The Effect of Seasonal Burning on Three Australian Native Orchids

by

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Declaration of Authenticity

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Dedication

This thesis is dedicated to my mother, Piyaseeli Abeysundera, my late father Dayananda Abeysundera, my husband Dileeka Jasinge, my daughters Sethma Jasinge and Methma Jasinge and my brothers Hesitha and Kosala Abeysundera.
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Abstract

In Australia, bushfires are common in the seasonally dry parts of the continent and prescribed burning is used to reduce fuel load and fire risk. Little information is available on the effects of fire, especially on terrestrial orchids and their mycorrhizal fungi. This project investigated the effects of seasonal burns on Australian native terrestrial orchids to find the best (least damaging) season for a burn in spring-flowering (*Glossodia major* and *Thelymitra pauciflora*) and autumn-flowering (*Pterostylis revoluta*) orchids. Mid seasonal burns in autumn, winter, spring and summer were conducted for each species and empirical data before and after the burns were used to compare against controls as a field standard. The effect of fire was compared based on plant (re-emergence, growth and reproduction) and fungal (colonisation, growth and biodiversity) response.

Based on plant responses, the least affected season for a burn was different for each species but coincided with seed dispersal or senescence. The least damaging burn season for the spring-flowering orchids (*G. major* and *T. pauciflora*) was spring or summer and winter for autumn-flowering (*P. revoluta*) orchids, with the number of emergent plants post-burn equal or higher than controls.

The isolation of *Rhizoctonia*-like fungi from post-burn plants resulted in 3-fold reductions for spring-flowering orchids but more than 3-fold increases for autumn-flowering orchids with up to 80% pelotons growing as *Rhizoctonia*-like fungi (summer post-burn). Based on *Rhizoctonia*-like fungi isolation success, the least damaging fire season for fungal isolation was autumn for spring-flowering orchids and summer for autumn-flowering orchids but was affected by phenology with plants in the vegetative stage (leafing) superior to reproductive stage (flowering).

The effect of fire on fungal growth was only observed on *G. major* isolates that was least affected by spring burns and had similar growth increases as controls coinciding with plant senescence. *Glossodia major* isolates were sensitive to smoke water with post-burn isolates from summer inhibited at high concentrations and may contribute to declining fungal populations if burns continued in this season. The season of fire and smoke water had little or no changes on fungal growth for *T. pauciflora* and *P. revoluta* isolates and may reflect the tolerance of fungi to fires.
Fire (irrespective of season) reduced the diversity of *Rhizoctonia*-like fungi from *G. major* and *P. revoluta* plants, with fungal variants as high as 10 individuals pre-burn reduced to only 2 individuals post-burn for *P. revoluta*. The season of least damage when post-burn fungi were the same as controls for *G. major* and *P. revoluta* was autumn and spring respectively, coinciding with leafing phenology. Fungal diversity for *P. revoluta* was 5 times greater than *G. major* and could reflect the advantages of mycorrhizal infidelity *in situ* to exploit available fungi or multiple sources of fungal reservoirs to re-establish post-fire.

The findings of this study are novel and critical for land management and conservation of endemic plants. The overall conclusion was that seasonal prescribed burnings at the correct time of the growth cycle were beneficial for plant responses but detrimental for mycorrhizal fungi. The best season (least damaging) for a fire was different depending on the plant host and a general compromise was late spring (late November) for all three orchids.
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Notations and abbreviations

ECM : Ectomycorrhizal
AM  : Arbuscular Mycorrhiza
OM  : Orchid Mycorrhizal Fungi
SE  : Standard Error
NS  : Not Significant
SD  : Significantly Different
MEA : Malt Extract Agar
SW  : Smoke Water
ANOVA: Analysis of Variance
$\chi^2$ : Chi-Square
Df  : Degrees of freedom
MSE : least square error from ANOVA
n   : replicate number
Chapter 1

Introduction and Literature Review
In Australia, bushfires are common in the seasonally dry parts of the continent and prescribed burning is used to reduce the fire risk. Comparatively little information is presented on the effects of this regime on conservation, particularly of terrestrial orchids. This thesis studied the effects of seasonal burns on Australian native terrestrial orchids in an attempt to find the best (least damaging) season for a burn in two spring-flowered and one autumn-flowered species in the common genera *Glossodia*, *Pterostylis* and *Thelymitra* and their associated mycorrhizal fungi in the genera *Sebacina*, *Ceratobasidium* and *Tulasnella* respectively. The following literature review therefore covers what is known about the topics of fire and the factors affecting its effect on forest habitats, in particular orchids and their mycorrhizal fungi.

### 1.1 Orchids

The Orchidaceae is one of the most fascinating and species-rich of all plant families (Backhouse & Jeanes, 1995). They are largely dependent on two entirely different types of organisms for their survival and reproduction: fungi and insects. The plants have highly modified structures that accommodate these dependencies, as detailed below. Of the more than 1000 species in Australia, 270 occur in Victoria, in a wide range of habitats, from wet alpine bogs to dry woodlands and heathlands (Backhouse & Jeanes, 1995; Jones, 2006).

#### 1.1.1 Habitat

Orchids are all herbaceous monocotyledons and have typical monocotyledonous features such as sheathing leaves with parallel venation (Backhouse and Jeanes, 1995; Jones, 2006). The roots of terrestrial taxa are often poorly developed and almost invariably colonised by endophytic mycorrhizal fungi. The species in the Orchidaceae are easily recognised to family because of their distinctive and sometimes bizarre floral structure.

The orchids are commonly divided into three groups: epiphytes, lithophytes and terrestrials. About 18% of Australian orchids are epiphytes or lithophytes while around 82% of the native Australian orchid species are terrestrials (Jones, 2006), the group that is the focus of this thesis. Many terrestrial orchids form underground
tubers that act as storage organs during a period of dormancy. Tubers and tuberoids allow terrestrial orchids to survive underground in extremely dry weather and during fires in summer/autumn (Backhouse and Jeanes, 1995).

Most terrestrial orchids are evergreen in the tropics and sub-tropics but seasonally deciduous and dormant in more temperate habitats. Soon after seed dispersal, the above-ground parts die back, leaving the underground tubers in dormancy in hot and dry or (less frequently) cold and wet conditions. On return of suitable conditions for growth, the dormant tuber produces new roots, leaves and flowers.

1.1.2 Sexual reproduction

Orchids have extremely unusual floral structures. Flowers have six perianth segments in two whorls of three and range from almost actinomorphic, e.g. *Thelymitra*, to strongly zygomorphic, e.g. *Pterostylis* (Backhouse and Jeanes, 1995; Jones, 2006). In the more strongly zygomorphic genera, the upper perianth segment in the inner whorl is larger and more brightly coloured than the others, frequently has prominent glandular hairs and is called the labellum. The stamens, style and stigma are combined above the inferior ovary into a central column in which the pollen is borne in clumps (pollinia) just above the stigma. In most orchid genera, the pedicel extends asymmetrically so that the flower appears ‘upside down’ (resupinate), e.g. *Glossodia*, with the labellum on the lower side, forming a platform, but in some, e.g. *Thelymitra*, this does not occur.

Some orchids appear to be self-compatible and largely self-pollinated, e.g. some species of *Thelymitra*, *Microtis* and *Caladenia* Section *Eucaladenia*, whereas most are almost or totally self-incompatible and depend heavily on pollination by specific pollinators, e.g. many species in *Caladenia* Section *Calonema* (Backhouse and Jeanes, 1995; Jones, 2006). Pollination is by various insects attracted to the flower either by rewards or for pseudo-copulation. Relatively few orchids have food rewards such as nectar, e.g. *Eucaladenia* species, and some have flowers that mimic nectar-rewarding species in their vicinity, e.g. the yellow-and-brown *Diuris* and ‘bacon-and-eggs’ pea flowers (Backhouse and Jeanes, 1995). Some attract insects, e.g. fungus gnats, by emitting odours resembling decomposition, e.g. *Acianthus*. 
Most bizarrely, many attract the pollinator by the flower imitating the female of a species of insect, e.g. thynnid wasp, thus attracting the male insects in the process of an effort to mate, e.g. *Calonema*, and then transferring the pollinia to another flower.

In terrestrial taxa about 6-8 weeks after ovules are fertilised, the ovary has developed into a capsule with thousands of minute, dust-like seeds that are dispersed when the capsule desiccates and splits longitudinally (Backhouse and Jeanes, 1995; Jones, 2006). Each seed is greatly underdeveloped and is little more than a cluster of undifferentiated cells surrounded by a dry testa. It contains little or no food reserves and in nature does not germinate until infected by a suitable mycorrhizal fungus (discussed later). It is thought that few seeds encounter the necessary combination of conditions that result in growth to maturity.

1.1.3 Asexual reproduction

Relatively few terrestrial species reproduce vegetatively. In most cases, the parent plant produces multiple tubers instead of only one and each produces a new plant. Such clonally reproducing species tend to occur in large clusters, e.g. mat-forming species of *Pterostylis* (Backhouse and Jeanes, 1995; Jones, 2006). Fire is said to stimulate the growth of tubers from dormancy and may explain the simultaneous appearance of large clusters of some orchids, e.g. *Pterostylis* species, after fire (Backhouse and Jeanes, 1995).

Some species of *Microtis* exhibit apomixes (ovules never undergo meiosis and so are diploid) and so individuals are clonal even though they produce seed from apparently normal flowers (Backhouse and Jeanes, 1995).

1.1.4 Nutrition

Orchids are totally reliant on their mycorrhizal fungi for seed germination and initial seedling growth (Backhouse and Jeanes, 1995; Jones, 2006). Once a suitable fungus has infected a specific group of cells in the seed, producing intracellular dense coils called pelotons, the seed swells, breaks open the testa and cell division produces an unorganised group of cells called a protocorm, though the relative order of infection and swelling may vary (Clements and Ellyard, 1979; Warcup, 1981).
Shortly afterwards, rhizoids appear over the surface of the protocorm and further organisation produces a leaf primordium. In green orchids, the leaf expands and turns green in daylight and presumably commences photosynthesis. It is some time later that roots are developed and often do not appear in culture until a dropper appears and grows downward, swelling at the end into a root tuber (tuberoid), the cells of which accumulate food reserves. Nutrition is provided to the orchid exclusively from the fungus until the leaf greens and even then the plants are not truly autotrophic, as there is still considerable exchange of both inorganic and organic compounds between the fungus and the orchid – a condition called ‘mixotrophy’ (Julou et al., 2005).

1.2 Mycorrhiza

A mycorrhiza is a mutualistic interaction between a fungus and a plant. The name mycorrhiza was given by Frank (1885) and literally means fungus-root (Stoian & Florian, 2009). Mycorrhizal fungi infected regions are roots and other underground organs such as stem-collars and tubers of orchids which allow the transfer of nutrients between plant and mycorrhizal fungus (Van der Heijden & Sanders, 2002; Brundrett, 2004; Cairney, 2005). Mycorrhizal fungi increase the tolerance to environmental stresses (Tommerup & Bougher, 2000) and facilitate the plant to defend from some pathogens (Zak, 1964; Marx, 1972).

Mycorrhizas are classified on the amount of fungal penetration into the root, the presence or absence of an external mantle (sheath) of hyphae and on inter- and intra-cellular characteristic structures they form inside the plant root (Brundrett et al., 1996). This divides the mycorrhizae into two broad groups: the ectomycorrhizae (ECM), in which the hyphae do not penetrate the cells, and the endomycorrhizae, in which hyphae penetrate the cells and form various structures. The ectomycorrhizae occur only in woody plants. Ectomycorrhizae are characterised by a sheath of hyphae surrounding the root. There is no penetration of hyphae into the root. Side protuberance of the sheath may go through the epidermal cells of the root and form the Hartig net, which increases the surface area for the interchange of nutrients between the host plant and fungi.
There are several types of endomycorrhizae: (vesicular-) arbuscular mycorrhiza (AM) (widespread in most plants, both woody and herbaceous), ericoid mycorrhiza (only in the family Ericaceae) and orchid mycorrhiza (only in the family Orchidaceae). The most common type of mycorrhiza is the Arbuscular mycorrhiza. They enter the cortical cells of the root and form arbuscules, which is unique (Brundrett, 2002). The plasmalemma of the host cell invaginates and encloses the arbuscule. Ericoid mycorrhizas penetrate into the epidermal cells of the fine roots of the host. Hyphal coils are produced in the cells and the fungi do not spread from one cell to another. Orchid mycorrhizas are characterised by hyphal coils (pelotons) in cortical cells of the underground organs of the plant.

1.2.1 Orchid mycorrhiza

All members of the family Orchidaceae are symbiotically associated with mycorrhizal fungi in their natural habitats. Orchids depend on mycorrhizal associations for seed germination, seedling establishment, plant growth and survival. If the mycorrhizal fungus does not stay alive, the orchid also dies out from the field (Perkins et al., 1995). At early stages of orchid seed germination, fungi provide both organic and inorganic nutrition to the developing seed and seedling. Some green and achlorophyllous orchid species rely on mycorrhizal fungi throughout their lives because they do not have widespread root system, have only few leaves or lack chlorophyll (Harley and Smith, 1983). Cameron et al. (2006) categorised the photosynthetic orchid-fungus relationship as a true mycorrhizal association because the photosynthetic orchid not only gains nutrition from its fungal partner but also supplies carbon nutrition to its fungi.

Australian terrestrial orchids associate with two major types of mycorrhizal fungi. The green (photosynthetic) orchids form mycorrhizal associations with Rhizoctonia-like fungi in Sebacina, Tulasnella, Ceratobasidium and Thanetophorus genera (Warcup & Talbot, 1971; Warcup, 1981; Perkins et al., 1995; Bougoure et al., 2005). By contrast, generally the non-photosynthetic orchids generally form mycorrhizal associations with homobasidiomycete fungi, for example Russula (Lee Taylor & Bruns, 1999) Laccaria (Kristiansen et al., 2001), Russulaceae (Bougoure et al., 2005) and also Ascomycota fungi (Rasmussen, , 1995; Currah et al., 1997a),
though the report of the association of a *Gastrodia* species with an *Armillaria* species (Campbell, 1962) now appears doubtful.

### 1.2.3 Fungal specificity in photosynthetic orchids

Comparatively few fungal genera associate with green orchids. This mycorrhizal fungi identified by isolation of the fungi from roots, tubers, stem collars and protocorms of orchid plants followed by genetic and morphological judgment with known teleomorphic test cultures (Lilja *et al.*, 1996; Roberts, 1999; Kristiansen *et al.*, 2001). Orchid mycorrhizal fungi are in the Basidiomycota fungal Division (mushrooms). Fungal endophytes are in the imperfect genus *Rhizoctonia* DC. (Basidiomycota) and have binucleate or multinucleate hyphae. These fungi have sexual stages in the teleomorphic genera *Ceratobasidium* D.P. Rogers, *Sebacina Tul. & C. Tul.*, *Serendipita* P. Roberts, *Thanatephorus* Donk and *Tulasnella* J. Schröt ((Warcup & Talbot, 1966; Warcup & Talbot, 1967; Warcup, 1971; Warcup & Talbot, , 1971; Warcup, 1973; Warcup & Talbot, 1980; Warcup, 1981; Warcup, 1991; Roberts, 1999).

Isolations of fungi from Australian wild orchids have shown specificity among the orchid and the associated mycorrhiza (specific orchids associate with specific fungi or a narrow range of fungi). There are patterns of specificity shared across species within the same genus and among different genera (Table 1.1).

<table>
<thead>
<tr>
<th>Orchid genera</th>
<th>Associated fungal genera</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pterostylis</em>, <em>Prasophyllum</em> and <em>Genoplesium</em></td>
<td><em>Ceratobasidium</em></td>
</tr>
<tr>
<td><em>Thelymitra</em>, <em>Calochilus</em>, <em>Diaris</em>, <em>Orthoceras</em> and <em>Caladenia</em>, <em>Eriochilus</em>, and <em>Glossodia</em></td>
<td><em>Tulasnella</em></td>
</tr>
<tr>
<td><em>Microtis</em>, <em>Spiranthes</em> and <em>Lyperanthus</em> (less specific)</td>
<td><em>Ceratobasidium</em>, <em>Tulasnella</em> or <em>Sebacina</em></td>
</tr>
</tbody>
</table>


Research that has used imperfect names is difficult to fit into this pattern. In *Pterostylis*, Perkins and McGee (1995) found three possible mycorrhizal fungi at an
Experimental site but only one was associated with adult plants and protocorms of *Pterostylis acuminata* in the field and in the germination trials. The fungus that had a specific association with *P. acuminata* was identified as *Rhizoctonia solani* by traditional hyphal anastomosis tests. The distribution and abundance of *P. acuminata* specific fungal association must have been influenced by asexual reproduction. By contrast, Irwin *et al.* (2007) used molecular techniques to study 15 *Pterostylis nutans* plants from six geographic areas to identify root associated fungi; two closely related *Ceratobasidium* fungi were associated with *P. nutans*.

Also, Perkins *et al.* (1995) collected mature roots and protocorms of *Microtis parviflora* from the field and isolated fungi. They found only two fungi, *Epulorhiza repens* and *Epulorhiza* sp., colonising the tissues of roots and protocorms and concluded that *M. parviflora* associated with a narrow range of fungi in the field (narrow ecological specificity) and that the same fungi were established in the protocorms. Without molecular data, it is difficult to place these isolates into the teleomorphic taxa.

1.2.4 Techniques for identification of orchid mycorrhiza

Orchid mycorrhizal fungi are usually studied by isolation of fungi and growth *in vitro*. This has allowed basic fungal identification and simple *in vitro* seed germination experiments with isolated fungi (Warcup 1971; Clements, 1988).

Identifying *Rhizoctonia*-like endophytes in culture is difficult because of the rarity of distinctive morphological characteristics and stable morphological features in culture (Andersen, 1990). Two methods have used to recognize them: morphological characters (Currah & Sherburne, 1992; Currah & Zelmer, 1992; Andersen, 1996; Currah *et al.*, 1997a; Currah *et al.*, 1997b) and anastomosis groups, similar to those used for plant pathogenic *Rhizoctonia* species (Carling *et al.*, 1999; Sen *et al.*, 1999), but they were unable to distinguish endophytes at the level of orchid specificity. The application of molecular methods (Kristiansen *et al.*, 2001) improved orchid fungal taxonomy. While orchid mycorrhizal fungi (OMF) had previously had to be isolated from orchids to study their metabolic and symbiotic capabilities, they have now been identified directly from infected regions of orchids (protocorms, roots, tubers and stems) as well as from cultured fungi using the ITS (internal transcribed spacer)-
based fungal-specific primers (Bruns et al., 1998; Bidartondo et al., 2000; Bidartondo & Bruns, 2001; Bougoure et al., 2005b; Martos et al., 2009). For orchid mycorrhiza recalcitrant to axenic growth, PCR amplification of colonized orchid tissues using fungus-specific primers has been used (Dearnaley & Le Brocque, 2006; Dearnaley & Bougoure, 2010), but does not distinguish physiologically different fungal isolates.

A problem in this type of molecular identification is the common occurrence of multiple fungal isolates from one orchid tissue (root or stem-collar) that vary in the ability to germinate seeds of the same orchid species and sustain its further growth (Rasmussen, 1995; Huynh et al., 2009). This suggests that more than one fungus inhabits a single orchid. The sequencing of single pelotons from the root cortex of Dactylorhiza majalis showed that more than one mycobiont was present (Kristiansen et al., 2001) and Huynh et al. (2004) showed that one peloton from Caladenia formosa contained more than one diameter of fungal hyphae by scanning electron microscopy. Sequencing of the ITS region of the ribosomal DNA is commonly used method to distinguish these fungal isolates but does not distinguish among the isolated fungi on their symbiotic effectiveness and this has been especially critical to orchid conservation procedures involving restorative work. At a broader taxonomic range, sequencing of the large subunit (LSU) of the nuclear ribosomal DNA of the Sebacinales, including common orchid mycobionts, gave high resolution separation of groups A and B, two major clades in this group (Weiβ et al., 2004; Selosse et al., 2009; Weiβ et al., 2011) in which the Sebacinales infecting orchids formed a separate clade (group B) from most of the other species. Huynh et al. (2009) showed that ITS sequencing did not sufficiently distinguish isolates of the ‘Sebacina vermifera’ complex (Sebacinales group B), which is a common mycobiont of spider orchids in Australia. Therefore, the application of more sensitive novel techniques is vital to distinguish closely related mycobionts of different symbiotic effectiveness.

Real-time or quantitative PCR (qPCR) allows simultaneous detection and quantification among specific DNA sequences (VanGuilder et al., 2008) by fluorescent dye that binds to the double-stranded DNA or a modified DNA probe that fluoresces when joins up with corresponding DNA. This facilitates the discovery and study of PCR products (Obrepalska-Steplowska et al., 2008) in a specified time reducing post-processing procedures and delays, and also reduces possible investigational errors (VanGuilder et al., 2008).
Melting curve analysis during or after qPCR cycles is an assessment of the dissociation-characteristics of double-stranded DNA during heating. During PCR, the double-stranded DNA is heated gradually until the DNA double strand detaches (or melts), which then, releases the fluorescent dye and as a result decreases the fluorescence. Melt curve graph is plotted using this decreased fluorescence and increased temperature, which is characteristic for that particular amplicon. These melt curves were converted into melt peak graphs. The information given from the melt peaks can be used to assume the presence and identity of the DNA used and to compare it with those of other related species. This is because G-C base pairing has three hydrogen bonds between bases while A-T base pairs have only two. DNA with a greater G-C content has a greater melting temperature than DNA with a lesser G-C and a correspondingly greater A-T content. The varying thermal stabilities between regions of the PCR amplicon, the denaturing of double stranded DNA sometimes takes place at different temperatures. This will result in a unique melt shape for the amplicon, with more than one peak (Robinson et al., 2006).

