5.441	4	0.000
Light	0.725	1	0.054
Plant part	67.048	4	0.000
Light x Harvest time	2.565	4	0.011
Plant part x Light	0.550	4	0.584
Harvest time x Plant part	12.255	16	0.000
Harvest time x Plant part x Light	7.341	16	0.003

Table 4.4 Estimates of percentage activity for ¹⁴C glyphosate activity (expressed as a percent of total absorbed activity) from treated leaves of *Rhododendron ponticum* to other parts of the plant. In ‘whole harvest’ all of the plant part was weighed whereas with ‘Estimate’ the plant parts were not and the calculation is an estimate based on representative subsamples.

Plant component part	Light (%)	Ambient (%)
<i>Whole harvest</i>		
Leaves above	0.80	0.43
Leaves below	0.67	0.50
<i>Estimate</i>		
Roots	11.2	1.18
Other shoots	0.27	0.09
Treated stem	2.44	0.46

¹⁴C-glyphosate in all the leaves on the stem above and below the treated leaves were 0.43 and 0.50% after 30 days under ambient light which increased to 0.80 and 0.67%, respectively under supplemental light (Table 4.4). Similar patterns were observed in other parts of the plant with approximate estimates for levels in the treated stem section, roots and other shoots being higher (11.2, 2.44 and 0.27%) under supplemental light than under ambient (1.18, 0.46 and 0.09%).

4.8 DISCUSSION

When applied to the lower (abaxial) leaf surface, with an appropriate surfactant, there was very high absorption of glyphosate into the treated leaves, even for plants grown in December. Over half of the applied glyphosate had been absorbed within the first six hours, and reached 88% after three days after which there was no significant increase in absorption. This rate of absorption is consistent with studies that have demonstrated an initial rapid absorption of glyphosate which has been reported to last a few hours in herbaceous plants (Sprankle *et al.*, 1975; Brecke & Duke, 1980) to a few days in woody species (Lund-Hoie, 1976). This initial phase has been followed by a longer phase of slower penetration (Sprankle *et al.*, 1975; Schultz and Burnside, 1980; Grangeot *et al.*, 2006). The duration of each of the phases is dependent on a number of factors including species, age, environmental conditions and concentration of the glyphosate and surfactant (Meritt,

1982; Neal & Skroch, 1985; Yonce & Skroch, 1989). Despite very low ambient light levels, there was remarkably little effect of high level supplementary lighting on either rate of absorption or total levels of ^{14}C -glyphosate after thirty days. However, the larger effect of supplementary lighting on the translocation of this ^{14}C activity implies that this lighting was having some impact on the physiology of the plants. This suggests therefore, that absorption of glyphosate across the cuticle of *R. ponticum* leaves is a physical process, largely independent of the physiology of the plants (particularly stomatal openings).

Results from the preliminary experiments showed that absorption following application to the upper (adaxial) surface of *R. ponticum* leaves was significantly lower (4%) than the lower (abaxial) leaf surface (88%) after seven days. This would suggest that leaf surface application is the primary factor in determining foliar penetration. The cuticle of rhododendron species has been shown to be well developed on the upper side with stomata present predominantly on the lower leaf surface (Roberts *et al.*, 1959; Mircea, 2005). Cross (1975) noted that the average density of stomata in *R. ponticum* leaves was significantly higher on the abaxial than the adaxial leaf surface. Many studies have shown that the presence of stomata can significantly increase the rate of foliar uptake of hydrophilic solutes, chiefly under conditions favouring stomatal opening (review Fernandez & Eichter, 2009 and references therein). Increased absorption by the lower leaf surface could therefore be due to higher stomatal density or a thinner leaf cuticle. Light is known to stimulate stomatal opening and various physiological processes in the plant such as photosynthesis or xylem flux, which may increase the rate of foliar uptake (Currier & Dybing, 1959; Jyung & Wittwer, 1964). However, since there was no effect of light on absorption in this study it is more likely that leaf cuticle thickness has the greatest effect on foliar absorption. The first limitation to absorption of a post-emergence herbicide is penetration through the epicuticular wax and cuticle which have been considered by to be the major barrier to absorption, especially to polar compounds such as glyphosate (Bukovac, 1976; Sherrick *et al.*, 1989; Wang & Liu, 2006). Several studies have attributed lower rates of ^{14}C -glyphosate foliar absorption to thicker cuticles and epicuticular wax (Wyrill & Burnside, 1976; Coupland *et al.*, 1978; Green *et al.*, 1992). Despite the addition of a surfactant, absorption of ^{14}C -glyphosate was much lower when applied to the adaxial surface. Significant

increased absorption by the abaxial surface was most probably due to a thinner cuticle, although higher stomatal density cannot be discounted as no measurements of gas exchange or stomatal openings were taken to test for the effect of light.

Although supplementary light had no effect on absorption it did increase translocation of ^{14}C -glyphosate from treated leaves to different parts of the plant. Schultz and Burnside (1980) also found that absorption of ^{14}C -glyphosate in *Apocynum cannabinum* was unaffected by light intensity seven days after treatment but translocation was enhanced by light intensity. Likewise Kells and Rieck (1979) found that in *Sorghum halapense* more ^{14}C was translocated in 'full' light than in shade or dark. In this study, the increase in translocation to other parts of the plant due to supplemental light was most notable at fourteen days after application with accumulation in the stem section on which the treated leaves were located, leaves on the same stem above the treated leaves and in the roots. Light intensity and photoperiod can affect physiological processes such as photosynthesis, transpiration or plant development resulting in enhanced glyphosate movement in the post spraying period (Caseley & Coupland, 1985). Photoperiod was found to have no significance on ^{14}C -glyphosate absorption or distribution in some studies (Caseley, 1972; Lund-Høie, 1983; Bowmer & Eberbach, 1993) but was linked to an increase (Whitwell *et al.*, 1980; Coupland) and decrease (Mejia & Romero, 1977) in efficacy in others. Research into the effects of environmental conditions on the performance of glyphosate in *Agropyron repens* by Coupland (1983) found that higher light levels, temperature and relative humidity all resulted in increases in herbicide toxicity by enhanced basipetal flow.

In this study, although only a small section of root was sampled levels of ^{14}C -glyphosate were comparatively high. Rapid translocation of the absorbed glyphosate to the roots, presumably via the phloem as the amount translocated was substantially boosted by supplementary lighting. This is typical of glyphosate translocation to roots which will subsequently be redistributed acropetally during periods of growth with the source sink phloem flow (Lund-Høie, 1976; Neal *et al.*, 1986; Green *et al.*, 1992). Results of this study also showed that there was some movement of ^{14}C -glyphosate up the stem, probably in the xylem as the amount translocated was substantially boosted by supplementary lighting. From its timing it could well have

been via loading into the xylem in the root system, but lateral transfer into the xylem within the stem cannot be excluded. However, it is notable that this transfer was much more rapid and greater into the leaves on the same stem above the treated leaves than those lower down on that stem or on separate stems. It is not possible to determine if this is because: (a) under the supplementary lighting the rate of transpiration, and thus water flow in the xylem from the roots, into the leaves high up the vertical stem was much greater than leaves lower down on the plant (which were more shaded), or (b) there was direct transfer into the xylem (and its upward water flow) in the stem adjacent to the treated leaves. This finding, however, may be general and not just confined to the present study: Satchivi et al. (2000) observed, in *Abutilon theophrasti*, that the largest portion of translocated ^{14}C moved to tissues above the treated leaf.

There was an accumulation of shikimic acid after seven days in both leaves and stem sections of plants that were treated with glyphosate compared to untreated control plants. Levels of shikimic acid were found to increase with increasing levels of ^{14}C -glyphosate, especially in the stem section. This work is in agreement with other studies that have found shikimic acid accumulation in plants in response to glyphosate application (Lydon & Duke, 1988; Pline *et al.*, 2002; Bresnahan *et al.*, 2003). Refining this technique for *R. ponticum* might prove useful in future trials for determining levels and extent of glyphosate damage in plants.