1.3 Fire

Australia has been a fire-prone continent for millions of years due to its geographical location, climate and vegetation. Many Australian ecosystems and plants within them have specialised adaptations to survive fire but some plants are at greater risk or endangered due to natural or, more recently, anthropogenic disturbances. In Australia, large proportion of orchids are at risk; 10% of orchids are uncommon or endangered in Victoria (Backhouse & Cameron, 2005). Most of the rare orchids have very small populations of less than 100 plants, therefore, conservation is a main challenge to conservation organizations (Coates et al., 2006).

1.3.1 Climate and vegetation

South-eastern Australia has four seasons: summer, autumn, winter and spring. The climate in Victoria can be categorized as cool in spring (September to November), temperate to hot in summer (December to February), less warm and wet in autumn (March to May) and cold and moist in winter (June to August). The gradual rise in temperature and the lengthening of the day in spring triggers the plants in
woodlands to form new leaves and flowers. In the warm to hot summer the plants dry out and increase the fuel load in the woodlands.

Woodlands in Victoria have similar vegetation structures in layers, with the tallest reached by eucalypts and a lower multilayered structure of tree ferns, acacias, shrubs, tussock grasses, and orchids. The floor cover consists of various grasses, non-flowering plants such as ferns and bryophytes. Victoria often experiences wildfire in these woodlands during the hot summer or in autumn from natural causes or by human activity, threatening human life, assets and ecosystems (Table 1.2).

Australian native plants, including eucalypts and tea tree (Leptospermum), have 50% oil glands in the leaf, which increases the intensity of fires and results in longer burning times and high flames (Mutch, 1970) reported that high crude oil content in eucalypts produced high heat content, which set the stage for more intense fires with greater flame heights (Table 1.3). Anderson (1968) stated that fuels that generated the tallest flames had the greatest spread rates, which increased the severity of the fire.

1.3.2 Management of wildfires

Fuel reduction burn is a planned and deliberate application of fire to woodland fuels (leaf litter, twigs, grass, shrubs, bark or other vegetation) under specified conditions to achieve well defined management objectives (Wade et al., 1989). The major reason to use prescribed burning is to reduce the severity of wildfires and limit the spread of fire by reducing the ‘fuel’ in forests and thereby minimise damage to infrastructure at the metropolitan boundary, which will eventually affect human safety (Haines et al., 1998). Other wide range of aims that can be achieved by fuel reduction burning are the preparation of locations for tree regeneration, wildlife environment management, biodiversity protection and management of weeds, insects and diseases (Kilgore & Curtis, 1987; Wade et al., 1989).
**Table 1.2** Significant bushfires and the death toll incurred in Victoria (Australia, 2010; DEPI 2013)

<table>
<thead>
<tr>
<th>Year and month</th>
<th>Season</th>
<th>Type of bushfire</th>
<th>Location</th>
<th>No. of human deaths</th>
<th>Area burnt (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1939 Jan.</td>
<td>Summer</td>
<td>Unknown</td>
<td>Black Friday - Warbuton, Omeo, Bright</td>
<td>-</td>
<td>2,000,000</td>
</tr>
<tr>
<td>1983 March</td>
<td>Autumn</td>
<td>Unknown</td>
<td>Cann River</td>
<td>-</td>
<td>140,000</td>
</tr>
<tr>
<td>1998 March</td>
<td>Autumn</td>
<td>Unknown</td>
<td>Trentham</td>
<td>-</td>
<td>3,500</td>
</tr>
<tr>
<td>2003 Jan.</td>
<td>Summer</td>
<td>Natural</td>
<td>North- east Victoria, Mt Buffalo, Bright, Dinner Plain, Benambra and Omeo</td>
<td>1</td>
<td>1,300,000</td>
</tr>
<tr>
<td>2006 Dec.</td>
<td>Summer</td>
<td>Unknown</td>
<td>Grampians, Kinglake, Moondarra, Yea and Anakie</td>
<td>1</td>
<td>160,000</td>
</tr>
<tr>
<td>2009 Feb.</td>
<td>Summer</td>
<td>Unknown</td>
<td>Black Saturday-Kilmore East, Marysville, Gippsland</td>
<td>173</td>
<td>430,000</td>
</tr>
</tbody>
</table>

**Table 1.3** Combustion tests of three litter fuels (constant loading, 0.048 g/cm² constant αλ, and varying fuel depth. Mean values: percentage expressed on dry weight bases) (Mutch, 1970)

<table>
<thead>
<tr>
<th>Item</th>
<th>Eucalyptus leaves</th>
<th>Ponderosa pine needles</th>
<th>Tropical leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude fat content (%)</td>
<td>19.1</td>
<td>9.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Heat content (cl/g)</td>
<td>5,598</td>
<td>5,081</td>
<td>4,747</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Flame height (m)</td>
<td>0.9</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Combustion residue (%)</td>
<td>7.3</td>
<td>11.6</td>
<td>18.5</td>
</tr>
<tr>
<td>Heat yield (cal g⁻¹)</td>
<td>4,990</td>
<td>4,371</td>
<td>3,947</td>
</tr>
<tr>
<td>Energy release rate (cal cm⁻²sec⁻¹)</td>
<td>6.7</td>
<td>5.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**1.4 Effect of fire on forest flora**

The response to fire within a population of plant species varies due to the size or stage of development of individual plants at the time of the fire, fire season, fire intensity, fire frequency and chemicals present in fire and smoke (Gill, 1981; Whelen, 1995).

**1.4.1 Fire season, intensity and frequency**

The intensity of fire depends on the amount of fuel available, air temperature, humidity, wind speed and topography of the site (Gill, 1981). In summer and autumn the vegetation from the previous spring is dense and dry and rainfall is low; therefore,
high intensity fires occur in these seasons and are naturally fanned by a hot dry northerly wind. Lamont and Runciman (1993), found that burnt *Anigozanthos pulcherrimus* plants reached 33–55°C at the rhizome level and it flowered more prominently after the fire, but when increased temperature using a microwave oven to reach 55–70°C at the rhizome level, no re-sprouting happened, which they assumed that soil heating to a certain point may induce flowering. Flowering of *Xanthorrhoea preissii* (grasstree) was 54%, 21%, 11% and 0% after summer, autumn, spring and winter burns respectively, and this was interrelated with the relative intensity of the seasonal burns (Lamont *et al.*, 2000). Also, Bowen and Pate, 2004 found that, for *Stirlingia latifolia* (shrub), 92% of plants flowered after summer or autumn burns but only 2% flowered after winter burns (Bowen & Pate, 2004).

### 1.4.2 Fire frequency

The fire frequency also affects the plants growth cycle. Frequent fires promoted the plant diversity of Australia (Morgan, 1999; Morgan & Lunt, 1999; Lunt & Morgan, 2002). Many terrestrial orchids have increased in number under management fire regimes (Calder *et al.*, 1989; Wheeler *et al.*, 1998; Goldman & Orzell, 2000; Norton & De Lange, 2003). Studies by Coates *et al.* (2006) on the orchid *Prasophyllum correctum* revealed that when fire intervals were less than three years, the amount of reproductive plants was higher (20% vs 3%) but when fire intervals were greater than three years, a higher number of plants remained dormant and non-reproductive. For non-emergent orchids, the threat of death rate increases as the period of dormancy lengthens (Wells, 1967; Tamm, 1972; Hutchings, 1987; Hutchings, 1989; Sieg & King, 1995). The response of *Prasophyllum correctum* was positive to fire to break the dormancy. Similarly, *Xanthorrhoea fulva*, produced three times greater flowering when fires at 4-5 year intervals compared to 9-17 year intervals (Taylor *et al.*, 1998). By contrast, the weed *Watsonia borbonica* burnt at 3-6 year intervals flowered half as compared to a population that has not being burnt for 29 years.

### 1.4.3 The plants size or stage of development

Plant communities respond differently to the time of burn, as do individual species and plants. Adaptations of plants to fire may correlate with the time of burning. Deciduous herbaceous perennials, such as orchids, are geophytes that are
above ground for only a limited part of the year. These store carbohydrates (usually starch) in the underground organ during periods of dormancy. Carbohydrate reserves in plants are necessary to commence growth above ground and shortly after breaking dormancy the stored carbohydrate levels are lowest (Harrington, 1989; De Groot & Wein, 2004). Energy of the stored carbohydrate is used for this rapid growth after breaking dormancy and these stored products are reloaded by the products of photosynthesis during the growing season. Plants might have a difficult time recovering from tissue loss if the fire occurs during the period when carbohydrate reserves are less than at other times of the year (Hough, 1968; Garrison, 1972; Volland & Dell, 1981). Temperate-climate geophytes were stimulated to flower more by summer to autumn (dry) burns than by winter to spring (wet) ones (Le Maitre, 1984; Jones, 2006). *Watsonia borbonica*, summer–autumn foliage removal was seven times more successful at stimulating flowering at the following year than winter–spring removal because the plants in spring removal had not formed sufficient resources in the corms for flowering in the next growing season (Le Maitre and Brown, 1992).

Also, tender tissues that emerge at the early stage of the growth cycle are more sensitive to heat (Bond & van Wilgen, 1996; DeBano et al., 1998). Fire in the late stages of the growth cycle can also kill the above-ground parts prior to seed production and seed fall, limiting reproductive capacity and therefore, fewer plants emerge the following year.

### 1.4.4 Fire adaptations of terrestrial orchids

Deciduous terrestrial orchids have developed remarkable adaptations that cope with high intensity fires (Jones, 2006) and have developed into an important part of orchids growth cycle. Orchids have underground organs (tubers and tuberoids) for storage of food reserves in dormancy. The tubers are situated 4-5 cm below the ground level, allowing orchids to survive in fires. After a hot summer fire, orchid tubers underground become active and form new plants. Some orchids re-sprout from tubers which may have lain dormant in the soil for up to 20 years (Parks and Wildlife Service Tasmania, 2008).
The orchid annual growth cycle consists of yearly tuber replacement followed by a period of dormancy during the hot dry summer season (e.g. orchids that flower in September–October are dormant in summer) results in most native orchid species avoiding the potentially lethal effects of fire, as most wildfires happen in summer (Table 1.1).

Seed germination in orchids is claimed to be stimulated by the heat of the fire, resulting in more plants and flowering after a summer burn (Jones, 1988; Jones, 2006). Fire might also cause a hormonal reaction which related to ethylene levels in the plant results in increasing flowering (Backhouse and Jeanes, 1995). Several orchid species require fire for the induction of flowering and many orchids flower more abundantly after a fire (Backhouse and Jeanes, 1995; Jones, 2006). For example, in Australia after the Ash Wednesday fires of 1983, rare terrestrial orchids blanketed the hillsides (Pyne, 1991). Also studies by Lamont et al. (2011) showed that orchids were the most represented family for fire-stimulated flowering. From a biological viewpoint, colonisation of bare ground after a fire increases the population and stimulation of flowering offers more chance for sexual reproduction and thus genetic recombination.

However, little is known about the effects of fires on terrestrial orchids’ populations and their mycorrhizal fungi. Though there is evidence that some orchids growth was improved or flowering was enhanced after fire, not all do, even in the terrestrial orchids (Backhouse and Jeanes, 1995). Some species, e.g. Leptoceras menziiesii, respond to both fire and ethylene by flowering. Others, e.g. species of Caladenia and Prasophyllum, flower after fire but respond just as much by clearing of competing vegetation. Yet others, e.g. species of Acianthus and Pterostylis, are reduced in numbers by fire, perhaps because their tubers are in the superficial leaf litter. Frequent fires can reduce the size of individual plants or reduce the rate of vegetative multiplication if the prescribed burns are not conducted at an appropriate time of year. Therefore, it is important for conservation purposes to investigate the effect of seasonal burns (prescribed burns) on native Australian plants.
1.4.5 Fire-stimulated flowering

Fire promotes the flowering of many perennials. Studies on fire-stimulated flowering in Australasia and South Africa/Madagascar by Lamont and Downes (2011) demonstrated that 40.2% of 386 species with fire-stimulated flowering were obligate (‘rarely’ flowered in the absence of fire) while 59.8% were facultative (Figure 1.1). This included 34 families recorded as having fire-stimulated flowering, headed by terrestrial orchids (45% of species studied) (Table 1.4).

Figure 1.1 Number of species with facultative (black) and obligate (grey) fire-stimulated flowering in Australia, New Zealand, Africa and Madagascar. Source: Lamont and Downes (2011)

Table 1.4 Plant families and number of species showing fire-stimulated flowering in Australia and South Africa compared with families with the largest numbers of species in the world. Families in common between the two are given in bold in the same colour. Source: Lamont and Downes (2011)

<table>
<thead>
<tr>
<th>Families with fire-stimulated flowering</th>
<th>No. of species</th>
<th>Families with largest numbers of species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Orchidaceae</td>
<td>173</td>
<td>1. Asteraceae</td>
</tr>
<tr>
<td>2. Xanthorrhoeaceae (includes Asphodelaceae)</td>
<td>42</td>
<td>2. Orchidaceae</td>
</tr>
<tr>
<td>3. Iridaceae</td>
<td>22</td>
<td>3. Fabaceae</td>
</tr>
<tr>
<td>4. Haemodoraceae</td>
<td>18</td>
<td>4. Rubiaceae</td>
</tr>
<tr>
<td>5. Asteraceae</td>
<td>16</td>
<td>5. Poaceae</td>
</tr>
<tr>
<td>7. Fabaceae (Faboideae)</td>
<td>14</td>
<td>7. Euphorbiace</td>
</tr>
<tr>
<td>8. Amaryllidace</td>
<td>9</td>
<td>8. Melastomace</td>
</tr>
<tr>
<td>10. Poaceae</td>
<td>7</td>
<td>10. Apocynaceae</td>
</tr>
<tr>
<td>11. Dasypogonaceae</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>12. Zamiaceae</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>13. Myrtaceae</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>14. Campanulaceae</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>15. Asparagaceae</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>16. Apocynaceae</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>17. Colchicaceae</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>18. Blandfordiace, Lamiaceae, Stylidiaceae, Cyperaceae</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>19. 13 families: Santalaceae, Ranunculaceae, Loranthaceae, Combretaceae, Doryantheaceae, Podocarpaceae, Goodeniaceae, Verbenaceae, Scrophulariaceae, Malvaceae, Ericaceae, Byblidaceae, Philyaceae</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
1.6 Effects of fire on soil properties

Fire can affect physical, chemical, mineralogical and biological soil properties of forests. Severe wildfires cause considerable amount of organic matter removal, weakening of porosity and structure, substantial loss of nutrients, leaching and erosion, ash entrapment in smoke columns, and noticeable changes in both quantity and specific composition of microbial communities, which degrades the soil properties. The intensity and duration of fire impact on soil. Fire Intensity is the rate at which a fire produces thermal energy. High fire intensities and longer burning times result in greater transfer of heat to underground.

The effect of fire on properties of soil strongly depends on soil moisture. In wet soil, heat is transported more rapidly and goes deeper into the soil. In mixed *Pinus ponderosa* – *P. menziesii* forest, three different levels of moisture in soil (-0.03, -1.0, and -1.5 MPa) experienced increasing levels of fire-induced loss of microbial biomass; this was due to faster heat transmission in moist soil than in dry soils. The latent heat of vaporisation avoids soil temperature increasing beyond 95°C until the water entirely vaporises (Campbell *et al.*, 1994), then the temperature rises to 200-300°C (Franklin *et al.*, 1997). The combustion and heat transfer produces sharp temperature rise in soil. At 5 cm in the mineral soil, the temperatures hardly ever increase above 150°C and usually below 20–30 cm no heating occurs (DeBano, 2000). Low to moderate prescribed burns increase the soil hydrophobicity, creating soil erosion (Neary *et al.*, 1999).

1.6.1 Effects of fire on microbial communities

Heating and oxidation of fire not only alters the physicochemical and biological setting, but also produces new sources of physicochemical and biological inputs into the soil such as charcoal, distillates, metal oxides, plant litter and some toxic organic compounds. Compounds such as polychlorinated dibenzo- p-dioxins (PCDDs), dibenzofurans (PCDFs), and polynuclear aromatic hydrocarbons (PAHs) are released and redistributed on and in the ground (Kim *et al.*, 2003). As a result, fire provides both direct and indirect influences to the forest ecosystem (Figure 1.2).
Figure 1.2 Conceptual model of the effects of fire on forest ecosystems. Dotted arrows denote immediate short-term effects of fire on vegetation and soil organisms, which are transitory in nature. These include selective mortality of plants and soil microorganisms and nutrient release from combustion of organic matter. The influence of fire on soil microclimate (temperature, moisture, insolation) is the midterm mechanism driving changes in the soil microflora and vegetation (denoted by thin, block arrows), as well as changes in rates of ecosystem processes such as decomposition and nutrient mineralization (denoted by medium, block arrows). However, over time, strong feedbacks develop among the soil microflora (composition and activity), decomposition and nutrient availability, and plant growth and functional composition (grass vs. shrub, vs. tree; N fixer vs. non-N fixer). These feedbacks (denoted by thick, block arrows), caused indirectly by the fire, are primarily responsible for the long-term stability of the ecosystem. In the absence of reoccurring fire, plant succession results in changes in plant growth and functional composition, altering these feedbacks, and creating a new ecosystem state. The relative strength of the longer term interactions is noted by the thickness of the block arrows. Source: (Hart et al., 2005).

1.6.2 Soil organisms different responses to heating (direct effect)

The immediate and direct impact of fire on soil microorganisms is the decrease of biomass due to the lysing of microbial cells and the change of microbial reproductive ability from soil heating (Covington et al., 1991). Soil microorganism abundances are most in the topsoil cover where most organisms exist in the soil (Neary et al., 1999). The maximum temperature of a fire often exceeds the required heat to kill most living organisms in the soil (DeBano et al., 1998). In high intensity fires, the topsoil can experience total sterilisation. In a forest with Pinus spp., immediately after a wildfire the microbial biomass in the 0–5 cm soil layer was almost
disappeared and in the immediate subsurface zone of 5–10 cm was reduced by 50% (Prieto Fernandez et al., 1998).

Some soil microorganisms are more sensitive to heating than others. Fire can cause selective death to special groups of soil microorganism, by changing the community makeup. Bacteria are more resistant to heat than fungal community (Dunn et al., 1985). Threshold temperatures for bacteria are 120°C for dry and 100°C for moist soils (Dunn & DeBano, 1977). Therefore, the bacteria to fungi ratio should increase after a fire. This predicted increase in bacteria to fungi ratios was established by Fuller et al. (1955) by selective culturing of microbial populations after both wildfire and controlled burns in ponderosa pine forests of northern Arizona, USA. Long-standing responses of the soil microorganisms to fire might be due to changes in plant composition and community as of the strong interrelationships between plants and soil microorganisms. A coniferous forest needed 12 years for microbial biomass to come back to its before fire stage (Fritze et al., 1993).

1.6.3 Mycorrhizal fungi and fire

The effects of fire on mycorrhizal fungi depend on factors such as fire intensity, duration of fire, soil and vegetation type. Most studies were paying attention on the effect of fire on AM and ECM; the effect of fire on orchid mycorrhizae is unknown (Warcup 1981).

Most studies have shown the negative impacts of fire on AM spores and rates of AM root infection. Klopatek et al. (1994) found that, in pinyon–juniper woodlands, the presence of AM was lower than before burn level even after 10 years of burning. Klopatek et al. (1988) recorded 100% death rate of AM at 94°C and Pattinson et al., 1999 reported a 100% loss of AM when field soils were heated over 80°C by simulated fires in the laboratory.

The direct effect of fire on ectomycorrhizae (ECM) appears to be more variable compared with AM fungi. The effects of burn on ECM fungi ranged greatly from significant decrease in propagule density and diversity in a jack pine (Pinus banksiana) stand after a wildfire (Visser, 1995) to no considerable alteration to ECM following a wildfire in a Bishop pine (Pinus muricata) stand (Baar et al., 1999). Low intensity fires usually have modest or no effect on ECM community (Korb et al.,
2003). Jonsson et al. (1999) speculated that the lethal temperatures of plant roots are around 50°C, which was more sensitive to heat than soil microorganisms (Hare, 1961). Hence, the capability of mycorrhizal fungi in plant roots to endure following fire depends on their capability to continue to exist in the absence of their hosts, which is likely to be more for AM than ECM fungi (Amaranthus & Perry, 1987; Allen, 1991) and to recolonise roots.

The response of AM and ECM fungi to fire are quite variable. After the removal of litter and soil organic matter by fire in Jarrah forest, the amount of mycorrhizal roots decreased noticeably in plants (Reddell & Malajczuk, 1984). Hart et al. (2005) hypothesized that considerable differences in AM and ECM survival after a fire may be influenced indirectly by the relative dominance of its respective host plants after a fire rather than the difference in lethal temperatures of these fungal groups.

1.7 Indirect effects of fire on microbial communities

Indirect effects of fire result in long-term changes to an ecosystem. These comprise of increased sunlight enter into the soil and interrelated alterations such as chemical and microclimate alterations of the mineral soil by forming hydrophobic surface conditions and the deposition of ash (mostly alkaline oxides) and charcoal.

1.7.1 Solar penetration and soil microclimate

Fire may indirectly affect the soil microorganisms by changing the soil microclimate. After a fire overstorey and understorey plant density was reduced, which allow more solar penetration to the ground surface during day and rapid heat loss at night. This will increase the soil temperature during the day time and cooler soil temperatures at night. These daily soil temperature changes due to fire extend to seasonal changes, with rapid warming of the soil during spring and summer months, and more rapid cooling during autumn and winter (Fisher & Binkley, 2000). Most biological reaction rates such as microbial processes (decomposition and nutrient release) are increased in warm soil temperatures following fire (Bissett & Parkinson, 1980; Covington & Sackett, 1984; Paul & Clark, 1996; Kaye & Hart, 1998b; Kaye & Hart, 1998a).
1.7.2 Formation of water-repellent soils

After a fire soil structure might have a temporary occurrence of increased water repellence due to heating of organic materials in the top surface (DeBano, 2000). The formation of hydrophobic conditions in soil after a fire usually occurs in the separated superficial layer of the soil (top 10 cm) that runs parallel to the mineral soil layer (DeBano, 2000). This hydrophobic soil state will increase the superficial surface flow, decrease the soil moisture, increase erosion of soil (DeBano, 2000; Robichaud, 2000) and alter activity of microorganisms (Letey, 2001). This hydrophobic condition due to fire will normalise in one or two years after a fire due to the microbial decomposition of the waxy, hydrophobic materials (Franco et al., 2000).

1.7.3 Charcoal

There are few studies done on the biological and chemical significance of charcoal on functions of ecosystem (Keeley, 1987; Zackrisson et al., 1996; Wardle et al., 1998); Glaser et al., 2002). These suggested that the increased water- and nutrient-holding capacities of charcoal increase the soil productivity. Studies on the effect of charcoal on soil microorganisms (Wardle et al., 1998; Pietikäinen et al., 2000) explain that charcoal has no significant effect on microbial activity but in late successional boreal forests, charcoal had a significant part in maintaining the nitrifying bacteria (DeLuca et al., 2002). Investigations in the laboratory have shown that charcoal taken from western Montana forest soils efficiently absorbs free phenolic compounds and enhances total nitrification (T.H. DeLuca, unpublished data; Hart et al., 2005).

1.7.4 Smoke

While fire directly stimulates flowering, several observations have revealed smoke stimulating flowering. Curtis (1998) observed a number of flowering of *Xanthorrhoea australis* in an unburnt area that was 200 m away from the fire and explained it by smoke flow from the fire. Linder and Kurzweil (1999) observed that South African orchids flowered well after smoke from fire passed over them. Keeley (1993) also proposed that *Cyrtanthus ventricosus* (lily) flowering was encouraged by smoke in unburnt stands surrounding the area of the fire. Similarly, when the corms
of the weed *Watsonia borbonica* were treated with smoke water, they flowered notably compared with untreated corms (Gill & Ingwersen, 1976; Light *et al.*, 2007).

The gas ethylene is a chemical in smoke that potentially promotes post-fire flowering, a common by-product of fire (Gill & Ingwersen, 1976). During a fire in banksia woodland, ethylene produced seeps into the soil to 12 cm and when ethylene was introduced into a dry soil which had *Drosera erythrorhiza* summer-dormant tubers, it stimulated protanth in the next autumn (Dixon & Barrett, 2003). *Pyrorchis nigricans*, a terrestrial orchid, which had not flowered for two decades, produced flowers after applying ethephon, which is an ethylene-producing compound (A. Batty, unpublished, via K. Dixon, pers. comm.; Lamont & Downes, 2011).

Smoke water produced by burning plant material and bubbling the smoke through water also improved germination, growth and promoted the growth of healthier plants (Van Staden *et al.*, 2004). Smoke water contains butenolide, derived from the burnt plant material, which also promoted the germination of lettuce at $10^{-9}$ M concentration (Flematti *et al.*, 2004; Van Staden *et al.*, 2004) and many other species (Soós *et al.*, 2009), including native Australian species (Dixon and Barrett, 2003).

The possible effects of smoke from the fires on microorganisms have received little attention, but several researchers have explored the fungitoxicity of smoke. Smoke from various fuels yields different types of compounds, many of which are antimicrobial (Frazier & Westhoff, 1967). Alam *et al.* (1999) also reported that smoke has antifungal activities and mycelial growth of *Botryis gemella*, *Penicillium expansum*, *Fusarium lateritium* and *Fomes annosus* was reduced when exposed to smoke (Parmeter & Uhrenholdt, 1975). This suggests that smoke/smoke deposits on plants might reduce the activities of important fungi. The phenolic compounds of smoke water inhibit the growth of microorganisms including soil-borne pathogens (Lin *et al.*, 2012).

### 1.8 Common Victorian orchids used for the study

*Glossodia major* R.Br. *Thelymitra pauciflora* R. Br. and *Pterostylis revoluta* R.Br. are the three terrestrial orchids selected for this study. All are common orchids and are most commonly found in drier inland zones of Victoria.
The orchids were chosen to have similarities and differences that might react differently to fires at different seasons. The first two are spring-flowered whereas the last is autumn-flowered. The annual growth cycles of *G. major* and *T. pauciflora* are similar, but *G. major* flowers a little earlier than *T. pauciflora*. *G. major* flowers in September and *T. pauciflora* flowers in October and both are dormant in December to February (summer). *Pterostylis revoluta* inflorescence in March to April (autumn) and is dormant in September to November (spring) (Table 1.2). It was hypothesised that there was a relationship between the season of the burn and the size/stage of development of individual plants.

Each genus of orchid was expected to be associated with a different genus of mycorrhizal fungi; *G. major* was expected to associate with *Sebacina*, *T. pauciflora* with *Tulasnella* and *P. revoluta* with *Ceratobasidium* (Table 1.1). It was hypothesised that there was a relationship between the season of the burn and the colonisation of the orchid by its mycorrhizal fungus. It was further hypothesised that the response of the orchid mycorrhizal fungi to fire and the smoke water ensuing from it would influence the recovery of the orchid from seasonal burning.

### 1.8.1 Glossodia major

The genus *Glossodia* R. Br. comprises only two species, *G. major* and *G. minor* R. Br. The common name for *G. major* is wax-lip orchid or Parson-in-the-Pulpit (Backhouse and Jeanes, 1995). It is a common slender hairy perennial terrestrial herb that is dormant underground in the hot months each year. Each plant has an erect single hairy broadly lanceolate leaf to 10 cm long x 20 mm broad. The flowering season of *G. major* is September to November; hot fires the previous summer were stated to enhance flowering (Backhouse and Jeanes, 1995). Each plant produces an inflorescence of one (or occasionally two-three) flowers on a stem about 20 cm to 30 cm tall, with the flower growing to about 5 cm across. Flowers are glossy mauve to purple though some rare plants produce all-white flowers. Perianth segments are up to 25 mm long, ovate and similar, with the dorsal tepal erect and the others spreading as in a star (Figure 1.3). The preferred growth conditions for *G. major* are drier and well drained soil in open forest and woodland. *G. major* is generally distributed in temperate habitats in south-eastern and eastern Australia (Figure 1.3) and its conservation status is secure.
Figure 1.3 Appearance and distribution of *Glossodia major* in Australia. The key gives the number of records in herbaria in Australia. Source: Atlas of Living Australia: [http://bie.ala.org.au/species/Glossodia+major](http://bie.ala.org.au/species/Glossodia+major).

The map is generated from a central database and cannot be changed, but is inaccurate in that there is no *Glossodia major* in Western Australia.
1.8.2 *Thelymitra pauciflora*

The genus *Thelymitra* J.R. Forst. et G. Forst. consists of about 130 species. At least 110 species are present in the wet habitats of south-western and south-eastern Australia, with only a few species found in northern Australia. Also some species are in Philippines, Indonesia, New Guinea and New Caledonia, and New Zealand has around 20 species (Royal Botanic Gardens Melbourne, 2010). The *T. pauciflora* complex was reviewed by Jeanes (2004). *Thelymitra pauciflora* was revised to *T. cyanapicata* Jeanes but with a much reduced distribution and herbarium records that did not include Greater Bendigo National Park. As voucher specimens have not yet been examined from the Greater Bendigo National Park, *T. pauciflora* will be used here and descriptions and distributions refer to the *T. pauciflora* complex.

*Thelymitra pauciflora* is a slender, erect, single-leaved summer-dormant perennial orchid that is commonly known as the slender sun orchid (Backhouse and Jeanes, 1995). Each plant has an erect single linear-lanceolate leaf up to 30 cm long x 20 mm broad (Figure 1.5). It flowers from September to December and is dormant in the hot months of the year. Bates (2010) commented that flowering of many species of *T. pauciflora* complex depended on good spring rainfall and that drought reduced the amount of flowering and seed set drastically. The peduncle grows up to 40-50 cm tall, with an inflorescence of (2)-3-5-(12) pale blue, but occasionally pink or white flowers (Backhouse and Jeanes, 1995; Jones, 2006). Each flower is up to 20 mm across, star-like and almost symmetrical, with elliptical perianth segments and a distinctive column with a yellow apex and white hair tufts. Flowers are generally autogamous and also cleistogamous, as self-pollination has observed while the flowers are still in bud (Jeanes, 2004). In cool weather, flowers may remain tightly closed throughout the flowering season. Autogamy is facultative, as flowers may open fully on hot days and native bees visits and transfer pollen (Bates, 1999; Jeanes 2004; Jones *et al.*, 2010). Its preferred habitat is *T. pauciflora* is generally distributed in temperate habitats in south-eastern and eastern Australia (Figure 1.3) and its conservation status is Secure (Backhouse and Jeanes, 1995), though some of the new species have as yet incompletely defined distributions.
1.8.3 *Pterostylis revoluta*

The genus *Pterostylis*, commonly known as greenhood orchids, comprises about 130 species. Most of the over 100 species occur in Australia (Jones, 1988), 23 species in New Zealand and a little in New Guinea and New Caladonia (Halle, 1977). *Pterostylis revoluta* (autumn greenhood) is one of the largest flowering orchids in the genus. *Pterostylis revoluta* reproduces vegetatively to form large aggregations of colonies by producing more than one tuber annually. The new tubers are borne at the ends of long underground stolons (up to 25 cm long) arising from the axils of stem bracts or scale leaves. Tubers have an apical bud that develops into a new plant and roots are produced at the base of the tuber. During mid- late summer, each mother tuber produces either a rosette of leaves (in a vegetative plant) or an inflorescence (in a reproductive plant) (Backhouse and Jeanes, 1995). Vegetative plants have 4-10 prostrate ovate leaves, each up to 25 mm long x 15 mm wide (Backhouse and Jeanes, 1995). Reproductive plants have an erect stem to 25 mm tall capped by a single flower up to 45 mm long that has alternate stripes of green and translucent white (Figure 1.5). The distinctive ‘greenhood’ flower has the dorsal sepal curved over with a point at the tip and the tips of the two lateral sepals erect on either side of the flower. *Pterostylis revoluta* is found in drier inland zones, usually growing on clay loams or sandy loams soil derived from sandstone or granite in open forest and woodland. *Pterostylis revoluta* is widely distributed in temperate habitats in south-eastern and eastern Australia (Figure 1.5) and its conservation status is secure.
Figure 1.5 Appearance and distribution of *Pterostylis revoluta* in Australia. Dots represents places with herbarium records of this species. Source: Atlas of Living Australia: http://bie.ala.org.au/species/Pterostylis+revoluta
1.9 Aims

The research reported in this thesis asked several important questions on the effects of prescribed seasonal burns on common species of three widespread genera:

1. What are the effects of fire on plant numbers and parameters of growth and reproduction (Chapter 2)?

2. What are the effects of fire on the colonisation of the orchids by their mycorrhizal fungi and the ease of isolating them from the orchids (Chapter 2)?

3. What are the effects of smoke water on the growth of the mycorrhizal fungi in culture (Chapter 3)?

4. What are the effects of fire on the genetic diversity of mycorrhizal fungi isolated from the orchids (Chapter 5)?
Chapter 2

Effects of prescribed burning on plant responses of *Glossodia major*, *Thelymitra pauciflora* and *Pterostylis revoluta*
2.1 Introduction

2.1.1 Fire

Fire is an important ecological factor in many countries across the world (Trabaud, 1987; Johnson, 1996). Australia is a fire-prone continent (Andersen et al., 2005) and fire has been a significant ecological feature in Australia for millions of years from natural and, more recently, from anthropogenic causes. South-eastern Australia in particular has fire-prone environments due to its geographical location, vegetation and climate (Cheney, 1995).

South-eastern Australia has four seasons: summer, autumn, winter and spring. The climate in Victoria can be summarised as warm-hot in summer when many plants senesce (December to February); mild in autumn when abscission happens in deciduous plants (March to May); cold and damp in winter when some plants undergo resting phases (June to August); and a gradual rise in temperature and day length in spring that triggers many plants to flower (September to November).

Recorded bushfires in Victorian woodlands have only occurred in summer or autumn, resulting in flora and fauna destruction (Australia, 2010; DEPI, 2013). In hot summers, many plants senesce and increase the fuel load in the vegetation. There are no reports of spring or winter bushfires in Victoria, though there are from other states, such as New South Wales in 2013.

Fires affect the vegetation structure of the habitat globally (Gill et al., 1981; Keeley, 1981; Trabaud & Lepart, 1981; Collins & Gibson, 1990). Vegetation is affected by fire frequency, fire intensity (Moreno & Oechel, 1991), the season of burning (Malanson & Trabaud, 1988; Lonsdale & Braithwaite, 1991), regularity of burning (Keeley, 1981) and plant size or stage of development (Kubiak, 2009). Bushfire intensity is influenced by vegetation type and structure as they affect fuel load and flammability. Woodlands in Victoria have similar vegetation structures in layers, with the tallest reached by eucalypts and a lower multilayered structure of acacias, tree ferns, shrubs and herbs, including orchids. The ground cover consists of various grasses, mosses and liverworts (DSE 2004).
2.1.2 Fire in Australia

Australia’s native plants have vegetative and reproductive adaptations that result in them surviving and regenerating after a fire, both above and below ground (Gill, 1981; Benson & McDougall, 1993; Whelan, 1995). Eucalypts produce epicormic shoots that emerge from the protection of thick-barked trunks and branches, whilst banksias and teatrees re-sprout from underground lignotubers. Eucalypts and native heath plants have sclerophyllous adaptations that include hard thick leaves resistant to moisture loss during extreme conditions and oil glands in the leaf epidermis. These oils result in high intensity fires with long burning times and fast rates of spread (Anderson 1968; Mutch 1970).

Eucalypts, banksias and hakeas store seed in woody, thick protective fruits that open as a result of desiccation after the fire. After fire, the seeds drop out, germinate and grow on the burnt ground with reduced competition from grasses and sedges (Parks and Wildlife Service Tasmania, 2008). Grass trees (*Xanthorrhoea australis*) flower prolifically after fire due to a fire-initiated release of the gas acetylene, which initiates the growth of the flower spike and the early release of seed (Parks and Wildlife Service Tasmania, 2008).

The season in which a fire occurs influences the post-fire flowering. Lamont et al. (2000) found that 75% of *Xanthorrhoea preissii* (grasstree) individuals flowered after a summer burn compared with only 1% flowering after a winter burn. Similarly, flowering of *Stirlingia latifolia* was greater after summer and autumn burns (ranged between 85% and 100% respectively) than after a spring burn (55% - 80%) (Bowen & Pate, 2004)

Smoke generated by burning is widely recognised as affecting plant species dynamics. Since the discovery by De Lange and Boucher (1990) that seeds of *Audouinia capitata*, germinated well at sites with smoke-fumigated soils, but not at smoke-free (control) sites, also, numerous studies have revealed higher potential of flowering after burns (Bowen & Pate, 2004; Keeley, 1993) and stimulation of somatic embryogenesis by smoke saturated water (Senaratna et al, 1999). Specific plants respond differently to fires. In the angiosperms, 77% of the monocotyledons are fire-stimulated and 44% of these are in Australia (Lamont et al., 2011). Of 34 families studied, the Orchidaceae represented the most fire-stimulated family in flowering.
(45%), with 174 species in Australia and South Africa, followed by 42 in the Xanthorrhoeaceae and 22 species in the Iridaceae (Lamont et al., 2011).

2.1.3 Fire and Australian orchids

Australian terrestrial native orchids are adapted to bushfires, with strategies that cope with high-intensity fires (Jones, 2006). *Pterostylis revoluta*, *Glossodia major* and *Thelymitra pauciflora* are herbaceous perennial geophytic terrestrial orchids found throughout Victoria that have such adaptations. These orchids have underground tubers to store nutrients. The plant grows actively in winter and survives as underground tubers in dry summer periods. Most native orchids that flower in spring are dormant by summer, when bushfires occur most frequently (Australia, 2010; DEPI, 2013). The tubers are situated 5-10 cm below the ground level, protected from fires and desiccation, resulting in re-sprouting in the next growing season. Orchid mycorrhizal fungi are also protected from fire as they colonise the roots of *T. pauciflora* or other underground organs are also 5 cm below the ground level.

Several orchids were reported to flower more profusely in the season following a hot summer fire (Jones, 2006). Spring-flowering orchids were reported to flower more prolifically after a summer wildfire (Quarmby, 1999). Conversely, the number of flowering plants reduced following a fire (Coates & Duncan, 2009). Some Australian native orchids sprout immediately after a fire from tubers that may have lain dormant in the soil for many years (Parks and Wildlife Service Tasmania, 2008).

2.1.4 Prescribed burning in Australia

Prescribed burns (also called control, controlled and fuel reduction burns) are a critical part of land management in Victoria thought to reduce the spread and intensity of bushfires. The goal of a prescribed burn is to reduce or remove the “fuels” (leaf litter, shed bark, dead grass etc.) that contribute to the intensity of bushfires (CFA, 2010). Each year, native vegetation in Victoria is burnt under this prescribed burning regime; in 2011, this amounted to 188,997 ha. The target set in the 2009 Victorian Fires Royal Commission (2010) report is 5% of public land in the state (415,412 ha), a target that is difficult to achieve with current financial and climatic restrictions and may have unpredictable consequences for conservation. The most
favoured seasons for prescribed burns are autumn and spring, when the correct combinations of fuel moisture content and wind speed, among other parameters, can most easily be met; autumn is favoured over spring because of the calmer conditions. The fuel is generally too wet to burn easily in winter and in summer the hazard of starting a wildfire is too great for burning off.

Despite the prescription of burns on such a large scale on habitats, information on their effects on particular species is surprisingly sparse, especially for small herbaceous plants like orchids that are minor components of the flora and may be dormant during surveys. Information on the best season for a prescribed burn for conservation of the ecosystem and particular species is often missing. It is important to fill this gap for conservation and land management authorities and private land owners to minimise the effects of bushfires on native plant populations, especially in orchids.

Terrestrial orchids in Australia fall into major groups linked together not only by morphology and conventional taxonomy but also by the endophytic fungi on which they depend and with which they form symbiotic relationships. Three of the most common genera are *Glossodia*, *Pterostylis* and *Thelymitra*, which form mycorrhizal relationships with the fungi *Sebacina vermifera*, *Ceratobasidium corinigerum* and *Tulasnella* spp. respectively (Table 2.1) (Warcup, 1981).

<table>
<thead>
<tr>
<th>Tribe</th>
<th>Subtribe</th>
<th>Genera</th>
<th>No. spp.</th>
<th>% examined</th>
<th>Sebacina vermifera</th>
<th>Tulasnella spp.</th>
<th>Ceratobasidium spp.</th>
<th>Thanatephorus spp.</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthuseae</td>
<td>Caledininae</td>
<td>Glossodia</td>
<td>2</td>
<td>100</td>
<td>15</td>
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<td></td>
<td>Pterostylidinae</td>
<td>Pterostylis</td>
<td>55*</td>
<td>&lt;29</td>
<td></td>
<td>83</td>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Neottieae</td>
<td>Thelymitrinae</td>
<td>Thelymitra</td>
<td>35</td>
<td>43</td>
<td>79</td>
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It is desirable to have any effects of fire studied on a site common to these genera to avoid the distortions that occur at different sites due to differences in moisture, soil, aspect, wind etc. Fire may affect these orchids differently because of several differences between them:

1. the season in which they flower relative to the season of the fire
2. the location of the tissue infected by the fungi relative to the soil line and so fire damage
3. the effect of the fire on the fungi themselves directly in the soil.

2.1.5 Aims

This study aimed to select orchids with different actively growing stages in different seasons to investigate the best (least damaging) season for prescribed burning on *Pterostylis revoluta* R. Br., *Glossodia major* R.Br. and *Thelymitra pauciflora* R. Br. populations and their mycorrhizal fungi in Victoria.

2.2 Materials and Methods

2.2.1 Site selection

For a study of the effects of season of prescribed burning on orchids and their mycorrhizal fungi, it was desirable to have both large numbers of the orchids studied and for those orchids to have large amounts of infection by orchid mycorrhizal fungi.

2.2.1.1 Criterion 1 – large numbers of orchids

Four sites around Bendigo were surveyed in March 2011 (autumn) to find the most suitable site with the maximum number of orchids for seasonal burns. Four local autumn-flowering orchids were recorded and numbered with self-designed and made tags (Table 2.1). The site with the greatest total number of flowering orchids was selected for the study.

2.2.1.2 Criterion 2 – orchids containing dense fungal colonisation

2.2.1.2.1 Autumn-flowering orchids in March 2011

Stem collars were collected for fungal isolation from the flowering orchids with the greatest numbers (*P. parviflora*, *P. revoluta* and *Genoplesium* sp.) and prepared for light microscopy to confirm fungal colonisation as the presence of pelotons (fungal coils). The stem-collars were surface-sterilised with 1% sodium hypochlorite for 1 minute and rinsed with sterile water three times. The stem-collar was hand-cut into 1 mm-thick longitudinal sections and sections were examined for pelotons using a
Leica MZ9.5 stereo microscope. Plants with stem-collars with frequent peloton occupation were selected for further study.

2.2.1.2.2 Spring-flowering orchids in September-October 2011

The Greater Bendigo National Park site was surveyed again in September to October 2011 to score the total number of spring-flowering plants. Orchids with the greatest number of flowering plants were selected for further study.