Clearly, there is no simple relationship between light and herbicide entry and movement, but results indicate that ^{14}C -glyphosate when applied to the leaves of *R. ponticum* travels both up and down the plant with the source-to-sink phloem flow and xylem transpiration flow. Glyphosate is rapidly absorbed into the leaves with 50-60% of the glyphosate being absorbed by 6 hours. The practical field implications of these results suggest that only a short window of time without rain is needed for effective treatment, but a further 3 days without rain would help increase this absorbance by half (i.e. add another 25%-35% of the applied glyphosate that is absorbed). A better understanding of the mechanisms behind physiological processes that affect xylem and phloem movement, and the factors that influence them might lead to reduced application rates or improved efficacy on invasive plant species such as *R. ponticum*.

CHAPTER IV – General Discussion

5.1 ADDRESSING THE UNDERLYING DYNAMICS OF *RHODODENDRON PONTICUM* INVASION

5.1.1 General Aims

An essential step in learning more about the underlying mechanisms of plant invasions is not only the identification of traits which make plant species invasive, but also investigating the relative importance of seed, habitat and microhabitat limitation. This information is critical in establishing which habitats are most at threat to invasion. It is not only of major importance in the prevention of invasion but also for management and prediction of future range expansion. The purpose of this study was to further understand the recruitment dynamics of the invasive species *Rhododendron ponticum* which was first introduced to the British Isles in 1763 as an ornamental plant in gardens and was also extensively planted as game cover in woodland (Elton, 1958). It since became widely established across Great Britain and Ireland where conditions were favourable. Local populations originated from *Rhododendron ponticum* L. subsp. *baeticum* in the Iberian Peninsula with some evidence of hybridisation with *R. catawbiense* and *R. maximum* (Milne & Abbott, 2000). The study area was focused on Snowdonia National Park in North Wales, UK, where the core distribution is associated with large estates having since spread rapidly into the surrounding countryside. The success of *R. ponticum* has been attributed to a combination of several factors: the climatic and edaphic suitability of the invaded habitat, the absence of natural predators, prolific seed production, widespread planting and habitat disturbance (Cross, 1981). A major factor thought to limit the spread of *R. ponticum* is the availability of suitable safe sites for seedling germination and survival (Rotherham, 1986, 2001).

The aims of these experiments were to answer fundamental questions about the *R. ponticum* microsite preferences. Firstly what seedbed substrates best promote or suppress seedling emergence and does it agree with distribution in the field. Secondly what are the effects of different light and watering treatments on germination and seedling survival and is there an interaction with seedbed substrate. Finally, is there any evidence for the effect of provenance of invasive *R. ponticum* populations from Britain and Ireland on germination in different light environments?

5.1.2 Functional interpretation and implications of key results

In this study, the germination of *R. ponticum* seeds was highly dependent on moisture and light availability, modified by the presence of a vegetative layer. Germination and seedling survival on exposed seedbed substrates was notably reduced by droughting; however, the presence of a thin pleurocarpous moss layer did eliminate this effect on seedlings once established. In its native habitat in the southern Iberian Peninsula where it exists only as relict populations, recruitment failure is attributed to the scarcity of safe sites free from drought for seedling establishment and it is restricted to the moist shady conditions provided by riparian forests (Mejías *et al.*, 2002, 2007). This contrast between its aggressive colonising ability in Western Europe and the lack of regeneration in southern Iberia (Erfmeier & Bruelheide, 2004) supports the hypothesis that moisture availability is a key limiting factor. Water also appears to play a part in long distance dispersal with *R. ponticum* seeds displaying adaptations such as surface wax, frills and folds to aid buoyancy. Schäfer and Böcker, 2006 also found that prolonged submersion in water (four days optimum) enhanced germination rate and therefore suggested focusing control measures on spreading corridors along streams and lakes in the British Isles. A similar result was found for the primarily wind dispersed invasive woody 'Tree of Heaven' (*Ailanthus altissima*), where seed germination was increased by 87% after floating in water for three days (Kowarik & Säumel, 2008). Other rhododendron species, such as *R. ripense* (endemic to Japan), are known to use rivers and waterways as a means of long distance dispersal (Kondo *et al.*, 2009).

In the present study germination and seedling survival rates were both highest in the low moss, bare soil and sown grass substrates and lowest in grass turf, leaf litter and deep moss. Combining both components of the process of seedling establishment, short moss carpets were the most suitable substrate. However, germination in the short moss carpet was lower than in either the bare soil or sown grass seedbed substrate especially in the no-shade treatment. Despite daily watering, the short moss had a tendency to dry out very quickly. Thus, in periods of drought even a thin moss cover may inhibit rather than facilitate seedling emergence. The presence of a simulated canopy above the bare soil and short moss carpet seedbed substrates greatly increased germination but had no effect on seedling survival. This suggests that in the absence of a shade canopy, moss carpets are highly vulnerable to desiccation with the effect of reducing germination. Various studies have reported higher seed germination on bare soil than in bryophyte layers (e.g. During & van Tooren, 1990; Zamfir, 2000; Jeschke & Kiesch 2008). Conditions affecting survival of seedlings in short moss appear to be different from those affecting seed germination. After 6 months growth in the greenhouse the greatest number of *R. ponticum* seedlings remaining were in thin moss carpets, which were also the healthiest. These results help in explaining the distribution of *R. ponticum* seedlings in the field (Cross, 1981; Stephenson *et al.*, 2006) on thin moss carpets and further support the positive effects of moss as a seedbed (McLaren & Janke, 1996; Parker *et al.*, 1997; Su *et al.*, 2009). In a related species, *Rhododendron hodgsonii*, at early life stages seedlings were found on all microsites but over time there was an increase in the number of seedlings found on bryophyte mats and a decrease on all other microsites (Gratzer & Rai, 2004). There are several factors that can alter the availability of moisture, light and nutrients such as, moss height, habit and density (During & van Tooren, 1990; Equihua & Usher, 1993; Serpe *et al.*, 2006). The result showing the poor performance in tall moss carpets, are in agreement with studies that found a decrease in germination and seedling emergence with an increase in moss thickness and height (Zamfir, 2000; Sedia & Ehrenfeld, 2003). On the other hand, dense carpets of the tall growing *Sphagnum* sp. have been found to have a positive effect on germinants (Duchesneau & Morin, 1999; Greene *et al.*, 2004) including *R. ponticum* (Jackson, 2008) due to the high water-retaining ability of this genus.

Although carpets of *Plagiothecium undulatum* (short moss) had a slight negative (in comparison to bare soil) affect on germination, a positive one developed on the post-germination performance of the seedlings, even under droughted conditions. This was not the case in bare soil, where the negative effect of periodic droughting continued from germination through to the seedling stage. The results suggest that as seedlings became rooted in the soil beneath the thin moss layer, a greater amount of soil moisture became available to them. Bryophytes are known to reduce rates of soil moisture loss (van Tooren *et al.*, 1985) and temperature fluctuations in soil (Richardson, 1958). Similar results were found by Equihua and Usher (1993) in a study on the effects of the invasive acrocarpous moss *Campylopus introflexus* on germination of *Calluna vulgaris* (common heather) seeds, a close relative of *R. ponticum* (both members of Ericaceae family). Carpets of *C. introflexus* had a depressive impact on germination but a positive one on seedling development which the authors suggested was as a result of light and/or moisture deprivation. Like *R. ponticum*, the seeds of *C. vulgaris* are small (0.55 – 0.65 mm) and positively photoblastic (Gimingham, 1972) and are thus vulnerable to burial and desiccation. Species with large seeds tend to have an advantage in more shaded habitats (Grime & Jeffrey, 1965; Salisbury, 1975; Foster & Janson, 1985; Leishman & Westoby, 1994). However, despite *R. ponticum* being a small-seeded species the shady conditions of woodlands is one of its favoured habitats. Brown (1954) found that germination of *R. ponticum* occurred in less than 1% full daylight. The results of this study showed that shade conditions (20% of ambient PPFD) imposed by both neutral and green canopies increased germination in the bare soil and short moss seedbed substrate but that a PPFD of $45 \mu\text{mol m}^{-2} \text{s}^{-1}$ did reduce germination on filter paper. Emergence could have been facilitated by several mechanisms, such as reduction of radiation and soil desiccation. The modification of the above-ground microclimate by a canopy species constitutes a major facilitation mechanism of seedling establishment in stressful environments (Kitzberger *et al.*, 2000; Gómez-Aparicio *et al.*, 2005). The ‘canopy effect’ did not influence seedling survival in either of the substrates but did result in a reduction in shoot and especially root biomass.