2.2.2 Construction of plots for plant survey and seasonal burns

Five plots, each 2 m × 2 m, were systematically selected in March 2011 to contain the maximum number of *P. revoluta* plants. One was a control plot and the other four plots consisted of one for each seasonal burn (spring, summer, autumn, winter). Each plot contained more than 20 flowering orchid plants. All plots were subdivided equally into three equal-sized rectangular quadrats (Figure 2.1) on the basis of abundance and distribution of orchid plants within each sub-plot. All plots were tagged and labelled using tent pegs, plastic tags and GPS co-ordinates.

![Figure 2.1](image)

Similarly, in September-October 2011, five plots each for *G. major* and *T. pauciflora* were formed and each plot was tagged and labelled as before. This gave a
The Effect of Seasonal Burns on Australian Native Orchids

total no. of quadrats = 3 species × 5 plots (spring summer, autumn, winter and control plots) × 3 quadrats = 45 quadrats.

2.2.3 Seasonal burns

Burns were conducted in plots in Greater Bendigo National Park in the middle of each season for spring, summer, autumn and winter based on suitable and safe conditions as advised by the Department of Sustainability & Environment and Parks Victoria (Table 2.2). A low-intensity burn was conducted on three plots in each season, one for each orchid species. One plot for each species was unburnt and retained as a control plot.

<table>
<thead>
<tr>
<th>Species</th>
<th>Flowering</th>
<th>Pre-burn sampling</th>
<th>Burns</th>
<th>Post-burn Sampling</th>
<th>Survey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spring</td>
<td>Summer</td>
<td>Autumn</td>
</tr>
<tr>
<td>P. revoluta</td>
<td>Autumn</td>
<td>23/03/11</td>
<td>18/10/11</td>
<td>24/01/12</td>
<td>27/04/12</td>
</tr>
<tr>
<td>G. major</td>
<td>Spring</td>
<td>19/09/11</td>
<td>18/10/11</td>
<td>24/01/12</td>
<td>27/04/12</td>
</tr>
<tr>
<td>T. pauciflora</td>
<td>Spring</td>
<td>18/10/11</td>
<td>18/10/11</td>
<td>24/01/12</td>
<td>27/04/12</td>
</tr>
</tbody>
</table>

Dates are day/month/year. Colour coding: green=unburnt, red=burnt, blue=spring and summer plots burnt but not autumn and winter.

A 1 m distance outside the 2 m × 2 m perimeter was raked to reduce the fuel load. Also, the perimeter of each plot was watered before the burn to wet the ground as a safety precaution to avoid the spread of fire to the adjacent area. The pre-burn and post-burn temperatures of the soil below 4 cm were measured in each quadrat on the day of the burn using a data logging system. The ground fire was ignited from the perimeter using a drip torch containing a mixture of gasoline and diesel, allowing the fire to spread to the centre of the plot. For plots that had incomplete or mosaic burns, the plots were ignited at several places. A thermal imaging camera (Fluke P3, U.S.A) was used to measure the maximum temperature of the fire.

2.2.4 Plant survey

Surveys for all three species were conducted during their respective flowering seasons before and after each burn; a total of 799 pre-burn and 1180 post-burn plants was surveyed (Table 2.5). Measurements recorded were number of plants; whether each was flowering or vegetative; plant height; stem width; flower length.
and width; number of buds; and leaf length and width as appropriate (Table 2.3). Measurements were recorded using electronic digital calipers and a conventional ruler (Figure 2.2).

Table 2.3 Plant data measurements for *Pterostylis revoluta*, *Glossodia major* and *Thelymitra pauciflora* pre-burn in 2011 and post-burn 2012/2013.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Number of plants</th>
<th>Flowering</th>
<th>Vegetative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plant height</td>
<td>Stem width</td>
<td>Flower length</td>
</tr>
<tr>
<td><em>P. revoluta</em></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><em>G. major</em>**</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><em>T. pauciflora</em>**</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
</tr>
</tbody>
</table>

* Flowering plants had no leaves and so leaf length and width were not recorded. Only one flower was present per plant and so the number of buds was not recorded.

** Flowers were only slightly asymmetrical and so the width of the flower was measured at the broadest part (Figure 2.2). There was only one flower per plant and so the number of buds per plant was not recorded.

*** During the survey many flowers were closed (*Thelymitra* spp. flowers only open on sunny days) and so the number of buds was counted instead of flower measurements. Leaf width was very narrow and leaves were half eaten by animals and so leaf length was not recorded.
Figure 2.2 Plant measurements taken for *Pterostylis revoluta* and *Glossodia major* at 2011 pre-burn and 2012 / 2013 post-burn survey. (A) *P. revoluta* flower length - Y and width – X, (B) *P. revoluta* stem width, (C) *G. major* leaf length - Y and width - X, (D) *G. major* flower width – X.
The *P. revoluta* pre-burn survey was conducted in March 2011 and the post-burn survey was in March 2013 when plants were flowering after the last burn in winter, July 2012. Plants were recorded as vegetative or reproductive (flowering plants); the sum of both indicated the total number of plants. Plant height, stem width and flower length and width were recorded (Table 2.2, Figure 2.2). Since *P. revoluta* flowering plants only produced one flower per plant and lacked leaves, the number of buds or leaves was not counted (Table 2.2, Figure 2.2).

The pre-burn surveys for *G. major* and *T. pauciflora* were conducted in September and October 2011 respectively and the post-burn surveys were in October 2012 when plants were flowering after the last burn in winter, July 2012. *G. major* plants were recorded as vegetative or reproductive as for *P. revoluta*. Plant height; stem width; flower width; and leaf length and width were recorded (Table 2.2, Figure 2.2). *Thelymitra pauciflora* vegetative plants were difficult to distinguish from other *Thelymitra* species without flowers and therefore only flowering plants were recorded and indicated as the total number of plants. The number of flowering plants, plant height, stem width and number of buds were recorded (Table 2.2).

### 2.2.5 Statistical analysis

The average number of flowering and vegetative plants and average plant measurements for each plot were graphed and analysed statistically using Minitab 16 statistical software. Significance was recorded at \( p \leq 0.05 \). Apart from plant numbers, zeros (absence) were not included in calculations; measurements therefore represent only plants where the feature was present, e.g. a vegetative plant of *G. major* had leaf length and width recorded but no zero for plant height, stem width or flower width was included in the calculations. Data were transformed as necessary to normality; transformations included \( \log_{10} \), square root and inverse square root. Where data could not be normalised, non-parametric tests, e.g. Kruskal-Wallis and Mood median, were used.

Plant numbers were compared for each species pre- and post-burn using \( \chi^2 \) tests for total numbers per plot and one-way Analysis of Variance (ANOVA) for mean (average) numbers per quadrat followed by Tukey’s family error tests. Plant measurements were compared using one-way ANOVA followed by Tukey’s family.
error tests. Least significant differences (LSD) were calculated using the formula LSD\(_{0.05} = t \sqrt{\frac{2\text{MSE}}{n}}\), where \(t\) was 2-tailed at \(p=0.05\) and had degrees of freedom; (df) = df of MSE; MSE = least square error from ANOVA and \(n\) = replicate number.

### 2.3 Results

#### 2.3.1 Site selection

##### 2.3.1.1 Criterion 1 – large numbers of orchids

Out of the four sites initially surveyed, the Greater Bendigo National Park site was selected for further studies because it had the greatest number of orchids, and *P. revoluta*, *G. major* and *T. pauciflora* were selected as the most numerous in the desired genera (Table 2.4, Figure 2.3). The site had good orchid diversity and plants were clustered in large colonies with more than 40 plants in each 2 m × 2 m plot. Plants were located close to the fence of the park boundary, which enabled easy access for fire trucks to the site, and the trees were sparsely spread 5-8 m away from each other for a uniform vegetation burn.

**Table 2.4** Site and autumn-flowering and spring-flowering plants around Bendigo, scored in March September and October 2011

<table>
<thead>
<tr>
<th>Site</th>
<th>Site details</th>
<th>GPS location</th>
<th>Orchard species</th>
<th>Total no. of flowering plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Car Hills Road</td>
<td>E144°16.693′ S36° 50.551′</td>
<td><em>Pterostylis revoluta</em> R. Br.</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Genoplesium sp.</em></td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. parviflora</em> R. Br.</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>Car Hills Road</td>
<td>E144°16.634′ S36°50.563′</td>
<td><em>P. revoluta</em></td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Genoplesium sp.</em></td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Diamond Hill</td>
<td>E144°15.625′ S36°49.233′</td>
<td><em>P. revoluta</em></td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. parviflora</em></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#<em>Thelymitra megcalyptra</em></td>
<td>3</td>
</tr>
<tr>
<td>**Site selected, *Species selected. **</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Site selected, *Species selected. **| #T. aristata var. megcalyptra (Fitz.) Rupp

*Acianthus pusillus* D.L.Jones 38
*Glossodia major* R. Br. 875
*T. ixioides* Sw. 12
*T. megcalyptra* 23
*T. pauciflora* R.Br. 51
Figure 2.3 Flowering plants of three orchid species selected for this study: Flowering plants of three orchid species selected for this study: *Pterostylis revoluta*: (A) flower, (B) cluster of flowering plants; *Glossodia major*: (C) flower, (D) group of flowering plants; *Thelymitra pauciflora*: (E) flower, (F, G) plants sparsely positioned at the Greater Bendigo National Park site in 2011.
The vegetation of the selected site, in Greater Bendigo National Park, included *Eucalyptus polyanthemos* Schauer (red box), *E. microcarpa* (Maiden) Maiden (grey box), *E. macrorhyncha* F. Muell. Ex Benth. (red stringy bark) and *E. melliodora* A.Cunn. ex Schauer (yellow box) in the top layer. The understorey bushes included *Exocarpus cupressiformis* Labill. (cherry ballart), *Daviesia ulicifolia* Andrews (gorse bitter pea), *Acacia pycnantha* Benth. (golden wattle), *Hibbertia basaltica* A.M.Buchanan & Schuh. (guinea flower), *Cassinia arcuata* R. Br. (Chinese scrub), *Dianella admixta* Gand. (black-anther-flax-lily) and *Xerochrysum viscosum* (Sieber ex DC.) R.J.Bayer (everlasting daisy) and was classified as Ecological Vegetation Class (ECV) 4.1 Box-Ironbark Forest (Department of Environment and Primary Industries Victoria, 2014).

### 2.3.1.2 Criterion 2 – orchids containing dense fungal colonisation

All three species of orchid had pelotons in the stem-collar region, with occupation ranging from 5% to more than 40% (Figure 2.4, Table 2.5). *P. revoluta* was selected for further studies because it had the greatest average percentage of cells occupied by pelotons in the three sections compared with the other two species. Peloton occupation was greatest in *P. revoluta* and least in *P. pauciflora* (Table 2.5).

![Figure 2.4](image.png)

*Figure 2.4* Hand-cut longitudinal sections of stem-collars from three orchid species. Fungal colonisation in (A) *P. parviflora*, (B) *P. revoluta* and (C) *Genoplesium* sp.. S = stele, C = cortex, circle = pelotons.
2.3.2 Seasonal burns

All plots burned; the summer plots burned readily but the winter burns required several supplementary ignitions (Figure 2.5). The summer burn plots had the greatest post-burn soil temperature and the greatest temperature difference between post-burn and pre-burn times at 4 cm below ground level (Table 2.6). The maximum surface temperature recorded, which was 387.7°C (729.9°F), was also from the summer burn plots (Figure 2.5E, Table 2.6). By contrast, the winter burn plots had the least average minimum post-burn soil temperature and the least temperature difference between post-burn and pre-burn times at 4 cm below ground level (Table 2.6). Spring and autumn burns were relatively low intensity and in a mosaic pattern compared with the summer burn (Table 2.6, Figure 5F).

After winter burns, the average temperature increase in soil below 4 cm was 0.5°C and maximum temperature reached was 11.9°C. After spring and autumn burns, the average temperature increases in soil below 4 cm were 6.5°C and 3.9°C respectively and the maximum average temperatures reached were 21.8°C and 22.9°C respectively. After the summer burns, the average temperature increase in soil below 4 cm was 17.8°C and the maximum temperature reached was 47.3°C.

Table 2.5 Peloton frequency in stem collar region of three orchid species

<table>
<thead>
<tr>
<th>Orchid</th>
<th>Occupation of pelotons in three longitudinal sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. parviflora</td>
<td>++</td>
</tr>
<tr>
<td>Genoplesium sp.</td>
<td>++</td>
</tr>
<tr>
<td>P. revoluta</td>
<td>+++</td>
</tr>
</tbody>
</table>

+ = Very low colonisation (0 - 20% occupation), ++ = Low colonisation (21 - 30% occupation), +++=Medium colonisation (31 -40% occupation), ++++ = High colonisation (> 41% occupation).

Table 2.6 Spring, summer, autumn and winter burn details including date of burn, ambient temperature, maximum temperature of fire and soil at 4 cm below ground, 5 minutes before and after burns in *Pterostylis revoluta*, *Glossodia major* and *Thelymitra pauciflora* plots in each season. Spring burn fire surface temperature was not measured.

<table>
<thead>
<tr>
<th>Season</th>
<th>Date of burn (dd/mm/yyyy)</th>
<th>Ambient temperature (°C)</th>
<th>Maximum fire surface temperature (°C)</th>
<th>Average soil temperatures at 4 cm below ground (°C) (5 minutes before and after burns)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P. revoluta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre-burn</td>
</tr>
<tr>
<td>Spring</td>
<td>18/10/2011</td>
<td>21</td>
<td>-</td>
<td>14.3</td>
</tr>
<tr>
<td>Summer</td>
<td>24/01/2012</td>
<td>28.1</td>
<td>387.7</td>
<td>28.6</td>
</tr>
<tr>
<td>Autumn</td>
<td>27/04/2012</td>
<td>22</td>
<td>189.6</td>
<td>19</td>
</tr>
<tr>
<td>Winter</td>
<td>24/07/2012</td>
<td>12</td>
<td>142.1</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 2.5 Field images of spring, summer and autumn burns of *Pterostylis revoluta*, *Glossodia major* and *Thelymitra pauciflora* in 2011/2012 showing methodology and temperatures of burns conducted. (A) Initial perimeter ignition spreading to the centre of the *P. revoluta* plot during intense summer burn, (B) Initial perimeter ignition of *G. major* plot during spring burn, (C) *P. revoluta* plot after summer burn, (D) *G. major* plot after spring burn, (E) Thermal image showing the maximum temperature (°F) of *T. pauciflora* plot during summer burn, (F) Thermal image showing the maximum temperature (°F) of *T. pauciflora* plot during autumn burn.
2.3.3 Glossodia major

2.3.3.1 Plant numbers pre-burn 2011 vs post-burn 2012

In 2011, before the burns, the control treatment had a significantly greater number of total plants than the autumn burn treatment, which was greater than those of the other treatments, which were not significantly different (Figure 2.6a). The average numbers of flowering plants were not significantly different among treatments but the average numbers of vegetative plants were significantly greater in the control and autumn burn treatments than in the others, which did not differ significantly. Flowering plants ranged from 8% (autumn plot) to 77% (summer plot).

In 2012, the winter burn treatment had no plants and the average number of plants decreased significantly in the control and autumn burn treatments but not others (Figure 2.6b). The average number of flowering plants did not change significantly but the average number of vegetative plants decreased significantly in the autumn burn treatment but not in the others or the control treatment. The total numbers of plants differed significantly pre- and post-burn but the proportion of flowering plants did not change significantly pre- and post-burn for any treatment ($\chi^2$ tests - results detailed under Figure 2.6).

After the burn, the control (unburnt) treatment had a significantly greater number of total plants than all burn treatments and the winter burn treatment had none (Figure 2.6b). The average numbers of flowering plants were not significantly different among treatments but the average numbers of vegetative plants were greater in the control treatment than in the others, which did not differ significantly. Flowering plants ranged from 14% (control) to 72% (spring burn).
Figure 2.6 Numbers of Glossodia major (mean± SE) flowering and vegetative plants in (a) 2011 pre-burn and (b) 2012 post-burn treatments. Control plots had no burn in 2012. No plants emerged after the winter burn. Bars on columns = 2 x SE. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. Bars at right: LSD_{0.05}: values from ANOVA: total plants =7.460, flowering plants = 4.992, vegetative plants = 9.489. \( \chi^2 \) for pre- vs post-burn: total plants among seasons = 42.166, \( p<0.001 \); in flowering vs vegetative plants for: control = 0.062, \( p=0.803 \); spring burn = 0.792, \( p=0.393 \); summer burn = 1.730; \( p= 0.188 \); autumn burn = 0.782, \( p=0.782 \).
2.3.3.2 Plant measurements pre-burn 2011 vs post-burn 2012

In flowering plants, plant height was not significantly different among treatments before or after the burns, apart from the winter burn, in which there were no plants (Figure 2.7a). There was also no significant difference in stem width among treatments before or after the burns (Figure 2.7b).

Flower width was not significantly different among treatments before the burns but decreased significantly after the burns only in the spring and summer burn treatments, though neither was significantly different from the control (Figure 2.7c).

In all plants (both flowering and vegetative), leaf length in 2011 was significantly greater in the summer burn treatment than the control and autumn burn treatments; the other treatments were intermediate (Figure 2.7d). Leaf length decreased significantly in 2012 in all treatments, including the control. After the burns, leaf length was not significantly different among treatments. Plants were absent from the winter burn treatment.

Leaf width in 2011 was also significantly greater in the summer burn treatment than in the control, autumn and winter burn treatments, with the other treatments intermediate (Figure 2.7e). Leaf width decreased significantly in 2012 after the burns in all but the control treatment. After the burns, leaf width in the control treatment was not significantly different from that in the spring burn treatment but greater than that in the others and the leaf widths in the other treatments did not differ significantly, apart from the winter burn where plants were absent.
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Fig. 2.7 continued overleaf

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Figure 2.7 Plant data measurements for *Glossodia major* plants in 2011 pre-burn and 2012 post-burn plots (spring, summer, autumn and winter seasonal burns) and control plots (had no burn in 2012). a. Plant height, b. stem width, c. flower width, d. leaf length, e. leaf width. Bars on columns = 2 x SE. ANOVA: plant height $F=15387$, $p<0.001$; stem width $F=15.83$, $p<0.001$; flower width $F=35.32$, $p<0.001$, leaf length $F=36.27$, $p<0.001$; leaf width $F=59.25$, $p<0.001$. LSD$_{0.05}$ from ANOVA: 66.817 (plant height), 0.422 (stem width), 5.974 (flower width), 10.839 (leaf length), 1.319 (leaf width). Means that do not share a letter are significantly different by Tukey’s family error test at $p=0.05$. 

---

**Leaf length**

<table>
<thead>
<tr>
<th>Fire treatment</th>
<th>Pre-burn</th>
<th>Post-burn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>d</td>
<td>bc</td>
</tr>
<tr>
<td>Summer</td>
<td>a</td>
<td>ab</td>
</tr>
<tr>
<td>Autumn</td>
<td>cd</td>
<td>bc</td>
</tr>
<tr>
<td>Winter</td>
<td>d</td>
<td></td>
</tr>
</tbody>
</table>

**Leaf width**

<table>
<thead>
<tr>
<th>Fire treatment</th>
<th>Pre-burn</th>
<th>Post-burn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>ef</td>
<td>b</td>
</tr>
<tr>
<td>Winter</td>
<td>c</td>
<td></td>
</tr>
</tbody>
</table>
2.3.4 *Thelymitra pauciflora*

2.3.4.1 Plant numbers pre-burn 2011 vs. post-burn 2012

In 2011, before the burns, the average number of (flowering) plants in the winter burn treatment was significantly greater than that in the summer burn treatment, with all other treatments intermediate (Figure 2.8).

In 2012, the average number of (flowering) plants decreased significantly in the autumn and winter burn treatments but not in the others (Figure 2.8). Only one plant was present across all quadrats after the winter burn. The total number of (flowering) plants differed significantly pre- and post-burn ($\chi^2$ tests - results detailed under Figure 2.8).

After the burn, the control (unburnt) and summer burn treatments had a significantly greater average number of (flowering) plants than the autumn burn treatment and the winter burn treatment had one, with the spring burn intermediate (Figure 2.8).

![Figure 2.8](image-url) Numbers of *Thelymitra pauciflora* (mean ± SE) flowering plants in (a) 2011 pre-burn and (b) 2012 post-burn treatments. Control plots had no burn in 2012. Bars on columns = 2 x SE. Means that do not share a letter are significantly different by Tukey’s family error test at p=0.05. Bar at right: LSD$_{0.05}$ values from ANOVA: total flowering plants $= 2.015$. $\chi^2$ for pre- vs post-burn: total flowering plants $= 10.933$, p=0.027.
2.3.4.2 Plant measurements pre-burn 2011 vs post-burn 2012

In flowering plants, the average plant height was not significantly different among treatments in 2011 before the burns (Figure 2.9a). It decreased significantly and uniformly across all treatments, including the control, such that in 2012 there was again no significant difference among treatments. Only one plant was present across all quadrats after the winter burn and so this estimate is unreliable.

There was no significant difference in the average stem width among treatments before or after the burns (Figure 2.9b).

The average number of buds per plant was not significantly different among treatments before the burns in 2011 and did not change significantly after the burns in 2012 except for the single plant from the winter burn (Figure 2.9c).

Leaf length in flowering plants in 2011 was not significantly different among the treatments (Figure 2.9d). In 2012, after the burns, leaf length decreased significantly in spring and autumn burn treatments (the winter burn treatment had only one plant).
The Effect of Seasonal Burns on Australian Native Orchids

**Fig. 2.9 continued overleaf**

**a** Plant height

![Graph showing plant height](image)

**b** Stem width

![Graph showing stem width](image)

**c** No. of buds per plant

![Graph showing number of buds per plant](image)

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2.3.5 Pterostylis revoluta

2.3.5.1 Plant numbers pre-burn 2011 vs post-burn 2013

Monitoring at flowering time extended for three years instead of two because the autumn and winter burns did not occur until 2012 (Table 2.2).