Germination and seedling survival of *R. ponticum* seeds sown simultaneously with lawn grass seed was the highest of any seedbed substrate, in the no-shade

treatment. On the basis of informal observations, the sown grass substrate was not prone to drying out as much as the other seedbed substrates. These results would suggest that the grass acted similar to the green canopy on the bare soil and short moss substrates in reducing the amount of solar radiation and soil surface evaporation. However seedlings were very stunted and seedling biomass and height were significantly lower than those growing with no vegetative canopy. Poor development could be as a result of competition with grass for light and nutrients, an explanation Stephenson *et al.*, (2006) offered for the absence of *R. ponticum* in a woodland environment. In this experiment, germination and seedling survival in the grass turf seedbed was very low, especially under a green canopy. Any seedlings observed were hard to find due to the dense sward and surviving seedlings after 6 months were again very stunted. Harris *et al.*, (2009) suggest the use of wide corridors of unsuitable habitat such as undisturbed grassland between infested and uninfested areas of *R. ponticum* as a containment strategy to prevent or slow down spread between the areas. The results of this study provide further evidence that competition for mainly light is prohibitively high in grassy substrates, especially dense high grass swards. Thus, while these results further support the positive effects of moss as a germination seedbed and the negative effects of dense grass cover (Siemann & Rogers, 2003; Dovciak *et al.*, 2008), it explicitly places these effects in the context of multivariate environmental gradients that can be encountered. Dovciak *et al.*, (2008) found that moss provided the best germination seedbed for the invasive species *Picea abies* into Western Carpathian grassland, whilst highly open areas with high direct light and high grass cover had low seedling density. Areas of shading by trees also decreased competition from grass, sufficiently enough to provide a suitable moist site for germination.

The results of this study indicate that there may be some degree of adaptation of genotypes from Britain and Ireland to differences in light level. The two populations growing at the edge of *Picea sitchensis* (Sitka spruce) forests (in Ireland and Scotland) were more sensitive to variations in light quantity and quality. Germination in the high PAR, high R:FR and low R:FR light was reduced; conversely germination in the low PAR, low R:FR was higher than the other populations. Although there was a reduction in germination for all populations in response to low levels of R:FR light, the two edge habitat genotypes were least

affected. There are three possible mechanisms for an exotic plant to be adaptive to the physical environment in its introduced areas: pre-adaptation to the new environments, greater phenotypic plasticity and environmental tolerance, and evolved adaptations through local ecotypic specialisations (Ren & Zhang, 2009). Since the populations that exhibited the highest germination in low light were from the same light environment, and were both young stands, the latter mechanism appears to be the most likely. The other populations were either old stands and/or from open habitats. Several studies have shown that species can become invasive by evolutionary adaptation to the physical environment, although such evolution often requires high levels of genetic diversity that may only exist in multiple-introduced species (Weber & Schmid, 1998; Lambrinos, 2004). This may be a contributing factor for the success and spread of invasive *R. ponticum* populations. There is evidence for multiple introductions and also hybridisation with other rhododendron species, such as *R. catawbiense* and *R. maximum* to increase its frost tolerance (Milne & Abbott, 2000). Previous studies by Erfmeier and Bruelheide (2004) found that invasive *R. ponticum* Irish populations differed from non-invasive (Georgian, Spanish) ones mainly in growth patterns and showed much higher rates of annual shoot growth in the field and higher rates of seedling recruitment. A later study Erfmeier and Bruelheide (2005) on native and invasive populations provided evidence for a genetic shift in invasive populations towards an increased investment in growth and towards a faster germination rate. In that study, Erfmeier and Bruelheide also refer to unpublished work which evaluates the relevance of rapid germination in context of the appropriate climatic conditions and conclude that the risk of experiencing severe losses due to climatic factors (such as drought, extreme temperature variation) in invasive genotypes is less likely than in the native ones. In an analysis of 133 invasive plant species (Ren & Zhang, 2009, phenotypic plasticity (either in environmental tolerance or in resource allocation) was responsible for the invasiveness of about 50% of the invasive plants. Alien species often exhibit a greater plasticity in their response to changes and disturbances in the environment and may therefore have a greater capacity to shift the physiological optimum to a range that is favourable in a changing climate (Verlinden & Nijs, 2006).

Prolific seed production, and widespread seed dispersal are often considered key traits contributing to invasion success (Mack *et al.*, 2000), but these results show

that microsite availability can be equally important. A key result in this study is in identifying the role that light, moisture and the interaction of seedbed substrate have on *R. ponticum* germination and seedling performance. In gaining an understanding of the mechanisms behind the site-specific factors that contribute to successful seedling emergence, an attempt can be made to identify vulnerable sites and predict future range expansion. *Rhododendron ponticum* reproduction is mainly by sexually produced seed with vegetative spread being very limited (Cross, 1975). Dispersal of *R. ponticum* by means of numerous small wind-dispersed seeds has proven very effective for expanding into new territory. Controlling seed set is a critical aspect of managing *R. ponticum*, which is a highly fecund species with seed production rates of up to one million seeds per plant per year. Another member of the rhododendron genus, *R. ferrugineum* that extensively colonises grazed or abandoned meadows in the northwestern Alps is a highly fecund species producing up to 2.4 million seeds per m² area of rhododendron cover annually (Pornon *et al.*, 1997). The authors suggest that the production of many small seeds over fewer large ones could be an adaptive response to the rareness of favourable sites, increasing the probability that a seed dispersed by wind or water reaches a safe site. Although the majority of *R. ponticum* seeds travel less than 10 m, a very small proportion can travel more than 50 m (Stephenson *et al.*, 2007). Management should aim to reduce seed production and dispersal of *R. ponticum* in already-invaded sites, and limit the number of microsites available by avoiding anthropogenic changes of habitat dynamics. Harris *et al.* (2009) developed spatially-explicit model to investigate the effectiveness of different control strategies for *R. ponticum* and found that a control strategy that involved removing the oldest plants first was the most effective strategy both in terms of the probability of successful eradication and the number of years taken to control. They were able to attribute this to the ability of the older (and taller) plants towards the core to produce more seeds that on average travelled further.

Although seed bank persistence of *R. ponticum* has been reported to be relatively transient (<1 year) (Thompson *et al.*, 1997; Cross, 1975) compared with many other weedy species, seeds persist long enough to be a management impediment. However, results from limited experiments by Jackson (2008) showed that a significant number of seeds remained viable after 18 months. He also observed germinating seedlings on sites which had been cleared three seasons before, where

no adjacent seed source was available. Having long-lived seeds can be advantageous for invasive species because propagules are effectively dispersed in time, ready to take immediate advantage when conditions become favourable for germination (Panetta, 2004). On the other hand, it may be equally advantageous to produce large amounts of short-lived seeds that germinate *en masse* in the spring, thus lessening the risk of prolonged exposure to seed predators and pathogens (Alvarez-Buylla & Martínez-Ramos, 1990). Particularly when coupled with effective spatial seed dispersal, this strategy produces numerous, widespread seedlings at the time of year most conducive to seedling survival. The results from this study indicate that *R. ponticum* employs the latter strategy, almost all seeds germinating within 70 days after sowing, regardless of microsite conditions. The chances of at least some of these seedlings emerging in sites where conditions are suitable for establishment are likely high, especially for such a fecund species as *R. ponticum*.

The ability of alien plant species to invade a region depends not only on attributes of the plant, but on characteristics of the habitat being invaded. Although all vegetation types are at risk of being invaded by alien plants, mesic communities have both greater numbers and higher frequencies of alien plants than drier communities (Larson *et al.*, 2001). In Britain and Ireland the climate is mild and moisture is unlikely to be a limiting factor to seedling recruitment. The experimental greenhouse-based results of this study are not only in agreement with the distribution of *R. ponticum* in the field as recorded by Cross (1981) and Stephenson *et al.*, (2006) but also help explain the underlying mechanisms. In a 2003 study by Dehnen-Schmutz *et al.*, it was estimated that 52,000 ha of land in the British Isles was affected by *R. ponticum* and that 89% of the total area was found in woodland. In the Snowdonia National Park, Wales, 1900 ha was recorded in 1986, a figure that has risen by 20% in 22 years in large part due to the colonisation of open land adjacent to stands especially in upland heaths, and an increase of stands in forestry plantations. Again the most affected habitat was woodland, with 70% of *R. ponticum* stands occurring within woodland especially coniferous where it can thrive in conditions of high acidity, high humidity and ground disturbance (Gritten, 1995). Stephenson *et al.* (2006) highlight the possible role that disturbance, in particular woodland and forestry management, is likely to contribute to the spread of *R. ponticum*. It is able to tolerate very low levels of light but often in the darker depths

of forestry plantations is limited to ground below canopy gaps. There is abundant evidence that natural or anthropogenic disturbance is a crucial factor for many plant species to invade new habitats (e.g. Hobbs, 1989; Hobbs & Huenneke, 1992; Burke & Grime, 1996) with invasive plants often benefitting disproportionately from disturbance compared with dominant native species (Smith & Knapp, 1999; Stohlgren *et al.*, 1999). Smaller-seeded species are especially more dependent on disturbance events within woodlands, such as gap creation, grazing and soil disturbance to encourage germination or establishment (Burke & Grime, 1996; Kobayashi & Kamitani, 2000; Loh & Daehler, 2007). Over the last decade, recognition of the conservation, amenity and landscape value of broadleaved native woodlands in the UK has increased their desirability over coniferous forests. Although this is a welcoming shift in policy, it could also promote the spread of *R. ponticum* due to the enhanced suitability of the light environment. With woodland being the habitat most affected by *R. ponticum* and forestry incurring the greatest control costs (Dehnen-Schmutz *et al.*, 2003), it is important to focus efforts on the sites most likely to become invaded and gain a further understanding of the factors, such as disturbance that may enhance site invasibility.

5.1.3 Limitations of the study, and need for further research

This study has brought new basic ecological knowledge to the underlying mechanisms of *R. ponticum* invasions, but as with most studies, particularly in ecology it has its limitations and has brought up further issues that need to be addressed. The following will highlight specific caveats about the data and methods used and outline possible areas of future research topics concerning invasion dynamics.

There are also several aspects of the experimental methodology that could be improved upon, such as the watering treatment in the greenhouse experiments. Some substrates, such as the bare soil and moss seedbed substrates had a tendency to dry out on hot days, despite daily watering. Seedlings were also occasionally uprooted by water drops so I would recommend using a finer rose or watering from below. Although the findings of this study indicate strongly the importance of light,

moisture and seedbed substrate in providing suitable microsites for *R. ponticum* seedling emergence, it would be even more significant if these findings were replicated in in-situ field trials. This could be done by establishing plots in different habitats and microhabitats and adding seed to them. Another level of complexity that could be incorporated is by applying different levels of disturbance to the plots. On the basis of informal observations in the field, it has become evident that subtle variations in habitat have a significant effect on the probability of *R. ponticum* colonisation. One of the key areas for potential future research is the role that disturbance and grazing have in *R. ponticum* colonisation. Disturbance can increase the invasibility of a community (Hobbs & Huenneke, 1992) and it has been noted that the disturbance of vegetation and soil by forestry management, grazing animals, or other events such as moorland fires, appear to considerably increase the availability of sites to *R. ponticum* (Fuller and Boorman 1977; Cross, 1981; Rotherham, 1986). Deer grazing in the Killarney oak woodlands increased the heterogeneity of the forest floor by reducing the cover of herbaceous and dwarf shrub layers, and promoting the development of bryophyte communities, thereby aiding establishment of *R. ponticum* (Cross, 1981; Kelly, 1981). The spread of *R. ponticum* in the Norfolk dunes was attributed to a decrease in grazing pressure in combination with soil disturbance from rabbits following the outbreak of myxomatosis (Fuller & Boorman, 1977).

Although *R. ponticum* contains grayanotoxins and is thus poisonous to herbivorous mammals, the unpalatability of a plant is thought to be learnt and not instinctively sensed (Freeland & Janzen, 1974). Despite this, there is evidence of small *R. ponticum* (with very small amounts of toxins) in grasslands, being grazed and uprooted by deer and sheep to such a degree as to control invasion into grass pastures (Jackson, 2008). Plants exhibit diverse responses to herbivory, and these responses determine the effectiveness of grazing for restoration. Thomson *et al.*, (1993) found evidence for sheep grazing impeding the establishment and spread of *R. ponticum* in the Beddgelert area of Snowdonia, North Wales, by mapping its distribution pattern over time (1968 and 1982). Grazing can be a valuable vegetation management tool and whilst the misuse of domestic livestock can increase populations of invasive plants (Mack *et al.*, 2000), proper grazing management can be used to promote desirable vegetation and reduce invasive plant populations

(DiTomaso, 2000). To date however, there has been no experimental based information on the effects of disturbance and grazing on *R. ponticum* spread, and hypotheses and the links between the invasion success of *R. ponticum* and factors such as physical disturbance and grazing, are not adequately understood and are based solely on field observations. Experimental studies on invasive plants have manipulated a range of factors to assess their relative importance for invasion success such as: soil disturbance (Burke & Grime, 1996; Kotanen, 1997), biomass removal (Rachich & Reader, 1999), canopy disturbance (Eschtruth & Battles, 2009), nutrients (Huenneke, *et al.*, 1990; Leishman & Thomson, 2005) and burning (King & Grace, 2000). The results of this study show that *R. ponticum* seedlings compete poorly with grass such as *Lolium perenne* mainly through competition with light. Oversowing pastures with perennial pasture plants and fertilizer to promote competition with emerging seedlings, followed by sheep grazing has been used to control gorse establishment following clearance (Rees & Hill, 2001). It would be valuable to test this in the field with *R. ponticum* as a possible post clearance management technique to prevent reinvasion.

The present study indicates an adaptation of ecotypes from forest edges to increased germination at low R:FR levels. There are several caveats that must be acknowledged in relation to this study, the first being the confounding effect of position in the growth chamber. In future studies I would advise creating a separate light environment for each petri dish by tinting the inside to create a green or neutral light filter and arranging them in a randomised block design within the growth chamber. The second is the small number of replication of populations of different origins in terms of both provenance and ecotype. Other factors that would need to be considered in future studies would be the age of the stand. A change in phenotype in response to environmental conditions that enhances the individual performance under the prevailing conditions is common in plants (reviewed in Schmitt *et al.*, 2003, Galloway & Etterson, 2007). There is some evidence that transgenerational plasticity has evolved in response to natural variation in light, and provides a flexible mechanism by plants to cope with heterogeneous environments (Galloway & Etterson, 2009).

5.2 THE USE OF GLYPHOSATE FOR CONTROL OF *R. PONTICUM*

5.2.1 General Aims

To date work on herbicide impacts on *R. ponticum* has largely been based on field and pot trials of different control practices. There are few well-controlled replicated studies of control methods that take into account all the variable factors that may have an impact on efficacy. There are also no records of any studies on the physiological mechanisms of glyphosate in *R. ponticum*. Assumptions on the mode of action of herbicides in *R. ponticum* have therefore been largely inferred from non-physiological trials in which only the effectiveness of killing treated plants has been monitored. The aims of this study were twofold: to determine glyphosate uptake and translocation in *R. ponticum*, and to test the effect of light and growth stage on these factors.