In 2011-2012, before the burns, the average number of plants in all the treatments was not significantly different (Figure 2.10a). The average numbers of flowering plants was significantly greater in the winter, spring and summer burn treatments than in the autumn burn and control treatments in 2011 and in the control, autumn and winter burn treatments in 2012; flowering plants were absent from the autumn and winter 2012 treatments. The numbers of vegetative plants were not significantly different. Flowering plants comprised 36-74% of the total in 2011 but decreased to 0-15% in 2012 for the control, autumn and winter pre-burn treatments.

In 2012-2013, the first year after the burns, the average number of plants increased significantly two- to five-fold in the spring and winter burn treatments.
respectively but did not change in the other treatments, including the control (Figure 2.10b). By 2013, the second year after the fire, there was no further change in average number of plants for the spring burn, but the average number of plants for the summer burn treatment had increased significantly to equal that pre-burn in 2011.

After 2011, all treatments had mostly or only vegetative plants post-burn, including the control, leading to significant differences in numbers and proportions ($\chi^2$ tests - results detailed under Figure 2.9). The average number of total and vegetative plants increased significantly in the spring and winter burn treatments in the first year after the burn but were unchanged from the previous year in the other treatments, including the controls in 2012 and 2013. The average number of flowering plants decreased significantly in the first year after the burn in all but the autumn burn treatment compared with 2011 but was no different from the previous pre-burn samples in 2012.

In the first year after the burn, the winter burn treatment had a significantly greater number of total and vegetative plants than the spring burn treatment and both were significantly greater than the control, summer and autumn burn treatments. The average number of flowering plants was very small (0-3% of total for burnt treatments and 15% for control) and was not significantly different among treatments.

In the second year after the burn for the spring and summer burn treatments, trends were as for the first year after the burn for the spring burn treatment but the average number of total and vegetative plants had increased significantly in the summer burn treatment.
Figure 2.10 Numbers of *Pterostylis revoluta* (mean ± SE) flowering and vegetative plants at 2011, 2012 and 2013 surveys. Control plots had no burn in 2012 or 2013. Bars on columns = 2 x SE. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. Bars at right: LSD$_{0.05}$: values from ANOVA: total plants = 32.31, p<0.001; flowering plants = 21.35, p<0.001; vegetative plants = 38.43, p<0.001. $\chi^2$ between years: 283.068, p<0.001; in proportions of flowering vs vegetative plants for: control = 68.318, p<0.001; spring burn = 183.765, p<0.001; summer burn = 168.094, p<0.001; autumn burn = 37.180, p<0.001; winter burn = 431.502, p<0.001.
2.3.5.2 Plant measurements pre-burn 2011 vs post-burn 2013

In flowering plants pre-burn in 2011, plant height was significantly greater in the summer, autumn and winter burn treatments than the others (Figure 2.11a). Post-burn in 2013, flowering plants were absent in the autumn and winter burn treatments and height had decreased significantly in the control, spring and summer burn treatments, though there was only one flowering plant post-burn in the summer burn treatment and so this estimate is unreliable. There was no difference in plant height post-burn among those treatments with flowering plants, including the control.

Pre-burn, stem width was significantly greater in summer burn treatments than other treatments apart from the control (Figure 2.11b). Stem width decreased in the control but was unchanged in the two other treatments with flowering plants after the burns. There was no significant difference in stem width among treatments after the burns.

Pre-burn, flower length was significantly less in the spring burn treatment than in the others (Figure 2.11c). It showed no significant change from the burns and post-burn plants had flower lengths that were not significantly different.

Flower width was significantly less in the spring burn treatment than in all others apart from the control treatment (Figure 2.11d). It increased significantly after the fire in the spring burn treatment but not the others that had plants present after the fire. After the burns, flower widths were not significantly different among the treatments with plants present, though there was only one flowering plant present in the summer burn treatment.
The Effect of Seasonal Burns on Australian Native Orchids

Fig. 2.11 continued overleaf
Figure 2.11 Plant data measurements for *Pterostylis revoluta* plants in 2011 pre-burn and 2013 post-burn plots (had no burn in 2012 or 2013). a. Plant height, b. stem width, c. flower length, d. flower width. Bars on columns = 2 x SE. ANOVA: plant height F=20.51, p<0.001; stem width F=38.07, p=0.001; flower length F=6.30, p=0.002; flower width F=5.91, p=0.003. LSD$_{0.05}$ from ANOVA: 43.357 (plant height), 0.149 (stem width), 15.456 (flower length), 5.257 (flower width). Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. *Estimate unreliable as only one plant.
2.3.6 Summary

In the spring-flowered G. major, burning was deleterious or had no effect on parameters (Table 2.7). Burning in winter had the greatest effect, as plants were absent in the following year, and burning in other seasons reduced flower and leaf width (Table 2.7). The effects of burning on total number of plants and leaf length need to be interpreted with caution because of corresponding effects in the unburnt control.

Similarly, in the spring-flowered T. pauciflora, burning was deleterious or had no effect on parameters (Table 2.8). Burning in autumn and winter had the greatest effect, as the number of flowering plants decreased to one in the following year (Table 2.8). The effects of burning on plant height and number of buds per plant need to be interpreted with caution because of corresponding effects in the unburnt control, the smaller number of plants initially, the lack of records of vegetative plants, and the sole plant present the year after the winter burn.

By contrast, in the autumn-flowered P. revoluta, burning in winter and spring increased plant number up to 4-fold but resulted in any season in mostly or entirely vegetative plants (Table 2.9). These effects were most pronounced in winter and spring. The effects of burning on flowering plant number and stem width need to be interpreted with caution because of corresponding effects in the unburnt control.

Thus the effects in the two spring-flowered species were similar but different from the effects on the autumn-flowered species.
Table 2.7 Summary of effects of burns on *Glossodia major* (2011 vs 2012)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Control</th>
<th>Burn season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
<td>Summer</td>
</tr>
<tr>
<td>Total plants</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Flowering plants</td>
<td>=</td>
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</tr>
<tr>
<td>Vegetative plants</td>
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<td>=</td>
</tr>
<tr>
<td>Plant height</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Stem width</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Flower width</td>
<td>=</td>
<td>↓</td>
</tr>
<tr>
<td>Leaf length</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Leaf width</td>
<td>=</td>
<td>↓</td>
</tr>
</tbody>
</table>

Key to symbols: = no change, ↓ decrease, ↑ increase, - absent

Table 2.8 Summary of effects of burns on *Thelymitra pauciflora* (2011 vs 2012)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Control</th>
<th>Burn season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
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<td>=</td>
</tr>
<tr>
<td>Plant height</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Stem width</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>No. of buds</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Leaf length</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Key to symbols: = no change, ↓ decrease, ↑ increase, - absent, *only one plant

Table 2.9 Summary of effects of burns on *Pterostylis revoluta* (2011 vs 2013)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Control</th>
<th>Burn season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring</td>
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<tr>
<td>Flowering plants</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>Vegetative plants</td>
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<td>↑</td>
</tr>
<tr>
<td>Plant height</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Stem width</td>
<td>↓</td>
<td>=</td>
</tr>
<tr>
<td>Flower length</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Flower width</td>
<td>=</td>
<td>↑</td>
</tr>
</tbody>
</table>

Key to symbols: = no change, ↓ decrease, ↑ increase, - absent, *only one plant
2.4 Discussion

This is the first study to investigate the effect of seasonal burning on orchids, using empirical data before and after the burns and comparing against a control as a field standard. Depending on season, the burns resulted in decreases in the spring-flowered orchids but increases in the autumn-flowered orchid. Based on this study, the best (least damaging) time to burn was consistently after seed dispersal in the orchid’s dormant stage. Therefore, there was a distinct correlation between the season of the burn and the stage of development of individual orchid plants.

2.4.1 Effects on individual orchids

2.4.1.1 Spring-flowered orchids – Glossodia major and Thelymitra pauciflora

The loss of emergent plants in *G. major* and their severe reduction in *T. pauciflora* suggests that burns in winter, and to a lesser extent autumn, are the most deleterious to both spring-flowered species. The active growth periods for *G. major* and *T. pauciflora* are autumn and winter, when new leaves emerge in autumn and are expanded in winter. If plants are burnt in autumn-winter, the new leaves and any flowers or buds are damaged by fire and most of the tubers do not have enough carbohydrate reserves for re-emergence (Figure 2.12) (Whigham, 1990). During winter, plants use most of the tuber carbohydrate reserves for the development of the leaves and flowers, and the new tubers are not yet fully formed. Therefore, there is a lack of carbohydrate reserves in the tuber for re-emergence in the next season if the plant is damaged by fire at this stage. This also applies to burns later in spring when the capsules release seed (typically about 2 months after flowering). By contrast, a burn after this, in late spring to summer, is likely to be less detrimental to the species because the tubers are fully formed and full of carbohydrate reserves and the plant is dormant underground (Figure 2.12).

This is somewhat analogous to the differences observed in seasonal patterns of root carbohydrate storage and capacity to re-foliate following experimental defoliations in south-eastern USA tree species, in which phenology is critically important in determining vulnerability of trees to fire (Hepting, 1945, Woods *et al.*, 1959; Robbins and Myers 1992; Wade and Johansen, 1986). The differences in
response among seasons reflected the phenology of the plant at the time of disturbance or the conditions in the environment following disturbance (Malanson, 2011). Reserves accumulated in early spring resulted in more vigorous growth following spring fires (Laude, Jones & Moon 1961; Plumb 1963). Similarly, orchids burned in late spring/early summer after plants have shed their seed and have accumulated their maximum carbohydrate reserves in the tubers are less damaged by burns than those burnt before reproduction and maximal accumulation of tuber reserves.

It is frequently stated that late spring to summer burns are beneficial to spring-flowering orchids (TSU, 2006). Several previous studies have observed that spring-flowering orchids flower more prolifically after a summer wildfire (Quarmby, 1999); such orchids flower in September to December and are dormant in summer with maximum carbohydrate reserves in the tuber. Keith (1996) noted that the orchid *Prasophyllum elatum* flowered more prolifically after a wildfire and Kubiak (2009) reported that *Prasophyllum elatum* and *Diuris maculata* flowering was stimulated by 1994 wildfire in Lane Cove River region in Sydney. *Glossodia major* flowering was stated as ‘enhanced by hot fires during the previous summer’ (Backhouse and Jeanes, 1995). *Thelymitra* species such as *T. bracteata*, *T. malvina*, *T. benthamiana* were stated as likely to be enhanced by summer fires (TSU, 2006), and summer was the least damaging season in which to burn *T. pauciflora* in this study.

Although late spring to summer burns are said to be beneficial to the species (TSU, 2006), there was no benefit to either of the spring-flowered orchids studied here from a spring or summer burn, as in each species there was no increase in plant number, the total number of plants declined and one or more parameters of plant size decreased following a burn. Also, the species were studied only in the year after fire and longer-term effects are unknown. In addition, Weston *et al.* (2005) conjectured that in the long term fires might reduce the populations, even though flowering was initially stimulated by high intensity fires in some orchid species; if fires are highly infrequent, then such fire-dependent species can decline (Keith, 1996).

2.4.1.2 Autumn-flowered orchid – *Pterostylis revoluta*

The massive increases in vegetative plants the year after winter and spring burns in the only autumn-flowered orchid studied, *P. revoluta*, suggests that burning
after it has released its seeds in late autumn to winter and become dormant in winter-spring was beneficial. As almost all plants were vegetative, it is not clear if this was as a result of stimulating dormant tubers to produce emergent foliage or of stimulating seed germination and seedling growth (Jones, 1988; Jones, 2006), as it almost eradicated flowering.

The season of active growth for *P. revoluta* was summer to autumn (Figure 2.12). During summer the plants emerge out from the soil and a burn in that season will damage the plants, leaving little carbohydrate reserves in the tuber. In autumn, *P. revoluta* plants formed flowers and used most of their tuber carbohydrate reserve for the development of the seed and by that time the new tuber was not fully formed, so that there was very little carbohydrate stored in the new tuber for the next season. A fire in late summer-autumn-winter when the plants are in active growth would therefore be expected to be more damaging than a fire in late autumn-winter-early spring when the plants are dormant. While the fires in summer and autumn produced little change in plant numbers, those in winter and spring not only did not reduce the plant numbers – they stimulated them. This may be due more to the effects of smoke water rather than to the temperatures reached. Smoke water has many stimulant effects, including seed germination, in many Australian species (Dixon *et al*., 1995), and may have stimulated either dormant tubers to emerge, seeds to germinate or protocorms to develop into leafy plants; this effect would depend on the amount of rainfall after the fire. Further research into the effect of smoke water on the development of *P. revoluta* would be worthwhile, both in the laboratory and in field sites.

A decline in rainfall, rather than the burns themselves, may explain the drastic decline in the proportion of flowering plants, as the trend started in early 2012, even before the autumn and winter burns, and was also observed in controls. Backhouse and Jeanes (1995) noted that vegetative plants of *P. revoluta* were commonly much more numerous in colonies than were flowering plants and that flowering appeared to be promoted by late summer rains, with flowering rare during drought conditions. Some other orchid species are sensitive to variation in annual rainfall; if less rainfall is received in the same or previous year, flowering is reduced (Inghe and Tamm 1988; Light and MacConnaill 1991) and flowering is enhanced if significant rainfall is received before the flowering season (Hutchings 1987; Light and MacConnaill 1991;
Kery and Gregg 2004). During the study period, the March to the following February rainfall decreased by two-thirds, from 1332.2 mm in 2010-2011, to 536.4 mm in 2011-2012, to 405.2 mm in 2012-2013. The corresponding decrease in *P. revoluta* flowering plants surveyed in March-April was 12-fold, from 235 in 2011, to 16 in 2012, to 24 in 2013. As the decrease in flowering plants was not proportional to the decrease in rainfall, both rainfall and burns may have contributed to this decline, and possibly other factors did also. Further research would be needed to elucidate the reason for the switch from 36-74% flowering in 2011, to 0-15% flowering in 2012, to 0-9% flowering in 2013.

### 2.4.2 Choice of season for prescribed burning

Damage to underground tubers attributable to heat generation during burning was unlikely at any season. Even in summer, the average soil temperature 4 cm below the ground, at the level of the tubers, did not rise much above ambient. This is similar to the finding that soil heating below 5 cm from the ground level was insignificant (Morgan, 1998). Even after the burns reaching high temperatures in summer, the numbers of plants did not change. Therefore, it can be assumed that fire did not damage the tubers directly but the after-effects, e.g. smoke water, increased insolation, may have stimulated tuber re-emergence or seed germination.

For all three species, with their different annual growth cycles, the best (least damaging) season for a burn was after seed dispersal (normally 2 months after flowering), when the plants are in the dormant stage with no above-ground parts present and when the maximum carbohydrate reserves are in the new fully formed underground tubers. The possibility of re-sprouting is related to carbohydrate reserves in the tuber (Smith, 1965). The season of the burn affects carbohydrate reserves; therefore the timing of the burn is critical for the plant to re-sprout (Jones and Laude, 1960). If the new tuber is fully formed at the time of burn, there should be enough carbohydrates (energy) for the developing plant to grow in the next season.

The most favourable season for burning varied considerably for each species. For the spring-flowered *G. major* and *T. pauciflora*, it was summer, whereas for the autumn-flowered *P. revoluta* it was winter. When considering the number of plants and plant measurements the most detrimental season for a fire for *P. revoluta* was
autumn followed by summer and for *G. major* and *T. pauciflora* it was winter followed by autumn (Figure 2.12, Table 2.10). For *P. revoluta*, when considering the number of total or vegetative plants, the most favourable season was winter but when considering the number of flowering (reproductive) plants) and plant measurements it was spring because flowering plants were absent following autumn and winter burns.

Table 2.10 Advantageous and disadvantageous burn seasons for *Pterostylis revoluta*, *Glossodia major* and *Thelymitra pauciflora* vegetative and flowering plants on the basis of number and condition of plants present pre-burn and post-burn

<table>
<thead>
<tr>
<th>Type of plant</th>
<th>Best/worst season to burn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. revoluta</em></td>
</tr>
<tr>
<td></td>
<td>Sum</td>
</tr>
<tr>
<td>Vegetative</td>
<td>-</td>
</tr>
<tr>
<td>Flowering</td>
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</table>

Clearly a compromise is needed for conservation of all three orchids. While for the spring-flowered orchids the best season to burn was late spring (*G. major*)-summer (*T. pauciflora*), the best season to burn was winter-spring. Therefore late spring is the best compromise for a burn on a site inhabited by all three orchids; it was the best or second-best season for the spring-flowered orchids and the second-best for the autumn-flowered orchid. This, however, is not the preferred season for a burn, which is autumn because of the ease of ignition and the calm conditions, though this was not the best time for conservation of these orchids.

From this study it is clear that there is a relationship between the season of the burn and stage of development of individual orchid plants. Future work examining the amount of carbohydrate reserves in the tuber before a burn, damage to the developing tuber and tuber emergence after the fire would be useful to explain the results of this study.
Figure 2.12 Effect of season of prescribed burns on the developmental stages (annual growth cycles) of (A) Glossodia major (B) Thelymitra pauciflora and (C) Pterostylis revoluta as observed in 2011-2013. Note that rosette form illustrated is symbolic and is not representative of the species.
Chapter 3

The effect of seasonal fire and plant developmental phase (flowering or vegetative) on the isolation of microbes from pelotons of *G. major*, *T. pauciflora* and *P. revoluta*
3.1 Introduction

Both fire and fungi are important factors to plants in Australian fire-prone ecosystems. Fire season, frequency and intensity, with other factors such as existing weather, fuel load and soil moisture, determine the level of fire disturbance of forest soil (Raison, 1979; Vázquez et al., 1993). Microorganisms can be influenced by fire either directly, such as by soil heating, or by indirect effects such as changes to soil physical (soil colour, texture, pH, bulk density and water-holding capacity) and chemical (soil organic matter and mineral nutrients) characteristics after a fire (Neary et al., 1999; Mao et al., 2002; Certini, 2005). Soil microorganisms are greatest in the upper few cm of soil, where organism abundance is highest (Neary et al., 1999).

The maximum temperatures of a fire often go beyond the heat required to kill most living beings in the soil (DeBano et al., 1998). Some studies indicate that fungal communities are more affected by fire than their bacterial counterparts (Raison, 1979; Bissett & Parkinson, 1980; Entry et al., 1986; Vázquez et al., 1993; Baath et al., 1995; Bergner et al., 2004; Palese et al., 2004). This could be due to most stems, roots and mycorrhizal fungi being destroyed directly by the fire, with a subsequent slow re-colonisation rate in the burned soil (Harvey et al., 1980; Baath et al., 1995). Mycorrhizal fungi (McMullan-Fisher et al., 2011) depend on the hosts for nutrition and so are affected by fire to the same degree as their host plant (Sugihara et al., 2006).

Many woody mycorrhizal plants, such as Eucalyptus and Pinus species, have root colonisation deeper in the soil than the Orchidaceae. Arbuscular mycorrhizae (AM) are the most widespread and common mutualistic fungi in Australian Eucalyptus plants (McMullan-Fisher et al., 2011) and are present in roots in 10 cm deep in the soil. The response of AM to fire is quite variable, from no effect (Anderson & Menges, 1997) to decreased (Dhillion et al., 1988) or increased (Bentivenga & Hetrick, 1991; Korb et al., 2003) root colonisation within 12 months of prescribed burning (Cairney & Bastias, 2007).

Ectomycorrhizal (ECM) roots are present in the top 10-20 cm of soil (Bastias et al., 2006a) and fire had no effect (Herr et al., 1994; Korb et al., 2003) or decreased (Korb et al., 2003; Smith et al., 2005) root colonisation within 12 months of fire (Cairney & Bastias, 2007). By contrast, the amount of ECM and the growth of E.
regnans seedlings were greater in burnt black soil compared with unburnt soil, which was attributed to changes in soil nutrition (increased phosphorus) and the presence of different ectomycorrhizal fungi (Launonen et al., 1999).

The importance of mycorrhizal fungi to the growth and survival of orchid species is well acknowledged (Rasmussen 1995, Dearnaley and Le Brocque 2006). Any environmental disturbance, such as fire, can imbalance this relationship and so increase or decrease the growth and survival of orchids. The effect of fire on above-ground orchid plant structures has been well studied and documented (Jones, 1988; Keith, 1996; Kubiak, 2009) but only a few studies (Huynh et al., 2009) have investigated below-ground plant structures and mycorrhizal fungi.

In Victoria, 370 orchid species are recorded and almost half (170) have a state conservation status of rare (R), vulnerable (V), endangered (E) or extinct (X), based on the World Conservation Union (IUCN 2001) listing (Duncan et al., 2005). Altered fire regimes are considered to be one of the causes of this status (McMullan-Fisher et al., 2011; Duncan et al., 2005).

Only one study has investigated the effect of fire on orchid mycorrhizal fungi (OMF) (Ramsay et al., 1986). They found that summer or early autumn fires increased root colonisation by OMF to 50% of cortical cells and 80% of the root length in four genera, including Thelymitra and Leporella. Fire also affected the viability and ease of isolation of OMF in Leporella fimbriata; only OMF from burnt sites grew in culture when isolated from pelotons. An understanding of the impact of fire on orchid mycorrhizae has an important role in successful orchid conservation because orchid mycorrhiza are present in the top 10 cm of soil and therefore fires can affect them significantly (Brundrett, 2006).