5.2.2 Functional interpretation and implications of key results

Results of both the field trial and laboratory experiments have brought basic knowledge to the absorption and translocation of glyphosate in *R. ponticum* and highlight the importance of treating the entire crown of *R. ponticum* to achieve complete control. In general, multiple mechanisms could be involved in the differential response to herbicides (Westwood *et al.*, 1997; Norsworthy *et al.*, 2001). Firstly, epicuticular wax and the cuticular layer have been considered to be the major barrier to foliar herbicide uptake (Eglinton, & Hamilton, 1967; Bukovac, 1976). In this study foliar absorption by the leaf abaxial surface was significantly higher than by the adaxial surface. Leaf cuticle thickness, which can vary between and amongst populations, can have a differential effect on the absorption of foliar-applied herbicides (Sherrick *et al.*, 1986; Huangfu *et al.*, 2009). A review of the major progress made between 1991 and 2006 by Wang and Liu in the understanding of pesticide uptake into plant foliage concludes that one of the most important ways to improve the efficacy of pesticides and minimise their impact on non-target organisms is through increasing the penetration of the active ingredient into plant

foliage. Results of this study indicate that despite the addition of an adjuvant, foliar uptake levels by the adaxial surface were still very low. Although application to the lower leaf surface substantially increased absorption this is not a method that could be practically applied in the field using knapsack sprayers. It may be possible to use control droplet application in this manner as the droplets essentially 'stick' to the leaf surface. This method can only be used with premixed Nomix glyphosate packs which are more costly than the unmixed knapsack sprayer glyphosate solution. However, considering the large difference in foliar absorption between the upper and lower leaf surfaces it would be worthwhile to conduct further research in this area with practical field application trials to estimate the cost difference and efficacy of application treatment. One of the benefits to using the control droplet technique is in limiting the amount of collateral damage to adjacent vegetation through drift and runoff associated with the foliar spray method (Pakatul, 2007).

Results of the ^{14}C -glyphosate experiments indicated that translocation out of the treated leaves to other parts of the plant was limited. There was a positive relationship between percentage crown sprayed and damage in the field trial, which could be attributed to a simple dose-response: the greater the number of sprayed leaves, the greater the total glyphosate dose received by the plant. A similar premise could explain the level of translocation: the greater the dose applied, the greater the amount absorbed and consequently translocated. The amount of ^{14}C -glyphosate translocated out of treated leaves to other parts of the plant was also proportional to levels of ^{14}C in the treated leaves. Koger and Reddy (2005) examined the effects of varying glyphosate dose, site of plant exposure to glyphosate spray on efficacy in pitted morning-glory (*Ipomaea lacunosa*). They found that control with glyphosate in pitted morning-glory was more affected by rate than the degree of plant exposure to glyphosate and that treating any one-third section of pitted morning-glory plants was as effective as entire plant exposure. Results from studies have shown that absorption and translocation increased with increasing herbicide concentration (or low spray volume) and that increased phytotoxicity was due to the concentration gradient between droplet and leaf (e.g. Sandberg *et al.*, 1978; Buhler & Burnside, 1987; Liu *et al.*, 1996). Increasing herbicide concentration could cause localized tissue injury and physiologically isolate the active ingredient at the deposit site, thereby reducing translocation from the treated leaves (Liu *et al.*, 1996).

Light had no significant effect on foliar absorption but did increase translocation of ^{14}C -glyphosate out of the treated leaves. Field trials also showed increased rates of translocation when glyphosate was applied to leaves in May and August compared with the November and February applications. Light regulates many aspects of plant growth and development through the effects of quantity of total energy and of photons, spectral quality, duration, and photoperiod (Holt, 1995). It is therefore likely that increased translocation in *R. ponticum* was more extensive due to an increase in metabolic activity in response to light. Similar results have been found by numerous studies (e.g. Neal & Skroch, 1985; Love & Anderson, 2009) where increased activity was experienced at times of the year when photosynthesis would have been highest.

There was an accumulation of shikimic acid in response to foliar application of glyphosate in treated leaves and especially stem sections of *R. ponticum* plants compared to untreated control plants. An increase in levels of shikimic acid due to glyphosate has been found in many other studies (Lydon & Duke, 1988; Pline *et al.*, 2002; Bresnahan *et al.*, 2003; Shaner *et al.*, 2005) Since shikimate accumulation is a direct result of herbicide inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, increased interest has arisen in using shikimate as a biomarker for glyphosate (Shaner *et al.*, 2005; Buehring *et al.*, 2007; Bonini, *et al.*, 2009). Refining this technique for *R. ponticum* might prove useful in future trials for determining levels and extent of glyphosate damage in plants.

5.2.3 Limitations of the study and need for further research

Although this study and others (Edwards, 2004) have shown the successful efficacy of foliar application at any time of year, the collateral damage due to drift can be considerable. Current practice for foliar application recommends spraying ‘till run off’. Since its introduction in the early 1970’s glyphosate has been favoured for its high efficacy, low mammalian toxicity and soil inactivity (Appleby, 2005). Broad-spectrum activity, efficacy and low cost make glyphosate an important chemical in eradication and ecological restoration programs (e.g., Wilkins *et al.*,

2003). However, unless stem injection is used, some herbicide inevitably reaches the soil when applied to weeds. Successful restoration includes not only the control of the invasive plant but also the establishment of high native diversity and appropriate composition of the plant community following invasive plant eradication. If efforts to control the invasive plant negatively affect native vegetation, then the treatment alone may not be worthwhile for restoration even though the method may be successful in eradicating the invasive plant (Love & Anderson, 2009).

In this study, translocation of glyphosate was determined both in a controlled environment using ^{14}C -labelled glyphosate and in field trials by measuring the damage caused. The size of the plants in both the ^{14}C -glyphosate and field trials were all mature plants between 0.3 and 1.5 m in height, and therefore do not reflect processes that might occur in larger plants. Evidence from stem treatment trials indicate that the 'pipe model theory' proposed by Shinozaki *et al.* (1964) to describe tree architecture, whereby a section of the stem, root and crown form ramets or linked units within a plant, becomes more applicable to larger, older bushes. In the Forestry Commission practice guide for managing and controlling invasive *R. ponticum* (Edwards, 2006), Edwards recommends choosing a position on the stem as close to the main root system as possible and at least below the last fork, as only branches above an application point will be controlled, but not those on adjacent stems. With larger stems (especially below a fork) it is necessary to treat two sides of the stem as treating one side will only result in the death of the branching stem directly above the treated area. Published studies from a variety of experimental situations generally indicate that both photosynthesis and stomatal conductance are reduced with the age (and therefore generally size) of shrubs and trees thereby reducing photoassimilate and consequently herbicide movement around the plant (Bond, 2000 and references therein). The 'hydraulic limitation hypothesis' explains the decreased productivity in aging trees to an increase in resistance of the hydraulic pathway through a reduction in the supply of liquid water for transpiration in turn limiting stomatal conductance and photosynthesis (Ryan & Yoder, 1997). A published review by Bond (2000) stresses the need for more well-controlled studies on photosynthesis in woody plants that are designed to separate the effects of age from other sources of variation (e.g. that owing to size or environment). Although quantitative measurements of glyphosate levels and extent of damage were recorded

in this study, it would be of even more value if photoassimilate distribution could also be followed. Several studies have linked the movement of glyphosate with that of photoassimilates in the phloem. (e.g. Gougler & Geiger, 1981; Dewey & Appleby, 1983; McAllister & Haderlie, 1985). An understanding of photoassimilate distribution at different times of year would be of great value for improving all control techniques that use glyphosate, especially the cut stump technique which has had variable success (Peter Jackson, Snowdonia National Park; Julian Miller, Kehoe Countryside, pers. comm.). Several studies have shown that the cut treatment of invasive woody species has been most effective in spring when total root non-structural carbohydrates are at their lowest (Lynn et al. 1979; Love & Anderson, 2009). Stenvall *et al.*, (2009) also showed that resprouting vigour in aspen (*Populus* spp.) was proportional to the amount of root carbohydrates which were lowest in early autumn. A study of glyphosate and photoassimilate translocation patterns in large *R. ponticum* bushes would therefore be valuable not only for practical field application but would contribute to the knowledge of photosynthesis related to the architecture of woody plants.

5.3 MANAGEMENT STRATEGIES FOR THE CONTROL OF *R. PONTICUM*

The main difficulty in dealing with widespread control of the plant species in this study is expense, and lack of funds can be a major factor limiting *R. ponticum* control efforts (Harris *et al.*, 2009). Costs of controlling *R. ponticum* can range between £150 for chemical control of re-growth, up to £10,000 (for complete removal) on steep sites although this does not take restoration costs into consideration (Dehnen-Schmutz, 2004). An alternative to reduce the labour intensive costs is the use of volunteers. This has major success in Florida's Blowing Rocks Preserve, where volunteers have helped remove Australian pine (*Casuarina equisetifolia*), Brazilian pepper (*Schinus terebinthifolius*), and other invasive plants and to plant more than 60000 individuals of 85 native species (Randall *et al.*, 1997). This scheme is also being successfully applied in Ireland where volunteers have helped clear more than 350 hectares of *R. ponticum* from the ancient semi-natural woodlands of the Killarney National Park over 28 years. Successful management

regimes for the long-term control of *R. ponticum* recommend a management programme of *R. ponticum* eradication in essentially three phases - an initial 'attack' phase, with two subsequent 'follow-up' phases - carried out over a period of between five to ten years (Barron, 2000; Edwards, 2006; Jackson, 2008).

There are some financial resources provided for clearing infested land of *R. ponticum*, but these tend to be limited and of unpredictable availability. A study undertaken by Wong *et al* (2002) into potential economic uses of rhododendron found that despite being a high quality firewood extraction costs were too high. The only product they found to be of economic value was the collection and sale of *R. ponticum* shoots for use by florists. In Wales, there are grants availability for the removal of *R. ponticum* from agricultural (Tir Gofal scheme) and woodland sites (Forestry Commission, Woodland Grants Scheme), but unfortunately these rarely cover the cost of clearance and are generally only once-off payments and therefore fail to take into account long-term nature of completion of any control programme (Jackson, 2008).

Other problems encountered are the lack of cross-institutional policy and tools for the planning or facilitation of landscape scale action which is required for effective control (Jenny Wong, Wild Resources; Peter Jackson, Snowdonia National Park Authority, pers. comm.). This becomes especially important when dealing with the concern of reinvasion of a cleared site by seed input from an adjacent infested one. Provided funds are available, which sites should be prioritised and what is the most economically efficient management strategy that will make the biggest impact? A general understanding of the traits that promote establishment is of major importance for the prevention of invasions, but can also assist in management recommendations and in the incorporation quantitative information into population models to predict future distribution and rate of expansion (Sakai *et al.*, 2001). A number of studies using simulation models have demonstrated the effect of initial control on invasive species success thus identifying the optimum manner in which to invest limited resources (Moody & Mack, 1988; Wadsworth *et al.*, 2000; Higgins *et al.*, 2002). For example, Moody and Mack (1988) emphasised the importance of satellite populations in range expansion. The strategy of first destroying outlying populations and then methodically reducing the area of the main infestations has

been recommended in the control of *Hakea sericea* and *Hakea gibbosa* in South Africa and *Euphorbia esula* in North America and is now employed in the control of *Centaurea diffusa* in Canada (Mack, 1985). In contrast to this model, more recent studies have found that targeting the larger core populations is more effective (Hulme, 2003; Harris *et al.*, 2009). Harris *et al.*, (2009) demonstrated that removing the older, taller plants at the core of the population, that produced the most seed and on average seed that travelled further, was the most efficient control strategy for *R. ponticum* in the British Isles. Whilst addressing current invasive species is vital Mack *et al.* (2000), stress that system management, rather than species management should be focus. For example, Love and Anderson (2009) tested different methods of controlling the invasive shrub Morrow's honeysuckle (*Lonicera morrowii*) and found that after reducing the cover of the target species new exotic species emerged, including aggressive invaders like Tree-of-heaven (*Ailanthus altissima*) and Spotted ladythumb (*Polygonum persicaria*). Continued restoration efforts, including follow-up treatments should therefore potentially incorporate the planting of native seeds and saplings, to favour the establishment of native seedlings and herbs and minimise the threat of reinvasion. In some nations, a broader strategic systematic approach aimed at reducing the impact of invasive species, are being put into place. South Africa has recently adopted a national strategy to clear all the invasive woody species from its river catchments in a 20-year, \$150 million programme. The multispecies strategy involves manual clearing of thickets, treatment of cut stumps with mycoherbicides, and the use of biological control to prevent reinvasion (Mack *et al.*, 2000).

5.3.2 Conclusions and Recommendations

This study has brought new basic knowledge to the absorption and translocation of glyphosate in *R. ponticum* the results of which stress the importance of treating the entire crown to ensure complete control. A better understanding of glyphosate and photosynthesis translocation patterns in large bushes could have profound implications for basic biology of woody plants as well as significant practical applications. Also, more attention should be directed toward not only concentrating the removal of *R. ponticum* from a site but also taking into consideration the restoration of the habitat to prevent reinvasion by *R. ponticum* or other invasive plant species. Results from this study indicate that moist open and woodland sites with thin layers of moss carpets are potentially the most vulnerable to invasion and that thick ground vegetation could be a possible management tool for preventing *R. ponticum* establishment. To fully achieve eradication of *R. ponticum*, control programmes must be co-ordinated at a regional or national scale, involve greater investment, and extend over a longer duration.

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APPENDICES

Chapter 2

Appendix 2.1 Univariate analysis of variance on the effect of light treatment (green shade v no-shade); watering regime (daily watering v periodic droughting) and seedbed substrate (bare soil, lawn grass, short moss, grass turf, leaf litter, tall moss) on final germination percentage of *Rhododendron ponticum* seeds after 21 in an unheated glasshouse.

Source of variation	Sum of Squares	df	Mean Square	F	p
Substrate	7900.642	5	1580.128	18.129	0.000
Light	56.033	1	56.033	0.643	0.425
Water	1562.408	1	1562.408	17.926	0.000
Block	1435.571	4	358.893	4.118	0.004
Substrate x Light	7876.967	5	1575.393	18.075	0.000
Substrate x Water	947.892	5	189.578	2.175	0.064
Light x Water	80.033	1	80.033	0.918	0.340
Substrate x Light x Water	313.417	5	62.683	0.719	0.611
Error	8018.629	92	87.159		
Total	28191.59	119			

Appendix 2.2 Univariate analysis of variance onr the effect of light treatment (green shade v no-shade); watering regime (daily watering v periodic droughting) and seedbed substrate (bare soil, lawn grass, short moss, grass turf, leaf litter, tall moss) on germination rate ($t_{1/2}$ = time taken for half of the seeds to germinate) of *Rhododendron ponticum* seeds in an unheated glasshouse.

Source of variation	Sum of Squares	df	Mean Square	F	p
Substrate	1699.676	5	339.935	6.761	0.000
Light	286.934	1	286.934	5.707	0.019
Water	942.995	1	942.995	18.755	0.000
Block	346.360	4	86.590	1.722	0.153
Substrate x Light	930.850	5	186.170	3.703	0.004
Substrate x Water	745.868	5	149.174	2.967	0.016
Light x Water	40.162	1	40.162	0.799	0.374
Substrate x Light x Water	621.148	5	124.230	2.471	0.039
Error	4223.439	84	50.279		
Total	9628.48	111			

Appendix 2.3 Univariate analysis of variance on the effect of light treatment (green shade v no-shade); watering regime (daily watering v periodic droughting) and seedbed substrate (bare soil, lawn grass, short moss, grass turf, leaf litter, tall moss) on seedling survival (percentage) of *Rhododendron ponticum* seeds after 21 in an unheated glasshouse.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Substrate	60009.510	5	12001.902	29.573	0.000
Light	17461.343	1	17461.343	43.025	0.000
Water	15.005	1	15.005	0.037	0.848
Block	1344.416	4	336.104	0.828	0.511
Substrate x Light	20827.970	5	4165.594	10.264	0.000
Substrate x Water	4400.597	5	880.119	2.169	0.065
Light x Water	245.315	1	245.315	0.604	0.439
Substrate x Light x Water	644.483	5	128.897	0.318	0.901
Error	34902.619	86	405.844		
Total	139261.563	113			

Appendix 2.4 Results of one-way analysis of variance tests on the effect of light treatment (green versus clear shade) on *Rhododendron ponticum* seedling growth during the first six months after seed sowing in bare soil/short growing moss.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
<i>Seedling height</i>					
Light	13.347	1	13.347	31.708	0.000
Error	29.466	70	0.421		
Total	42.813	71			
<i>Seedling shoot weight</i>					
Light	0.018	1	0.018	9.750	0.003
Error	0.126	70	0.002		
Total	0.143	71			
<i>Seedling root weight</i>					
Light	0.008	1	0.008	28.788	0.000
Error	0.020	70	0.000		
Total	0.028	71			
<i>Seedling root:shoot</i>					
Light	2.596	1	2.596	56.699	0.000
Error	3.204	70	0.046		
Total	5.800	71			