The present study was conducted on three species of orchids: G. major, T. pauciflora and P. revoluta. The mycorrhizal infections of P. revoluta and G. major occur in the stem-collar region, which is close to the soil surface below the litter layer and therefore more likely to be affected directly by fire than the mycorrhizal infection of T. pauciflora, in which the mycorrhizal infection occurs in the roots, which are 4-5 cm deeper in the soil and so are less likely to be affected by fire, since soil is not a good heat conductor.
The aims of this study were to:

1) Investigate the effect of seasonal burns on the growth of *Rhizoctonia*-like fungi isolation from pelotons of *P. revoluta*, *G. major* and *T. pauciflora*

2) Compare the growth of *Rhizoctonia*-like fungi from pelotons in flowering and vegetative plants of *P. revoluta*.

### 3.2 Materials and Methods

#### 3.2.1 Pre- and post-burn collection of *P. revoluta*, *G. major* and *T. pauciflora* collars and roots

In 2011, stem-collars of *P. revoluta* and *G. major*, and roots of *T. pauciflora* were collected during their flowering season before the burns (Table 3.1) from the 15 selected plots (3 species × 5 plots – 4 plots for each seasonal burn and 1 plot as a control for each species) as described in Chapter 2. In the five *P. revoluta* plots, there were more than 15 flowering and vegetative plants in March 2011. There were also more than 15 flowering plants in each *G. major* plot in September 2011 and more than 8 flowering plants in each *T. pauciflora* plot in October 2011 (Table 3.1). In the 2011 (pre-burn) sampling, samples were collected only from *T. pauciflora* flowering plants; samples were not collected from vegetative plants because they were difficult to distinguish from grasses and other *Thelymitra* species present in the same plot. Three samples of stem-collars/roots were collected for each species at the plant’s flowering stage from each plot (5 plots - 4 plots for each seasonal burn and 1 plot as a control × 3 species × 3 replicate plant samples from each plot = total 45 samples) for fungal isolation (Table 3.1).
Table 3.1 Pre-burn and post-burn numbers of *Glossodia major*, *Thelymitra pauciflora* and *Pterostylis revoluta* plants present in each plot on the sample collection date and number of samples collected from five plots. Abbreviations: C=control, Spr=spring, Sum=summer, Aut=autumn and Win=winter. Colour key: unburnt and burnt.

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<th>Plant</th>
<th>Life stage</th>
<th>Colonised region</th>
<th>Collection dates</th>
<th>No. of plants present in each plot on the collection date</th>
<th>No. of samples collected</th>
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<td></td>
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<td>C</td>
<td>Spr</td>
<td>Sum</td>
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</table>
For G. major and T. pauciflora, after the four seasonal burns were completed in July 2012, samples of stem-collars and roots respectively were collected in their next flowering season in September and October 2012 respectively. From each control, spring, summer and autumn burn plot, three flowering plant samples of each species were collected (Table 3.1). No samples of G. major were collected from the winter burn plot because no plants (flowering or vegetative) were present post-burn (Table 3.1). Only one plant was collected from the T. pauciflora winter burn plot because only one plant was present (Table 3.1).

For P. revoluta, after the 2011 spring (October) and 2012 summer (January) burns, stem collars were collected in the next flowering season in March 2012. There were only 6 flowering plants in the control plot and 10 in the spring burn plot and no flowering plants in the summer burn plot and the autumn (unburnt) and winter (unburnt) plots, but there were more than 30 vegetative plants in each plot (Table 3.1). Since flowering plants were absent in summer, autumn and winter plots, only vegetative plants were collected. Therefore, three flowering plants and three vegetative plants were collected from the control and spring burn plots, but only three vegetative plants from each summer, autumn and winter (unburnt) burn plot were collected in 2012 (total of 21 samples) (Table 3.1).
3.2.2 Sampling and fungal isolation

About 5 cm radius around the plant was excavated with a trowel to a depth of 6 cm around each plant (Fig. 3.1). The stem-collars of *P. revoluta* and *G. major* plants were excised 2 cm below and above the collar region using a sterile blade (Figure 3.1G). The roots of *T. pauciflora* were traced using a brush (Figure 3.1E) and excised similarly. The collars or roots were each placed in separate sterile Eppendorf tubes for each plant and transported to the laboratory for use within 24 hours for fungal isolation.

Collars and roots were washed with running tap water until all soil was removed (Figure 3.1G-H). In a laminar flow cabinet, stem-collars/roots were immersed in 1% NaOCl for 3 minutes and then washed three times with sterile water. Each collar/root section was further cut into 1 mm-thick longitudinal sections using a stereomicroscope. Single pelotons, which were opaque and circular-oval (Figure 3.2A), were isolated (Huynh et al., 2009) and washed five times with sterile water before culturing in antibiotic free, Fungal Isolation Medium (Clements et al., 1986) using a separate sterile Pasteur pipette for each plate (Huynh et al., 2009). Each plate contained approximately 10 (±3) pelotons. Plates were sealed with Parafilm™ and incubated at 25°C. After 3 weeks of growth, fungi were stained with lactophenol cotton blue (LPCB) and observed using a Leica MZ9.5 Stereomicroscope and an Olympus compound microscope.

Pelotons were cultured from each plant species, season (control, spring, summer, autumn and winter burns) and treatment (pre-burn and post-burn) (Table 3.2). Isolations from pelotons were categorized according to Huynh et al. (2009): 1) *Rhizoctonia*-like fungi, 2) contaminants or other sporing or pigmented fungi (e.g. *Trichoderma, Aspergillus, Penicillium*), 3) bacteria (e.g. *Pseudomonas*) or 4) dead/no growth (Warcup, 1973).
Table 3.2 The number of pelotons cultured from pre-burn and post-burn plots for *Pterostylis revoluta*, *Glossodia major* and *Thelymitra pauciflora*

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Pterostylis revoluta</em></td>
<td><em>Glossodia major</em></td>
</tr>
<tr>
<td>Control</td>
<td>97</td>
<td>165</td>
</tr>
<tr>
<td>Spring</td>
<td>107</td>
<td>162</td>
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<td>Summer</td>
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<td>156</td>
</tr>
<tr>
<td>Autumn</td>
<td>114</td>
<td>173</td>
</tr>
<tr>
<td>Winter</td>
<td>110</td>
<td>158</td>
</tr>
</tbody>
</table>

*Unburnt at time of sampling in 2012, - no plants.

Isolated fungi were labelled in the following order: name of the plant (P= *P. revoluta*, G= *G. major* and T= *T. pauciflora*), treatment (B = burnt or U = unburnt), fungal collection year (1=2011, 2=2012), plot ID (C=control plot, Sp=spring plot, Sum=summer plot, A=autumn plot, W=winter plot), plant number (1 to 3), fungal isolate (a letter from A to Z for each peloton), e.g. PB2Sp2C (*P. revoluta*, burnt, 2012, Spring plot, plant No. 2 and isolate C).

Percentage growth of each category pre-burn and post-burn was analysed statistically using Minitab 16 statistical software after any necessary transformation to normality. The proportions in each category were also compared using $\chi^2$. Significance was recorded at $p \leq 0.05$. 
Figure 3.1 Pterostylis revoluta flowering plant (A), P. revoluta vegetative plant (B), Glossodia major flowering plant (C), Thelymitra pauciflora flowering plant (D), excavation and careful tracing of T. pauciflora roots (E), T. pauciflora infected roots positioned at 4-5 cm below the soil surface (F), washed and cleaned cut collar sections of P. revoluta (G) and G. major (H). Key: S and horizontal white line = soil surface; F-fungal colonisation region; R–roots; T-tuber.
3.2.3 Fungal observation and peloton morphology

Cut sections of pre-burn *P. revoluta* and *G. major* were prepared for scanning electron microscopy (SEM) by fixing overnight in 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0, and post-fixed with 1% osmium tetroxide in water for 2 hours. Fixed sections were dehydrated in an ethanol series (10, 20, 30, 50, 70, 90, 100, 100%) at 15 minute intervals. Samples were critical-point dried using carbon dioxide as the transition liquid, mounted onto double-sided sticky tabs, sputter-coated with gold and viewed with a FEI XL30 scanning electron microscope to examine fungal occupation and peloton morphology. Peloton morphology was observed and categorised as intact (healthy) or collapsed. Post-burn samples were fixed to score fungal occupation and peloton morphology but were not examined due to the timing of the burns and time restrictions in the study.

3.3 Results

3.3.1 Fungal growth observations

Pelotons were observed in the tissues (Figure 3.2A) and pelotons grew fungi and bacteria (Figure 3.2B). Fungal colony growth was observed when there was sufficient growth for scoring: for *P. revoluta* at 5 days, *G. major* at 6 days and *T. pauciflora* at 10 days (Figure 3.2B) for both pre-burn and post-burn isolations. Two types of fungi were observed for *P. revoluta* cultures. The slow-growing fungi had monilioid cells (Figure 3.2C), which are characteristic of *Rhizoctonia*-like orchid mycorrhizal fungi, and the fast-growing fungi had characteristic sporulation or clamp connections (Fig. 3.2D). *Glossodia major* produced two morphologically different types of fungi but *T. pauciflora* had only one type, all of which formed monilioid cells.
Figure 3.2 Growth and hyphal morphology of *Rhizoctonia*-like fungi from *P. revoluta* at the flowering stage in 2011. Longitudinal section of the stem-collar with pelotons (circled) (A), *Rhizoctonia*-like fungi growing from isolated pelotons (B), monilioid hyphae of *P. revoluta* (C) fast-growing fungus with clamp connections (D).
3.3.2 *Glossodia major*

Pre-burn pelotons grew mainly *Rhizoctonia*-like fungi and bacteria but very few other fungi. No isolations from winter burn pelotons were possible because burning eliminated the plants.

The majority of pelotons grew *Rhizoctonia*-like fungi (50-70%) except in control plots (40%) (Figure 3.3). All four seasonal burns almost halved (30%) the proportions of isolated pelotons that grew *Rhizoctonia*-like fungi and doubled the proportion that grew bacteria (Figure 3.3). This was different from the 2011 and 2012 control plots, which had similar proportions of *Rhizoctonia*-like fungi (30-40%) and bacteria (55-30%).

In pre-burn plots, the pelotons that failed to grow (no growth) comprised <5% of the total except for the winter plot (27%) (Figure 3.3). In post-burn spring, summer and autumn plots, pelotons had similar proportions of no growth but in the control plot the proportion of pelotons with no growth increased to 30% (Figure 3.3).

Isolation of ‘other fungi’ was similar in all five plots, both pre-burn and post-burn. There were no other fungi isolated from all five pre-burn plots but 1-2% of other fungi were isolated in post-burn spring and summer burn plots (Figure 3.3). Fungi isolated from pelotons were identified as *Fusarium* species.
Figure 3.3 Pre- and post-burn isolation of microorganisms from five treatments (control and spring, summer, autumn and winter burns) from *Glossodia major* flowering plants. Control treatments had no burn. No plants were present in the post-burn winter treatment. Bars on columns = 2xSE. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. Bars at right: LSD$_{0.05}$ = *Rhizoctonia*-like fungi 11.129, other fungi NS, bacteria = 11.546, no growth=7.936. $\chi^2$ among seasons: pre-burn = 104.352, p<0.001; post-burn = 104.352, p<0.001; pre- vs post-burn: control = 20.134, p<0.001; spring = 20.194, p<0.001; summer = 29.799, p<0.001 autumn = 15.705, p<0.001.
3.3.3 *Thelymitra pauciflora*

In pre-burn plots, 60% of pelotons grew either *Rhizoctonia*-like fungi (20-25%) or bacteria (40%) and almost 40% of pelotons produced no growth. These proportions were uniform across all five pre-burn plots, including the control (Figure 3.4).

The proportion of pelotons growing *Rhizoctonia*-like fungi in 2011 and 2012 control plots was the same (27%) (Figure 3.4). By contrast, all four seasonal burns halved the proportions of pelotons that grew into *Rhizoctonia*-like fungi and increased either bacteria or no growth (Figure 3.4) (although the winter data are less reliable because they are from the only plant that emerged after the winter burn). Isolation of bacteria increased from 36% in pre-burn autumn plots to 82% in post-burn plots but decreased in the winter burn plot from 47% to 7% (Figure 3.4).

The proportion of pelotons that failed to grow in the control, spring burn and summer burn plots were similar pre-burn and post-burn (35-40%) but decreased in autumn burn plots from 43% in pre-burn to 6% post-burn as the proportion that grew bacteria increased (Figure 3.4).

There were no other fungi isolated from all pre-burn plots and less than 1% of the total growths from post-burn spring, summer and autumn plots (Figure 3.4). The fungi isolated were assignable to *Fusarium* and *Trichoderma*. 
Figure 3.4 Pre- and post-burn isolation of microorganisms from five treatments (control and spring, summer, autumn and winter burns) from *Thelymitra pauciflora* flowering plants. Control treatments had no burn. Bars on columns = 2xSE. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. Bars at right: LSD$_{0.05}$ = *Rhizoctonia*-like fungi = 10.373, other fungi = NS, bacteria = 16.552, no growth=16.296. $\chi^2$ among seasons: pre-burn = 6.487, p=0.593; post-burn = 118.507, p <0.001; pre- vs post-burn: control = 1.578, p=0.454; spring = 11.241, p=0.004; summer = 48.326, p<0.001; autumn = 48.326, p<0.001; winter = 27.492, p<0.001.
3.3.4 *Pterostylis revoluta*

3.3.4.1 Fungal observation and peloton morphology

Pre-burn fungal occupation for *P. revoluta* flowering plants showed 30-40% of fungal occupation in the cortex in plants from all five plots (Figures 3.5, 3.6). There were more collapsed (digested) pelotons (than intact (healthy) pelotons (Figures 3.5, 3.7).

![Figure 3.5](image)

**Figure 3.5** Electron micrographs of *Pterostylis revoluta* showing different peloton morphology at flowering stages. Section of the collar showing 40% of peloton occupation (A), intact/healthy pelotons (B), collapsed pelotons (C, D).
**Figure 3.6** Percentage of *Rhizoctonia*-like fungal occupation of cortical cells in *Pterostylis revoluta* pre-burn plots (control, spring, summer, autumn and winter).

**Figure 3.7** Morphology of pelotons in *Pterostylis revoluta* observed from pre-burn plots.
3.3.4.2 All plants

In pre-burn plots, only 10-30% of pelotons yielded *Rhizoctonia*-like fungi; the remainder was dominated either by bacteria (20-80%) or no growth (0-70%) (Figure 3.8). The proportions of pelotons that grew bacteria or had no growth were inversely related. Pelotons from the control, winter burn and spring burn plots had a smaller proportion of pelotons that grew bacteria than did those from the summer burn and autumn burn plots.

Burns in spring and summer more than doubled the proportions of pelotons from which *Rhizoctonia*-like fungi were isolated, but this occurred even in the unburnt autumn and winter plots. All summer-autumn winter burn plants in 2012 were vegetative as were two-thirds of the spring samples. By contrast, the control plot had no change in the proportions of growth.

The greatest post-burn change was the decrease in the proportion of pelotons growing bacteria. The post-burn isolation of bacteria in all five plots decreased significantly compared with the pre-burn samples.

No ‘other fungi’ were isolated from the plants in 2011 and 2012 (Figure 3.8).
Figure 3.8 Morphology of pelotons in *Pterostylis revoluta* observed from pre-burn plots. Control treatments had no burn. Sampling in autumn and winter plots was before 2013 burns. Bars on columns = 2xSE. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. Bars at right: LSD$_{0.05}$ = *Rhizoctonia*-like fungi = 17.01, other fungi = N/A (all zero), bacteria = 13.860, no growth=18.598. $\chi^2$ among seasons: pre-burn = 224.079, p<0.001; post-burn = 100.429, p<0.001; pre- vs post-burn: control = 0.549, p=0.760; spring = 36.430, p<0.001; summer = 119.978, p<0.001; autumn = 122.560, p<0.001; winter = 45.882, p<0.001.
3.3.4.3 Comparison of flowering and vegetative plants

In flowering plants, there was a decrease in the proportion of pelotons that grew *Rhizoctonia*-like fungi compared with pre-burn and a corresponding increase in the proportion of pelotons that did not grow (Figures 3.8, 3.9). Vegetative plants had more than twice the proportion of pelotons growing into *Rhizoctonia*-like isolates and less than half that did not grow compared with flowering plants (Figure 3.9). The There were no ‘other fungi’ isolated from the flowering or vegetative plants in 2011 and 2012 (Figure 3.9).

![Figure 3.9](image)

Figure 3.9 Pre- and post-burn isolation of microorganisms from flowering and vegetative plants of *Pterostylis revoluta* after the spring burn in 2012. Bars on columns = 2xSE. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05. Bars at right: LSD_{0.05} = *Rhizoctonia*-like fungi = 26.797, other fungi = N/A (all zero), bacteria = N/A (NS), no growth = 23.306. \( \chi^2 \) for proportions between flowering and vegetative plants = 49.035, p<0.001.
3.4 Discussion

The contrasting results in the spring-flowered orchids *G. major* and *T. pauciflora* from those of the autumn-flowered orchid *P. revoluta* suggests that the differences are not directly related to the time of year of the burn but rather to the effects of fire on the plants themselves or their mycorrhizal fungi.

3.4.1 *Rhizoctonia*-like fungi

The effect of fire on the proportion of pelotons growing *Rhizoctonia*-like fungi was different for different genera, with decreases for *G. major* and *T. pauciflora* but increases in *P. revoluta* from spring and summer burns, largely because of the greater proportion of vegetative plants.

Fire at all seasons (spring, summer, autumn and winter) significantly decreased the isolation of *Rhizoctonia*-like fungi for *G. major* and *T. pauciflora*. Some studies have indicated that fungal communities are more sensitive to fire than their bacterial counterparts (Raison, 1979; Vazquez et al., 1993; Baath et al., 1995; Bergner et al., 2004; Palese et al., 2004). There were overall reductions in the soil fungal population after a burn compared with that in unburnt soil (Vazquez et al., 1993, Wright and Tarraunt, 1957; Renbuss, 1973) reported that fungal growth was very slow in burnt soil. Post-burn mycorrhizal re-infection from soil may be reduced for these reasons. The fire burns the partially to fully decomposed organic material between the litter-lichen-moss stratum and mineral soil layer (duff) more completely than it burns the surface organic soil. Fungi are often present in the duff layer, and so fungal populations are more affected by fire than bacterial populations. A shift from a microbial community dominated by fungi to one dominated by bacteria can affect plant species composition by favouring non-mycorrhizal dependent species or plant species dependent on mycorrhizal fungi that survive or colonise rapidly in changed sites (Sugihara 2006). Therefore, orchids that depend heavily on mycorrhizal fungi throughout their growth cycle may not be re-colonised rapidly in burnt areas and are threatened by fires.

The proportion of isolation of *Rhizoctonia*-like fungi from flowering *P. revoluta* plants after the spring burn was less post-burn than pre-burn, suggesting that the fire
affected them similarly to *G. major* and *T. pauciflora*. The reason for the apparent increase in isolation of *Rhizoctonia*-like fungi from *P. revoluta* is that in other treatments (apart from the control) only vegetative plants were found and *Rhizoctonia*-like fungi were isolated in greater proportions from vegetative plants than from flowering plants. This suggests that the orchid developmental phase affected success in isolation of *Rhizoctonia*-like fungi. Similarly, in *Caladenia formosa* a larger proportion of *Rhizoctonia*-like fungi were isolated at leafing and budding stages than in the later developmental phases of flowering, fruiting, senescing and dormancy (Huynh *et al.*, 2009). The increase in isolation of *Rhizoctonia*-like fungi from *P. revoluta* was therefore largely attributable to a difference in life stage rather than a difference in response of the orchid or its mycorrhizal fungi to the fire.

Re-colonisation of orchid mycorrhizal fungi on site after a burn has not been reported. It may be assumed that the fast-growing *Ceratobasidium* mycorrhizal fungi colonised the site quickly from reservoirs in deeper soil or from higher plant roots deep down the soil profile and infected previous or new *P. revoluta* plants, as the proportion of *Rhizoctonia*-like fungi isolated pre-burn and post-burn increased after the spring and summer burns. Similarly, Ramsay *et al.* (1986) found that summer or early autumn fires increased root colonisation by OMF (as judged by peloton occupation of cortical cells) to 50% of cortical cells and 80% of the root length in four genera, including *Thelymitra* and *Leporella* Unfortunately, time precluded SEM examination of collected and fixed tissues post-fire and it is recommended that they be examined to see if similar trends are visible. Fire also affected the viability and ease of isolation of root-located OMF in the autumn-winter (March-July)-flowered *Leporella fimbriata* (a potential fire-following species of orchid (K. Dixon, pers. comm.)); only OMF from burnt sites grew in culture when isolated from pelotons (Ramsay *et al.*, 1986), which appears contrary to the responses here.

### 3.4.2 Bacteria

The effect of fire on the isolation of bacteria was different for different genera and burn seasons. Isolation of bacteria from *G. major* flowering plants after all seasonal burns (spring, summer, autumn and winter) increased significantly compared with pre-burn, and similar effects were observed for *T. pauciflora*. By contrast, the isolation of bacteria from *P. revoluta* decreased post-burn (2012)
compared with pre-burn (2011), but also occurred in the 2012 pre-burn samples. This may be related to rainfall, as the rainfall reduced by more than half in 2012 compared with 2011. At the time *P. revoluta* was flowering during the sample collection period (January-March 2011), rainfall at the Bendigo airport site was 376 mm, but in January-March 2012 it was 187 mm (Australian Government Bureau of Meteorology 2013).

Bacterial populations in soil are more resistant to fire than fungal populations (Raison, 1979; Vazquez *et al*., 1993; Baath *et al*., 1995; Bergner *et al*., 2004; Palese *et al*., 2004). For example, the bacterial population was 25 times more in burnt soil than in unburnt soil after one month (Vazquez *et al*., 1993). Bacterial populations may increase due to enhanced availability of N and K in the burnt soil (Saravanan *et al*., 2013). Wet or moist soils encourage bacterial growth (Wilkinson *et al*., 1989; Tsavkelova *et al*., 2003) but this does not explain why bacterial contamination was greater in these orchid plants, as the rainfall after the fires was less than half that in the preceding year. It is more likely that the burns damaged the orchid tissues, allowing bacteria to penetrate and this should be investigated further.

### 3.4.3 No growth

There was no consistent effect of fire on the proportion of pelotons that failed to grow. The proportions were similar pre-burn and post-burn in all three species of orchids, except for isolated increases or decreases in all three species. Remarkably, 20-40% of pelotons failed to grow in pre-burn and post-burn plots of the spring-flowered orchids but this reached up to 70% in *P. revoluta* pre-burn, in which the lack of growth was possibly due to the large proportion of collapsed pelotons (Huynh *et al*., 2004).

### 3.4.4 Other fungi

The small proportions of pelotons growing other fungi were very small and similar from pre-burn and post-burn plots. There were no other fungi isolated from *G. major* and *T. pauciflora* pre-burn but post-burn 2% of other fungi were isolated; these belonged to common soil genera such as *Fusarium* and *Trichoderma* (Huynh *et al*., 2004), suggesting little invasion by other fungi into the orchid tissues.
3.4.5 Summary

Isolation of *Rhizoctonia*-like fungi after seasonal burns was different for different genera with decreases for *G. major* and *T. pauciflora* but increases for *P. revoluta*. On the basis of this study, spring, summer and autumn burns had similar effect on isolation of *Rhizoctonia*-like fungi.

In *G. major* and *T. pauciflora*, the effects on isolation of *Rhizoctonia*-like fungi after spring, summer or autumn burns were similar. There was no ‘best season’ for a fire after which to isolate fungi as all declined equally, but the most deleterious season was winter because there were few or no plants available after fire. Autumn is the leafing stage of *G. major* and *T. pauciflora* and it may be preferably to isolate then as plants at this stage typically have a larger proportion of *Rhizoctonia*-like fungi than in later developmental phases (Huynh *et al*., 2009). Fire at this stage might have reserved active healthy fungi (intact) in protected reservoirs in roots or tubers (5 cm below the soil). Ramsay (1986) noted that in root-infected orchids (including *Thelymitra*) endophyte occupation was best developed following a summer or early autumn fire. The best season for a burn after which to isolate fungi from *P. revoluta* was summer followed by spring (only spring and summer burns were conducted). Similarly, summer was the leafing stage of *P. revoluta* when it had a large amount of *Rhizoctonia*-like fungi in its tissues. This suggests that burning in late spring-early autumn was the least damaging to the *Rhizoctonia*-like fungi in these orchids.

Future work examining pre-burn and post-burn peloton occupation and morphology using scanning electron microscopy would be useful to investigate further the outcomes of this study.
Chapter 4

Effect of fire treatment and smoke water on growth of fungal isolates
4.1 Introduction

Terrestrial orchids lack extensive root systems, have few leaves and some have no chlorophyll for photosynthesis; therefore the efficacy of their mycorrhizal associations is important for orchid nutrition (Harley and Smith 1983; Perkins, 1995). Terrestrial orchids have fluctuations in symbiotic infection because they undergo a dormant period in their annual growth cycle where no nutrients are required but the fungus is still present in the surrounding litter and in underground tubers during dormancy in at least one *Caladenia* species (Huynh *et al*., 2004). New organs emerging the following season of active growth rely on reinfection by mycorrhizal fungi, including roots, collars and leaves (Jones 1988; Dixon 1991).

4.1.1 Fire, fire season and orchids

Fire results in charred tree stumps, cleared exposed soil, eroded ecosystems (Chuvieco, 1999) and sterilisation of the microflora of the soil, including mycorrhizal fungi, in the fire-affected depths of soil (Mataix-Solera *et al*., 2009). Fire changes the chemical composition of soil, in particular the concentrations of nitrogen (Anderson *et al*., 2004), phosphorus (Cade-Menun *et al*., 2000) and carbon (Fernandez *et al*., 1997).

Mycorrhizal fungi are located in the soil as natural flora and are affected by environmental and anthropogenic activities, including fire. Fire affects microbial and fungal activities that are essential for the efficient and healthy functionality of all ecosystems because it partially sterilises the soil (Prieto Fernandez *et al*., 1998). Several factors in fire that affect the presence and growth of mycorrhizal fungi include the temperature and severity of the fire (intensity and fire duration) (Certini, 2005) and gaseous smoke (Parmeter and Uhrenholdt, 1975). These factors are affected by the season of the fire and the amount of litter on and in the top layers of soil, with summer wildfires resulting in greater temperatures for longer durations. Prieto-Fernandez *et al*. (1998) found that immediately after a severe wildfire, microbial biomass (bacteria and fungi) was absent in the surface layer (0-5 cm) and reduced by 50% in the subsurface zone (5-10 cm) (reviewed (Certini, 2005)).
The orchid life cycle stage at which the fire occurs would be expected to affect the outcomes for both the orchid hosts and their mycorrhizal fungi. Fires that occur during the orchid’s dormancy period (usually in summer) might be expected to be less damaging to the subterranean orchid but the greater heat and smoke and the greater soil sterilising effect generated may be more deleterious to its mycorrhizal fungi. With the greater emphasis on fire management of orchids and ecosystems, including the increased demands for fuel-reduction prescribed burns, it is important to investigate the effects of burning at different seasons not only on the orchids themselves but also on the mycorrhizal fungi on which they depend. If the fungi must re-infect the orchid annually, their rate of growth after a burn may determine when and if the orchids that survive the burn are infected and establish symbiotic associations with their mycorrhizal fungi.

The different mycorrhizal fungi associated with different orchids may also react differently to fire and so affect the outcomes for their host orchids. The fungi normally associated with the three genera studied here are listed in Table 1 (Warcup, 1981) and typically have different growth rates. These different genera are likely to have different types of wood-rotting enzymes (discussed below) and so to be differentially affected by fire.

<table>
<thead>
<tr>
<th>Orchid</th>
<th>Mycorrhizal fungus species</th>
<th>Relative rate of growth</th>
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<tbody>
<tr>
<td>Glossodia major</td>
<td>Sebacina vermifera</td>
<td>Slow</td>
</tr>
<tr>
<td>Thelymitra pauciflora</td>
<td>Tulasnella sp.</td>
<td>Slow</td>
</tr>
<tr>
<td>Pterostylis revoluta</td>
<td>Ceratobasidium sp.</td>
<td>Fast</td>
</tr>
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**4.1.2 Wood, smoke and smoke water**

Wood is a major component of the carbon on and in the soil that is affected by fire and is composed mainly of cellulose, hemicelluloses and lignin. The thermal degradation of wood produces a complex mixture of compounds with antibacterial properties (Guillén & Manzanos, 1999a; Milly *et al.*, 2005; Wei, 2010) that reduce the growth of microorganisms(Clifford *et al.*, 1980; Maga, 1987; Montazeri *et al.*, 2013). These compounds include anhydroglucose, carbonyl-containing compounds and
furans from cellulose; anhydroglucose, carbonyl-containing compounds and furans, acetic acid and carbon dioxide from hemicellulose; and phenolic compounds from lignin (Miler & Sikorski., 1990). The antibacterial compounds also have fungal inhibition properties through the action of phenolic compounds and changes in pH from the acetic acid produced (Lin et al., 2012).

Smoke by-products can exist as three phases: gaseous, liquid or solids. These can be produced artificially and are used in industry to germinate seeds of many native Australian plants (Roche et al., 1998) and to reduce pathogenic microorganism growth, since they have antimicrobial properties (Lin et al., 2012). The structural effects of the fungi static and fungicidal activities of smoke water were observed using SEM on Pythium species and showed a loss of structural integrity, abnormal degradation, deformation, abnormal lysis, cytoplasmic leakage, and mycelial slimming (Lin et al., 2012).

Gaseous smoke can be native heated (500-600°C) or cooled to ambient temperature (22°C). Gaseous smoke is produced by burning plant material and the smoke is used directly (native heated) or passed through a stove pipe to cool to ambient temperature. Cooled gaseous smoke was fungicidal to Fusarium oxysporum (Alam et al., 2004) and Rhizoctonia solani, Fusarium solani and other soil-borne fungi (Zagory and Parmeter, 1984) in as little as 15 minutes exposure. No experiments have been conducted on native heated gaseous smoke which will have greater effect due to heat-sterilisation.

Liquid smoke can be liquid smoke condensate (LSC) or smoke water (SW) and mixed with the growth medium to determine the fungistatic and fungicidal effects. LSC is produced when the smoke condenses and settles (Toledo, 2007). SW is produced by igniting dry plant material in a stainless steel barrel, bubbling the compressed smoke air through a graduated cylinder filled with distilled water for 45 minutes and passing the liquid through filter paper (Boucher & Meets, 2004a; Lin et al., 2012). LSC was fungicidal to Mucor, Aspergillus and Penicillium species (Wolkowska & Lapszin, 1962), Rhizoctonia solani, Fusarium solani and other soil-borne fungi (Zagory and Parmeter, 1984). SW reduced the growth of Stromatini acepivopra (Tehranchian, 2011), Pythium species (Lin et al., 2012), L. lepideus and F. cajanderi (Zagory & Parmeter Jr, 1984). The effects of SW on fire-affected orchid
mycorrhizal fungi are unknown but would be expected to be deleterious and to vary with orchid genera because of the specificity of their associations with different mycorrhizal fungi.

4.1.3 Aims

This chapter investigated the effects of burn season and smoke water on the growth of orchid endophytic fungi isolated before and after burns in four seasons (spring, summer, autumn and winter) for three orchid species: (G. major, T. pauciflora and P. revoluta) in situ. There were three main research questions:

1. Was there a difference in fungal growth with burns at different seasons?
2. Was there a difference in fungal growth with concentration of smoke water?
3. Was there an interaction between burn season and smoke water, in that different fungi had different responses to smoke water?

4.2 Materials and Methods

Forty-five samples from pre-burn plots (3 species × 3 samples x 5 plots, 4 plots for each seasonal burn and 1 plot as a control) and 46 samples from postburn plots were collected from sites and species described previously in Chapters 2 and 3. Single pelotons were isolated and grown in fungal isolation medium (FIM) (Chapter 3, Table 3.1). Rhizoctonia-like cultures were cut into 5 mm diameter agar blocks and sub-cultured onto malt extract agar (MEA) medium in 9 cm diameter Petri plates (Oxoid, 17g/L malt extract agar and Gelita, 8g/L agar). Isolated fungi were labelled as in Chapter 3 in the following order: name of the plant (P=P. revoluta, G=G. major and T= T. pauciflora), treatment (burnt=B or unburnt=U), fungal collection year (1=2011, 2=2012), plot number (preburn / postburn: control=C, spring burn=Sp, summer burn=Sum, autumn burn=A, winter burn=W), plant number (1 to 3), fungal isolate (A to Z). For example, isolatePB2Sp2C was P. revoluta, burnt, 2012, Spring plot, plant 2 and isolate C. Plates were sealed using Parafilm and incubated at 25°C in darkness.

After 3 weeks, fungal isolates were selected systematically for different growth rates and 5 mm diameter mycelial blocks were sub-cultured on malt extract agar in Petri plates containing one of six dilutions of smoke water (Regen-2000®) at 0,
10, 100, 1000, 5000 and 10000 µL/L. This experiment was performed with three replicates for each isolate and each treatment. The radius (mm) of each mycelial colony was measured using electronic digital callipers at different days after subculturing depending on the rate of growth as follows: *P. revoluta* (6 days), *G. major* (10 days) and *T. pauciflora* (14 days).

Data were analysed using Minitab 16 statistical software. Significance was recorded at $p \leq 0.05$. Where data were normally distributed or could be normalised by transformation, the parametric Analysis of Variance (ANOVA) and regression analysis were used (the latter with indicator variables for fire treatments). The non-parametric Mood median test was used as well or instead for non-normalised distributions.

### 4.3 Results

The effects of the fire treatment and smoke water on growth of the fungal isolates were more generally similar for the two spring-flowering orchids (*G. major* and *T. pauciflora*) than either was to the autumn-flowering orchid (*P. revoluta*). A notable feature of the data was the almost universal significance of differences in growth among isolates from the same treatment in how their growth responded to smoke water. This is reported in more detail for each orchid species below.

Initially, the isolates were categorised by growth rate (Table 4.2) although subsequent analysis supported this division only for *G. major* (see individual orchids).

#### 4.3.1 Glossodia major

No plants emerged at flowering time after the winter burn and so no isolation or growth of fungi was possible.

#### 4.3.1.1 Without smoke water

The mean radius of fungal isolates ranged from 15-27 mm at 10 days on MEA (Figure 4.1). There were significant differences among fire treatments (ANOVA, $F=10.77$, $p<0.001$; Mood median test, Chi-Square = 36.55, $p<0.001$). The growth of fungi isolated pre-burn varied significantly with plot, from the control to the summer
burn. Growth of the 2012 (post-burn) control was greater than the 2011 (pre-burn) control. The effect of the burns was inconsistent; for the spring burn, growth after the burn was greater than before but the opposite was true for the summer burn and growth of isolates was unchanged after the autumn burn. The effect of the winter burn was qualitative (no plants or fungi) and so was not included in the analysis.

Table 4.2 Categories of fungi from Glossodia major, Thelymitra pauciflora and Pterostylis revoluta grown in malt extract media without smoke water for 6, 10 and 14 days respectively. Numbers are highlighted in red for the most frequent growth type and blue for equally frequent growth types. – No plants emerged and so no fungi were isolated. There were no fungi in the very slow category (<11 cm diameter) and so that column has been omitted.

<table>
<thead>
<tr>
<th>Orchid</th>
<th>Treatment</th>
<th>Detail</th>
<th>Slow (11-20 cm Diameter)</th>
<th>Fast (21-30 cm Diameter)</th>
<th>Very fast (31-40 cm diameter)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glossodia major</td>
<td>Control</td>
<td>2011</td>
<td>6</td>
<td>3</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>1</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>Pre-burn</td>
<td>7</td>
<td>2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-burn</td>
<td>0</td>
<td>9</td>
<td>9</td>
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</tr>
<tr>
<td></td>
<td>Summer</td>
<td>Pre-burn</td>
<td>3</td>
<td>6</td>
<td>9</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Post-burn</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Autumn</td>
<td>Pre-burn</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-burn</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>Pre-burn</td>
<td>0</td>
<td>8</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-burn</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Thelymitra pauciflora</td>
<td>Control</td>
<td>2011</td>
<td>7</td>
<td>2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Spring</td>
<td>Pre-burn</td>
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<td>Post-burn</td>
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<td>Summer</td>
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<td>Post-burn</td>
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<td>Autumn</td>
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<td>Post-burn</td>
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<td>Winter</td>
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<td></td>
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<td>Post-burn</td>
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<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Pterostylis revoluta</td>
<td>Control</td>
<td>2011</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>3</td>
<td>6</td>
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<tr>
<td></td>
<td>Spring</td>
<td>Pre-burn</td>
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<td>7</td>
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<td></td>
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<td>Post-burn</td>
<td>1</td>
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<td>9</td>
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<tr>
<td></td>
<td>Summer</td>
<td>Pre-burn</td>
<td>4</td>
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<td>Post-burn</td>
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<tr>
<td></td>
<td>Autumn</td>
<td>Pre-burn</td>
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<td>8</td>
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<tr>
<td></td>
<td></td>
<td>Pre-burn</td>
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<tr>
<td></td>
<td>Winter</td>
<td>Pre-burn</td>
<td>2</td>
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<td></td>
<td></td>
<td>Pre-burn</td>
<td>6</td>
<td>3</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>
4.3.1.2 With smoke water

Smoke water reduced the growth of all fungi in all treatments by up to 100% at the greatest concentration of 10000 µL L\(^{-1}\) (Figure 4.2). Excluding the winter data, for which there was no matching post-burn sample, there were significant effects in two-way ANOVA of fire treatment (F=2.88, P<0.001), smoke water concentration (F=116.5, p<0.001) and there was a significant interaction between fire treatment and smoke water concentration (F=1.98, p=0.001).

The growth inhibition by smoke water varied with fire treatment, but was greater in pre- than post-burn isolates. The spring and autumn isolates declined by 55-60% in pre-burn isolates compared with 44-45% in post-burn isolates. The controls behaved similarly, showing greater inhibition (74%) in pre-burn isolates but less (61%) in post-burn isolates. The effect on summer burn isolates was much greater than on those from other seasons, with decreases of up to 92% in pre-burn and up to

![Figure 4.1](image-url)
100% in post-burn isolates. This greater than average decrease was also shown by the pre-winter burn isolates (93%).

Growth inhibition by smoke water was dose-dependent. Smoke water concentrations of ≤100 µL L\(^{-1}\) had no significant effect but growth was reduced with ≥1000-5000 µL L\(^{-1}\) and was greatest at 10000 µL L\(^{-1}\). With pre-burn isolates, growth inhibition was greatest (up to 93%) with summer and winter burn isolates and least with spring and autumn burn isolates (55-60%). With post-burn isolates, growth inhibition showed greater variation and was greatest (100%) with summer burn isolates and least (44%) with autumn burn isolates.

### 4.3.1.3 Individual isolates

A striking feature of the data was that individual isolates within any treatment were significantly different by ANOVA in their responses to smoke water at \(p<0.001\) (Figure 4.3).

The radius of the fastest-growing isolates from any individual treatment was commonly 2-3 times that of the slowest. In some treatments the radius of the isolates overlapped between pre- and post-burn treatments, e.g. autumn burn, whereas in others the pre-burn isolates were mostly clustered and separate from the post-burn isolates, e.g. spring and summer burns. There was also evidence that the fungi without smoke water could be categorised into two groups of radius ≤20 cm and ≥21 cm (\(\chi^2 = 36.293\), \(p<0.001\)).

The treatments also varied in the responses of their individual isolates to smoke water. Within some treatments, isolates were uniformly sensitive, e.g. winter pre-burn, whereas within others isolates varied in sensitivity, e.g. summer pre-burn. The least concentration of smoke water at which growth was reduced varied in isolates within treatments, e.g. winter pre-burn.
Figure 4.2 Effect of smoke water, burn and season of burn on growth of fungi isolated from *Glossodia major* on malt extract agar after 10 days’ growth. a. Pre-burn sampling, b. post-burn sampling. Bars = 2xSE. LSD_{0.05} = 4.676.
Figure 4.3 Effect of smoke water on growth of individual fungal isolates (n=18) extracted from *Glossodia major* in 2011 and 2012 grown on MEA without and with smoke water. Green = 2011 fungal isolates (pre-burn) and red = 2012 fungal isolates (post-burn). The horizontal axis is non-linear to show the responses at the smallest concentrations clearly.
4.3.2 Thelymitra pauciflora

As only one plant emerged after the winter burn, only three isolates were tested.

4.3.2.1 Without smoke water

The mean radius of fungal isolates ranged from 16-24 mm at 14 days on MEA (Figure 4.4). There were significant differences among fire treatments by ANOVA (F=3.64, p=0.001) but not by the Mood median test ($\chi^2=14.67$, p=0.101), reflecting lesser differences than for G. major. The only significant difference between years was that the 2012 (post-burn) control was greater than the 2011 (pre-burn) control (post-burn). The growth of all other fire treatments did not change with the burn.

![Figure 4.4](image)

*Figure 4.4* Effect of burn and season of burn on growth of fungi isolated from *Thelymitra pauciflora* on malt extract agar after 14 days' growth. Bars = 2xSE. LSD$_{0.05}$ = 3.504. Means that do not share a letter are significantly different by Tukey's family error test at p=0.05.
4.3.2.2 With smoke water

Smoke water reduced the growth of all fungi in all treatments by up to 50% at the greatest concentration of 10000 µL L\(^{-1}\) (Figure 4.5). Excluding the winter sample (unbalanced number of replicates) there were significant effects in two-way ANOVA of fire treatment (F=13.26, P<0.001) and smoke water concentration (F=75.05, p<0.001) but there was no significant interaction between fire treatment and smoke water concentration (F=1.18, p=0.223).

The pattern of growth inhibition by smoke water was similar with all fire treatments and inhibition varied from 28-50% with the greatest smoke water concentration.

Growth inhibition by smoke water was dose-dependent. Smoke water concentrations of ≤1000 µL L\(^{-1}\) had no significant effect but growth was reduced with 5000 µL L\(^{-1}\) (except for the pre-summer burn isolates) and was greatest at 10000 µL L\(^{-1}\). With pre-burn isolates, growth inhibition was greatest (up to 47%) with spring burn isolates and least with summer burn isolates (32%). With post-burn isolates, growth inhibition was greatest (49%) with spring burn isolates and least (28%) with summer burn isolates.
Figure 4.5 Effect of smoke water, burn and season of burn on growth of fungi isolated from *Thelymitra pauciflora* on malt extract agar after 14 days' growth. a. Pre-burn sampling, b. post-burn sampling. Bars = 2xSE. LSD_{0.05} = 2.716.
4.3.2.3 Individual isolates

Apart from the post-winter burn isolates, individual isolates within any treatment varied significantly by ANOVA in their responses to smoke water at p<0.001 (Figure 4.6), though the differences were smaller than with G. major isolates.

The radius of the fastest-growing isolates from any individual treatment was commonly 1.5-2 times that of the slowest. In all treatments the radius of the isolates overlapped between pre- and post-burn treatments, e.g. autumn burn isolates. There was no evidence that the fungi without smoke water could be categorised into two groups of radius ≤20 cm and ≥21 cm ($\chi^2 = 8.650$, p<0.470).