Appendix 2.5 Results of one-way analysis of variance tests on the effect of light treatment (no v green shade) and seedbed substrate (bare soil/short moss v lawn/turf grass) on *Rhododendron ponticum* seedling height during the first six months under no shade.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Substrate	7.933	1	7.933	43.274	0.000
Error	12.833	70	0.183		
Total	206.690	72			

Appendix 2.6 Results of one-way analysis of variance tests on the effect of light treatment (no v green shade) and seedbed substrate (bare soil/short moss v lawn/turf grass) on *Rhododendron ponticum* seedling shoot biomass during the first six months under no shade.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Substrate	0.052	1	0.051670	31.111	0.000
Error	0.116	70	0.00161		
Total	0.168	71			

Appendix 2.7 Univariate analysis of variance on the effect of light treatment (green, neutral and clear) and seedbed substrate (bare soil, sown lawn grass and tall moss) on germination capacity (cumulative seeds germinated / total seeds sown (%)) of *Rhododendron ponticum* seeds, 12 weeks after sowing in an unheated glasshouse.

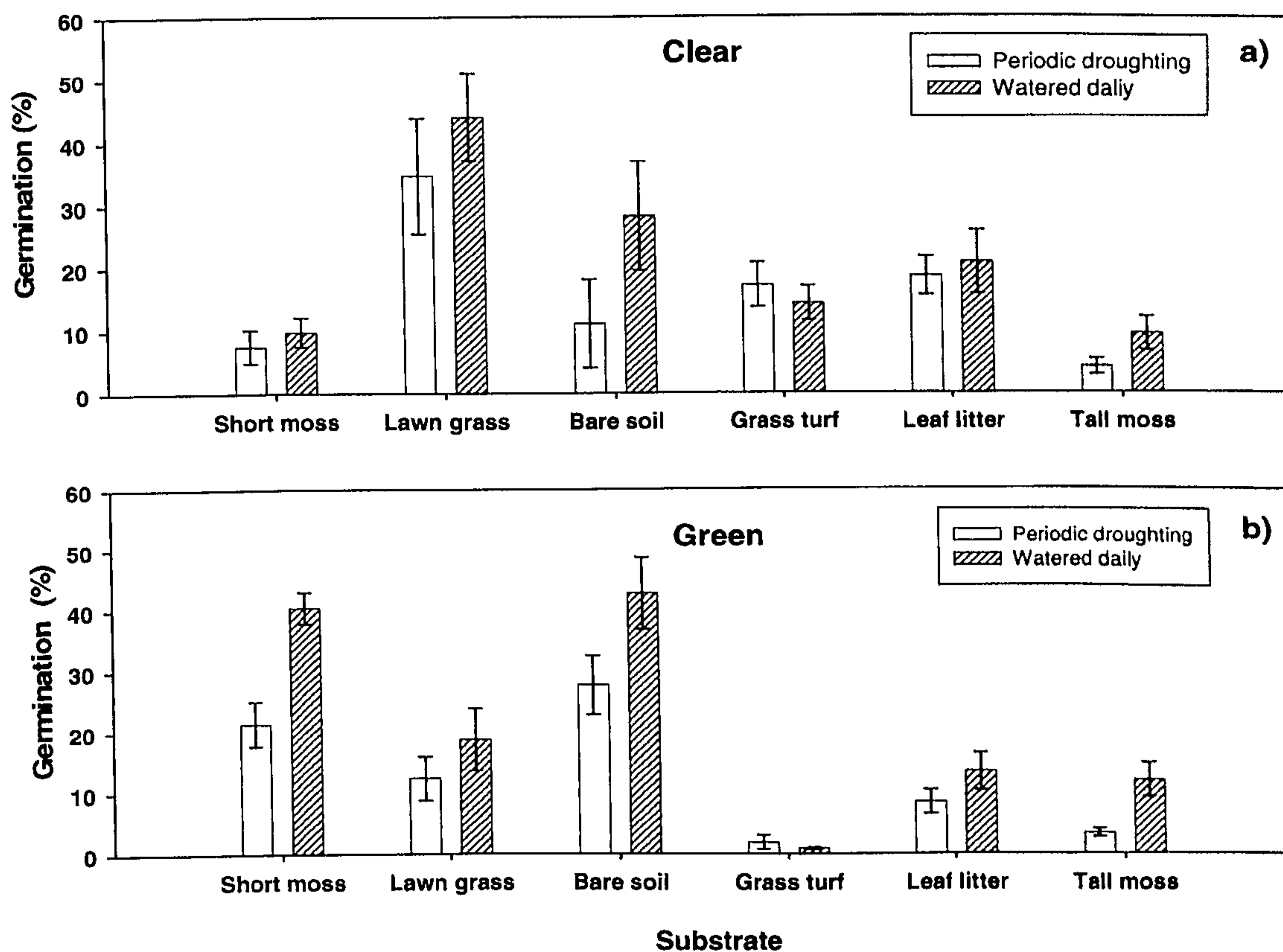
Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Substrate	8201.144	2	4100.572	38.128	0.000
Light	1610.811	2	805.406	7.489	0.002
Block	1052.867	4	263.217	2.447	0.066
Substrate x Light	1650.622	4	412.656	3.837	0.012
Error	3441.533	32	107.548		
Total	38896.000	45			

Appendix 2.8 Univariate analysis of variance on the effect of light treatment (green, neutral and clear) and seedbed substrate (bare soil, sown lawn grass and tall moss) on germination rate ($t_{1/2}$ = time taken for half of the seeds to germinate) of *Rhododendron ponticum* seeds, 12 weeks after sowing in an unheated glasshouse.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Substrate	163.808	2	81.904	2.577	0.094
Light	203.929	2	101.964	3.208	0.056
Block	480.429	4	120.107	3.778	0.014
Substrate x Light	172.458	4	43.114	1.356	0.274
Error	890.079	28	31.789		
Total	1877.596	40			

Appendix 2.9 Univariate analysis of variance on the effect of light treatment (green, neutral and clear) and seedbed substrate (bare soil, sown lawn grass and tall moss) on *Rhododendron ponticum* seedling survival, 12 weeks after sowing in an unheated glasshouse.

Source of variation	Sum of Squares	df	Mean Square	F	p
Substrate	4685.302	2	2342.651	4.022	0.029
Light	7613.274	2	3806.637	6.536	0.005
Block	4030.307	4	1007.577	1.730	0.171
Substrate x Light	3986.177	4	996.544	1.711	0.176
Error	16308.006	28	582.429		
Total	37541.262	40			



Appendix 2.10 The interaction effect of watering and light treatment on final germination percentage of *Rhododendron ponticum* 21 weeks after sowing seed. Vertical bars are standard errors of the

Chapter 3

Appendix 3.1 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on the percentage of live shoots remaining at various times after application.

Source of variation	Sum of Squares	df	Mean Square	F	p
<i>Measurement 1: July 2008</i>					
Block	631.114	7	90.159	.713	0.661
Dose	200001.56	4	50000.392	395.585	0.000
Season of application	2883.404	3	961.135	7.604	0.000
Dose x Season	4054.144	12	337.845	2.673	0.004
Dose x Block	1091.715	28	38.990	.308	1.000
Season x Block	3104.743	21	147.845	1.170	0.299
Error	10617.257	84	126.396		
Total	222383.94	159			
<i>Measurement 2: December 2008</i>					
Block	1239.753	7	177.108	1.041	0.409
Dose	205720.08	4	51430.021	302.189	0.000
Season of application	1469.176	3	489.725	2.877	0.041
Dose x Season	1869.357	12	155.780	.915	0.536
Dose x Block	2814.452	28	100.516	.591	0.942
Season x Block	3707.759	21	176.560	1.037	0.430
Error	14296.097	84	170.192		
Total	231116.67	159			
<i>Measurement 3: May 2009</i>					
Block	1388.578	7	198.368	1.022	0.422
Dose	344949.70	4	86237.425	444.306	0.000
Season of application	1178.301	3	392.767	2.024	0.117
Dose x Season	3106.558	12	258.880	1.334	0.215
Dose x Block	4100.946	28	146.462	0.755	0.798
Season x Block	5071.424	21	241.496	1.244	0.238
Error	16303.937	84	194.094		
Total	376099.44	159			

Appendix 3.2 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) percentage shoots producing new growth on *Rhododendron ponticum* plants < 1.5 m, 1 and 2 growing seasons after application.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
<i>Measurement 1: July 2008</i>					
Block	1154.102	7	164.872	1.224	0.299
Dose	202026.450	4	50506.612	374.832	0.000
Season of application	585.305	3	195.102	1.448	0.235
Dose x Season	1491.705	12	124.309	0.923	0.529
Dose x Block	3654.881	28	130.531	0.969	0.520
Season x Block	3579.997	21	170.476	1.265	0.223
Error	11318.541	84	134.745		
Total	223810.980	159			
<i>Measurement 2: May 2009</i>					
Block	2194.597	7	313.514	1.142	0.345
Dose	592716.643	4	148179.161	539.797	0.000
Season of application	681.624	3	227.208	0.828	0.482
Dose x Season	4596.968	12	383.081	1.396	0.184
Dose x Block	8552.959	28	305.463	1.113	0.345
Season x Block	7697.861	21	366.565	1.335	0.177
Error	23058.756	84	274.509		
Total	639499.408	159			

Appendix 3.3 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on percentage flowering shoots on *Rhododendron ponticum* plants < 1.5 m, 1 and 2 growing seasons after application.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Measurement 1: July 2008					
Block	422.777	7	60.397	2.412	0.027
Dose	4954.335	4	1238.584	49.461	0.000
Season of application	33.225	3	11.075	0.442	0.723
Dose x Season	765.616	12	63.801	2.548	0.006
Dose x Block	916.015	28	32.715	1.306	0.176
Season x Block	740.246	21	35.250	1.408	0.138
Error	2103.501	84	25.042		
Total	9935.715	159			
Measurement 2: May 2009					
Block	493.636	7	70.519	.974	0.455
Dose	14290.168	4	3572.542	49.358	0.000
Season of application	714.556	3	238.185	3.291	0.025
Dose x Season	1495.821	12	124.652	1.722	0.076
Dose x Block	1433.813	28	51.208	.707	0.849
Season x Block	1766.293	21	84.109	1.162	0.306
Error	6079.877	84	72.379		
Total	26274.163	159			

Appendix 3.4 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on the percentage of live shoots remaining on *Rhododendron ponticum* plants < 1.5 m, approximately one year after treatment.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Block	2206.372	7	315.196	1.192	0.328
Dose	22507.547	2	11253.773	42.562	0.000
Season of application	3066.060	3	1022.020	3.865	0.016
Dose x Season	1419.044	6	236.507	0.894	0.508
Dose x Block	2203.000	14	157.357	0.595	0.853
Season x Block	6376.482	21	303.642	1.148	0.342
Error	11105.251	42	264.411		
Total	48883.756	95			

Appendix 3.5 Univariate analysis of variance on the effect of percentage of the crown treated (dose) and seasonal timing of glyphosate treatment (spraying with 2% glyphosate, 2% Mixture B) on growth of new shoot growth (shoot length, new leaf length) on *Rhododendron ponticum* plants < 1.5 m, after one growing season.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
<i>New shoot growth</i>					
Block	511.361	7	73.052	16.303	0.000
Season	129.230	3	43.077	9.614	0.000
Dose	1276.884	3	425.628	94.990	0.000
Season x dose	258.201	9	28.689	6.403	0.000
Error	1801.266	402	4.481		
Total	3994.034	424			
<i>New leaf growth</i>					
Block	256.705	7	36.672	1.887	0.070
Season	89.932	3	29.977	1.542	0.203
Dose	1411.271	3	470.424	24.201	0.000
Season x dose	261.595	9	29.066	1.495	0.147
Error	7814.226	402	19.438		
Total	9755.487	424			

Chapter 4

Appendix 4.1 Univariate analysis of variance on the effect of foliar application of glyphosate to levels of ^{14}C glyphosate in the leaves and related stem section of *Rhododendron ponticum* woody shrubs 30-50 cm in height.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Treatment	82.366	1	82.366	25.399	0.000
Plant part	72.698	1	72.698	22.418	0.000
Treatment x Plant part	72.698	1	72.698	22.418	0.000
Error	278.883	86	3.243		
Total	579.342	89			

Appendix 4.2 Univariate analysis of variance on the effect of foliar application of glyphosate to levels of shikimic acid in the leaves and related stem section of *Rhododendron ponticum* woody shrubs 30-50 cm in height.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Treatment	1.000	1	1.000	11.826	0.001
Plant part	0.001	1	.001	0.010	0.921
Treatment x Plant part	0.093	1	.093	1.101	0.297
Error	6.765	80	.085		
Total	7.858	83			

Appendix 4.3 Univariate analysis of variance on the effect of light treatment on foliar absorption of ^{14}C -glyphosate applied to *Rhododendron ponticum* plants 30 – 50 cm in height over 30 days.

Source of variation	Sum of Squares	df	Mean Square	F	<i>p</i>
Light	567.940	1	567.940	2.394	0.128
Harvest time	14786.252	4	3696.563	15.582	0.000
Light x Harvest time	238.384	4	59.596	0.251	0.908
Error	11861.589	50	237.232		
Total	27454.165	59			

Appendix 4.4 Univariate analysis of variance on the effect of light treatment on the translocation to different plant parts of ¹⁴C-glyphosate applied to the leaves of *Rhododendron ponticum* plants 20 – 50 cm in height over 30 days.

Source of variation	Sum of Squares	df	Mean Square	F	p
Harvest time	5.441	4	1.360	7.056	0.000
Light	0.725	1	0.725	3.760	0.054
Plant part	67.048	4	16.762	86.958	0.000
Light x Harvest time	2.565	4	0.641	3.327	0.011
Plant part x Light	.550	4	0.137	0.713	0.584
Harvest time x Plant part	12.255	16	0.766	3.974	0.000
Harvest time x Plant part x Light	7.341	16	0.459	2.380	0.003
Error	48.190	250	0.193		
Total	144.114	299			

Appendix 4.5 Tukey HSD on the effect of plant component part on ¹⁴C-glyphosate activity applied to the leaves of 30-50cm *Rhododendron ponticum* plants.

Plant part	Plant part	Mean Difference	p	95% Confidence Interval	
				Lower Bound	Upper Bound
Treated stem	Leaves above	1.1898*	0.000	0.9696	1.4101
	Leaves below	1.2160*	0.000	0.9957	1.4362
	Leaves other shoot	1.2300*	0.000	1.0097	1.4502
	Roots	1.0461*	0.000	0.8258	1.2663
Leaves above	Treated stem	-1.1898*	0.000	-1.4101	-0.9696
	Leaves below	0.0261	0.998	-0.1941	0.2464
	Leaves other shoot	0.0401	0.987	-0.1801	0.2604
	Roots	-0.1438	0.380	-0.3640	0.0765
Leaves below	Treated stem	-1.2160*	0.000	-1.4362	-0.9957
	Leaves above	-0.0261	0.998	-0.2464	0.1941
	Leaves other shoot	0.0140	1.000	-0.2062	0.2343
	Roots	-0.1699	0.215	-0.3901	0.0504
Leaves other shoot	Treated stem	-1.2300*	0.000	-1.4502	-1.0097
	Leaves above	-0.0401	0.987	-0.2604	0.1801
	Leaves below	-0.0140	1.000	-0.2343	0.2062
	Roots	-0.1839	0.150	-0.4042	0.0364
Roots	Treated stem	-1.0461*	0.000	-1.2663	-0.8258
	Leaves above	0.1438	0.380	-0.0765	0.3640
	Leaves below	0.1699	0.215	-0.0504	0.3901
	Leaves other shoot	0.1839	0.150	-0.0364	0.4042