The treatments also varied in the responses of their individual isolates to smoke water. Within some treatments, isolates were uniformly sensitive, e.g. spring pre-burn, whereas within others isolates varied in sensitivity, e.g. autumn pre-burn isolates. The least concentration of smoke water at which growth was reduced varied in isolates within treatments, e.g. control–pre-burn. In one treatment, there was no effect of even the greatest concentration of smoke water, e.g. winter post-burn isolates. In others, there was much variation in sensitivity of individual isolates, e.g. autumn post-burn isolates.
Figure 4.6 Effect of smoke water on growth of individual fungal isolates (n=18) extracted from *Thelymitra pauciflora* in 2011 and 2012 grown on MEA without and with smoke water. Green = 2011 fungal isolates (pre-burn) and red = 2012 fungal isolates (post-burn). N/A = not applicable, NS=not significant. The horizontal axis is non-linear to show the responses at the smallest concentrations clearly.
4.3.3 *Pterostylis revoluta*

4.3.3.1 Without smoke water

The mean radius of fungal isolates ranged from 18-27 mm at 6 days on MEA (Figure 4.7). Including all samples, there were significant differences among fire treatments by ANOVA ($F=3.88$, $p<0.001$; Mood median test ($\chi^2=17.11$, $p=0.047$), and lesser differences than for *G. major*. The only significant difference between years was that the growth of the 2012 winter sample was less than that of the 2011 sample (both unburnt). The growth of all other fire treatments was the same before and after the burn.

![Figure 4.7](image)

*Figure 4.7* Effect of burn and season of burn on growth of fungi isolated from *Pterostylis revoluta* on malt extract agar after 6 days' growth. Samples for spring and summer in 2011 and 2012 are pre- and post-burn respectively; all others are pre-burn. Bars = 2xSE. LSD0.05 = 4.429. Means that do not share a letter are significantly different by Tukey’s family error test at $p=0.05$. 
4.3.3.2 With smoke water

Smoke water reduced the growth of all fungi in all treatments by up to 44% at the greatest concentration of 10000 µL L⁻¹ (Figure 4.8). Excluding the autumn and winter data, for which there were no matching post-burn samples, there were significant effects in two-way ANOVA of fire treatment (F=20.37, P<0.001) and smoke water concentration (F=39.05, p<0.001) but there was no significant interaction between fire treatment and smoke water concentration (F=0.53, p=0.918).

The pattern of growth inhibition by smoke water varied with fire treatment and was 19-43% with the greatest smoke water concentration.

Growth inhibition by smoke water was dose-dependent. Smoke water concentrations of ≤1000 µL L⁻¹ had no significant effect but growth was reduced with 5000 µL L⁻¹ (except for the pre-summer burn isolates) and was greatest at 10000 µL L⁻¹.

There was greater variation among 2012 than among 2011 isolates. With pre-burn isolates, growth inhibition was greatest (39%) with summer burn isolates and least with control isolates (19%). With 2012 isolates, growth inhibition was greatest (43%) with winter unburnt isolates and least (22%) with summer post-burn isolates.
Figure 4.8 Effect of smoke water, burn and season of burn on growth of fungi isolated from *Pterostylis revoluta* on malt extract agar after 6 days' growth. a. 2011 sampling, b. 2012 sampling. Samples for spring and summer in 2011 and 2012 are pre- and post-burn respectively; all others are pre-burn. Bars = 2xSE. LSD 0.05 = 3.876.
4.3.3.3 Individual isolates

Except for the 2011 winter and the 2012 summer samples, the individual isolates within any treatment varied significantly by ANOVA in their responses to smoke water at p<0.001 (Figure 4.9).

The radius of the fastest-growing isolates from any individual treatment was commonly up to double that of the slowest. In some treatments the radius of the isolates overlapped between pre- and post-burn treatments, e.g. autumn and winter burns, whereas in the pre-summer burn sample the isolates were mostly clustered and separate from the post-burn isolates. Although in some samples there was an apparent grouping by growth rate, e.g. autumn and winter burns, there was no evidence that the fungi without smoke water could be categorised into two groups of radius ≤20 cm and ≥21 cm ($\chi^2= 11.354$, p<0.252).

The treatments also varied in the responses of their individual isolates to smoke water. Within some treatments, isolates were uniformly sensitive, e.g. autumn burn samples, whereas within others isolates varied in sensitivity, e.g. 2011 control. The least concentration of smoke water at which growth was reduced was 5000-10000 µL L$^{-1}$ and varied in isolates within treatments, e.g. winter pre-burn. In two treatments, there was no effect of even the greatest concentration of smoke water, e.g. summer post-burn and winter pre-burn isolates. In others, there was much variation in sensitivity of individual isolates, e.g. autumn post-burn isolates.
Figure 4.9 Effect of smoke water on growth of individual fungal isolates (n=18) extracted from Pterostylis revoluta in 2011 and 2012 grown on MEA without and with smoke water. Green = 2011 fungal isolates (pre-burn) and red = 2012 fungal isolates (post-burn for spring and summer). N/A = not applicable, NS=not significant. The horizontal axis is non-linear to show the responses at the smallest concentrations clearly.
4.3.4 Summary

The effects of the season of burn on the growth of fungi varied with the species of orchid from which they were isolated and are summarised in Table 4.3. The fungi isolated from *G. major* (especially those from the summer post-burn treatment) were more sensitive to the burns and to smoke water than those from the other two species, LC50 values were less.

**Table 4.3** Summary of effects of burns on *Glossodia major*, *Thelymitra pauciflora* and *Pterostylis revoluta*

<table>
<thead>
<tr>
<th>Orchid</th>
<th>Control (2012 vs 2011)</th>
<th>Season of burn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spring</td>
</tr>
<tr>
<td><em>Glossodia major</em></td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>(2011 SW LC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>7.8</td>
<td>9.6</td>
</tr>
<tr>
<td>(2012 SW LC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>&gt;10</td>
<td>2.3</td>
</tr>
<tr>
<td><em>Thelymitra pauciflora</em></td>
<td>↑</td>
<td>=</td>
</tr>
<tr>
<td>(2011 SW LC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>&gt;10</td>
<td>10</td>
</tr>
<tr>
<td>(2012 SW LC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td><em>Pterostylis revoluta</em></td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>(2011 SW LC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>(2012 SW LC&lt;sub&gt;50&lt;/sub&gt;)</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

↑ increase, ↓ decrease, = no change, - no data, *taking into account the reduction in orchid emergence and hence lack of fungi, # taking into account the increase in orchid emergence, smoke water LC<sub>50</sub> = concentration of smoke water (µL L<sup>-1</sup>/1000) at which growth was reduced by 50%.
4.4 Discussion

The enhancement or lack of effect of spring or autumn burns and the deleterious effects of summer or winter burns on the growth of these orchid endophytic fungi suggests that in general the best seasons for a prescribed burn were spring or autumn. The most deleterious seasons for spring-flowering orchids were winter or, for *G. major*, summer. This may be because the greater quantities of litter and the consequently greater temperatures of the burn result in greater quantities of the toxic metabolites from smoke water that inhibited the growth of these fungal isolates.

4.4.1 Effect of burn and burn season

The effect of fire on these orchid endophytic fungi varied depending on the orchid species and the season of burn. For the spring-flowering *G. major*, the growth of endophytic fungi was increased after a spring burn but decreased after a summer burn. For the spring-flowered *T. pauciflora* and the autumn-flowered *P. revoluta*, there was no significant effect of a burn at any season on fungal growth. There are no published studies of the effect of seasonal burns on orchid mycorrhizal fungi. Though Bruns et al. (2002), found that ectomycorrhizal fungi were rare or absent after a wildfire (summer burn), there was no study of the subsequent rates of growth of the fungi isolated.

The possible reason for the deleterious effect of a summer burn on the growth of fungi from *G. major* may be that in summer large amounts of dry and easily flammable litter accumulate on the ground and fire causes smoke that dissolves and produces smoke water in the soil. This is intensified when fire authorities use water to control the fire, so that smoke water spreads further and deeper into the ground. This may explain why the worst season for a prescribed burn was summer for *G. major*. More than 95% of wildfires have occurred in summer and therefore it is vital to have prescribed burns before or after summer, preferably in spring as that enhanced growth of fungi from *G. major*. 
4.4.2 Effect of smoke water

Orchid endophytic fungi are sensitive to smoke water, as shown by the significant reduction of growth by 19-100% at the greatest concentration of smoke water. This inhibition could be due to the phenolic and imidazole compounds present in smoke water (Chumpookam et al., 2012). Phenolic compounds can chelate transition metals and lower the reactivity of metal ions by forming inert metal-ligand complexes (Wong & Kitts, 2006) that alter the fungal cell wall (Ciafardini & Zullo, 2003; Lin et al., 2012). Imidazole compounds present in smoke water inhibit the growth of fungi by damaging their membranes at high concentrations (Sanglard et al., 1996; Jain et al., 2010). Scanning electron microscopy by Lin et al (2012) revealed that 3% (300 µL L⁻¹) smoke water caused structural integrity, abnormal degradation, deformation, abnormal lysis, cytoplasmic leakage and hyphal slimming to the mycelia of *Pythium* species, which are soil-borne pathogenic fungi. In nature after a burn or wildfire, smoke water produced in the soil increases phenols and Imidazole compounds, which increases the fungal toxicity to fungi and may reduce the growth of orchid mycorrhizal fungi and their re-colonisation of host orchids.

4.4.3 Effect of orchid and endophytic fungus

Different isolates of fungi may metabolise the compounds in smoke water in different ways, as the least significant reductions in growth were noted at different concentrations of smoke water. In *G. major* and *T. pauciflora*, fungal growth was significantly decreased at 1000 µLL⁻¹ compared with the 5000 µL L⁻¹ noted in *P. revoluta*. Also, >50% of *G. major* fungal isolates were completely inhibited by 10000 µL L⁻¹ of smoke water but none of the *P. revoluta* or *T. pauciflora* fungal isolates exceeded 50% mean inhibition. Furthermore, the LC50 values for growth of fungi from *G. major* were much less than those from the other orchids. Therefore, *G. major* fungal isolates were the least tolerant to smoke water.

This different strength of inhibition by different fungi suggests that they may differ qualitatively or quantitatively in enzymes capable of metabolising compounds in smoke to toxic metabolites. Lindberg (1949) used two mycorrhizal fungi and litter-decomposing fungi and found that fungi that did not have laccase (polyphenol
oxidase), e.g. brown rot fungi grew in the presence of phenolic acid (gallic acid) while those with laccase, e.g. white rot fungi, were strongly inhibited (Zagory and Parmeter, 1984). White rot fungi secrete laccase in the presence of phenols and the oxidised phenols (ortho-quinones) are more fungitoxic than the native reduced forms.

Some *Rhizoctonia*-like orchid mycorrhizal fungi, including *S. vermifera*, produce polyphenol oxidases, including laccase (Rasmussen, 1995; Zelmer et al., 1996). The activity may also vary with strain, in that cultures of *S. vermifera* isolated from four *Caladenia* species showed greater activity of laccase than those isolated from *Microtis uniflora*. The greater sensitivity of fungi from *G. major*, which would be expected to be *S. vermifera*, to smoke water than those from other orchids suggests that they have active laccase and that is the reason for their sensitivity. There is no information on laccase and other polyphenol oxidases in the fungi from the other two orchids (expected to be *Tulasnella* sp. from *T. pauciflora* and *C. cornigerum* from *P. revoluta*) but their lack of sensitivity to smoke water suggests that they do not have active enzymes capable of producing toxic metabolites from smoke water.

The different sensitivities of the fungi isolated from *G. major* at different seasons suggests that the activity of such enzymes varies seasonally and is greatest in summer and winter, as these fungi were more sensitive than those from other times. In summer, there may also be greater concentrations of smoke water left in the soil after a burn, which would be inhibitory to *S. vermifera* growth. This may also explain why *G. major* plants did not emerge from dormancy after the summer burn. If *S. vermifera* needs to re-invade the dormant tubers to activate growth and its growth is inhibited by smoke water, there may be insufficient growth to re-establish the orchid mycorrhiza and the orchid may not emerge from dormancy. Similar but lesser sensitivity may explain the reduced emergence of *T. pauciflora* after the summer burn. By contrast, the relative lack of sensitivity in fungi from *P. revolute* may mean that it outgrows the inhibited competing fungi and may explain the increased emergence of *P. revoluta* after fire.

In nature, fire changes the soil composition by smoke, fly ash and charcoal and the mycorrhizal fungi of *P. revoluta*, *G. major* and *T. pauciflora* may respond similarly as in these *ex-situ* experiments. The fungi from *P. revoluta* and *T.
pauciflora may have less polyphenol oxidase activity and so avoid the toxicity of oxidised phenolic acids from fire compared with fungi from G. major.

The large amount of variation among fungi within almost all treatments makes it difficult to predict how a given fungal isolate will behave from a study of the means and suggests that even within one orchid there is a difference in the relative activity of polyphenol oxidases in the fungi in the pelotons. This may reflect the substrate on which the fungus is feeding, as decomposition of wood and litter, which the fungi are believed to degrade, requires active polyphenol oxidases for complete digestion. Other authors have noted large variation in the morphology and efficacy of fungi from the same orchid (Rasmussen, 1995; Huynh et al., 2009) and this is another example. Exactly why the isolates are so diverse is puzzling and further research is desirable.

4.4.4 Conclusion

In this study, there was an impact on the isolated fungi by fire and a distinct correlation between the types of mycorrhizal fungi and their reactivity to smoke water. Future work examining the enzymatic activity on smoke water of different types of orchid mycorrhizal fungal would be useful to examine the importance of phenol metabolism in smoke water in the ability of the fungi to survive fire. Studies of the relative activities of laccase and similar polyphenol oxidases in these fungi may also help to predict outcomes from prescribed burning.
Chapter 5

Fungal diversity and differentiation
5.1 Introduction

Mycorrhizal fungi are diverse and important for the survival of orchids in situ for growth and development (Harley & Smith, 1983; Rasmussen, 1995). The specificity of this relationship is variable, ranging from low to high selectivity (Harley & Smith, 1983). Some orchids are associated with a wide range of fungi (McCormick et al., 2004; Jacquemyn et al., 2012; Tan et al., 2012) whilst others are only associated with fungi from the same orchid species from the same site (Wright, Magali et al., 2010); pelotons (McKendrick et al., 2002; Taylor et al., 2003); roots (Taylor et al., 2003); plants (Bougoure et al., 2005a); or populations (Chen et al., 2013). This may be further complicated by orchids that require different fungi at different developmental stages (Rasmussen, 1995; Sharma et al., 2003) with some protocorms able to survive without mycoheterotrophy (McCormick et al., 2006). The association with fungi can result from simultaneous or concurrent infections with one type of fungus (monogamous) or multiple types of fungi (polygamous) and is dependent on mycoflora availability in the soil. Fungal polygamy can be an advantage, particularly during times of environmental and ecological stress when specific fungi are limited or unavailable. For example, adult Goodyera pubescens was highly monogamous with Tulasnella fungi but switched to other types of Tulasnella during drought conditions even though the fungal infidelity resulted in high mortality for younger seedlings (McCormick et al., 2006).

Fire changes soil nutrient profiles with short term increases to nutrient availability, particularly of ammonium and phosphate (Schafer & Mack, 2010) that both mycoflora and plants utilise to re-establish. The impact of fire on mycoflora is devastating, with ectomycorrhizas eradicated (Bruns et al., 2002; Dahlberg, 2002) but can vary due to the mosaic patterns of fires (Stendell et al., 1999). It is assumed that fires affect orchid endophytes since orchid host plants are smaller and the number of flowering plants reduced following a fire (Coates & Duncan, 2009), which may be indicative of inferior or reduced mycoflora diversity and availability.

Seasonal fluctuations influence mycorrhizal dynamics in arbuscular mycorrhizae (AM). Fungal communities are highly sensitive and closely related to host phenology, climate variations and vegetation (Bentivenga & Hetrick, 1992; Rosendahl & Rosendahl, 1992; Sanders & Fitter, 1992; DeMars & Boerner, 1995; Allen, 1996).
Seasonal variations in spore density and their relationship to host phenology and water availability are well known in AM mycorrhiza (Ebbers et al., 1987; Gemma et al., 1989; Allen, 1991; Bentivenga & Hetrick, 1991; Rosendahl & Rosendahl, 1992; Sanders & Fitter, 1992; Blaszkowski, 1994; DeMars & Boerner, 1995; Sigüenza et al., 1996). The greatest AM spore density was found in the dry seasons (autumn and winter) of mountain grassland in Argentina and coincided with a lack of flowering and fruiting and the end of the growth season. The opposite was found in wet seasons (spring and summer) (Lugo & Cabello, 2002; Lugo et al., 2003). Some studies on arbuscular mycorrhizae found that intra- and extra- matrical mycelium increases during the rainy season, because spore germination is favoured and, as a result, mycorrhizal colonization increases and spore abundance decreases (Mason et al., 1992; Ragupathy & Mahadevan, 1993) but the effects on orchid mycorrhizal activity and density of seasonal changes are unknown.

The effect of seasonal fluctuations and their interaction with fire on fungal availability is unknown. In Victoria, Australia, autumn–winter is likely to be the most important period for the growth of spring-flowered orchid species. Increased moisture in April is thought to promote rapid development of leaf primordia, larger plants, greater tuber development and high mycorrhizal activity (Raleigh, 2005). Therefore, for the conservation of orchids, it seems logical to avoid burning when the orchid and their mycorrhizal fungi are actively growing. Most natural fires occur during summer when spring-flowering plants have completed active growth but whether or not this is the least damaging season for a burn to improve or maintain fungal communities conducive to orchid symbiosis is unknown. Using molecular methods to detect mycoflora diversity will improve our understanding of the baseline seasonal fungal variability and the impact of fire, which will lead to improved conservation efforts and fire management regimes.

This study examined the fungal populations associated with orchids that have different active growth seasons: autumn flowering (P. revoluta) and spring-flowering (G. major and T. pauciflora) plants pre-burn and post-burn. The aim was to investigate if fire, and specifically in what season, affected the fungal type and diversity based on qPCR peak melt temperatures and ITS-PCR restriction digests of fungi isolated from orchids.
5.2. Materials and Methods

5.2.1. Isolate details

The plan was to isolate fungi from three plant species, *P. revoluta*, *G. major* and *T. pauciflora* (chapter 3) pre-burn (2011) and post-burn (2012) comprising two fungal isolates from each of the plant in control, spring, summer, autumn and winter burn plots (3 plants × 2 isolates × 5 plots = total 30 isolates). Thirty fungal isolates from *P. revoluta*, *G. major* and *T. pauciflora* were used for pre-burn studies (2011) but the post-burn (2012) numbers varied according to the availability of plants. Fungi were initially isolated and grown in malt extract agar plates. Two isolates were systematically selected for growth in 60 mL liquid oatmeal in 200 mL glass jars in an orbital shaker at 20 rpm at 22°C. After 6-8 weeks, the fungi were prepared for DNA extraction, ITS-qPCR and RFLP digestion of the ITS product.

5.2.2. DNA extraction

Mycelia (100-125 mg) were harvested and ground to a fine powder using liquid nitrogen. DNA was extracted using a Favor Prep™ Plant Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer’s instructions. DNA extracted (total 100 µL) was quantified by gel electrophoresis as 2 µL of the DNA extract with 2 µL gel loading dye (Fermentas, Germany) and 2 µL sterile MilliQ® water against a GeneRuler™ 100bp DNA Ladder Mix (Fermentas, Germany) in 1.4% (w/v) agarose (Bioline, Australia) gel at 100 V for 1 h and post-staining with 50 ng/L ethidium bromide. Bands were visualized over ultraviolet light in a Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad, Australia). DNA was stored at -20°C until required.

5.2.3. qPCR amplification and melting temperature analysis

Two isolates were randomly selected from each plant of each species (*G. major*, *P. revoluta* and *T. pauciflora*) and treatment (unburnt, burnt and control). DNA samples were amplified using a Biorad Mini Opticon and a Qiagen Rotor-Gene qPCR with the universal primers (Table 5.1) targeting the ITS region of the nuclear ribosomal DNA (White *et al.*1990). Primer concentrations were optimised (1 µM, 2 µM, 4 µM and 6 µM) initially with each reaction containing 1 µL DNA (1 ng), 12.25 µL
Sybr Green mix (Bioline Sensimix, Australia) and made to 25 µL with DEPC-treated water. A negative control with water replacing the DNA was included in the reaction (NTC). Cycling parameters were as follows: 95°C for 10 min; 35 cycles of 95°C for 60 s, 55°C for 60 s and 72°C for 60 s. Temperature melt curve increments were optimised (0.02, 0.1 or 1°C) and used in subsequent reactions. Melting temperatures were rounded up to the nearest unit and scores were plotted using Minitab 16 software multivariate principal components analysis. Isolates that were further apart were considered as genetically diverse for fire treatment (pre-burn and post-burn) and the season of fire (autumn, spring, summer and winter) compared with pre-burn and unburnt controls.

Table 5.1 Primers and enzymes used for real-time polymerase chain reaction (qPCR) and restriction fragment length polymorphism (RFLP) analysis.

<table>
<thead>
<tr>
<th>Type</th>
<th>Label</th>
<th>Details</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>ITS1</td>
<td>Universal forward</td>
<td>TTCGTAGGTGAACCTGCGG</td>
</tr>
<tr>
<td>Primer</td>
<td>ITS4</td>
<td>Universal reverse</td>
<td>TCCTCCGCTTTATGGATATGC</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Hin61</td>
<td>24 h at 37°C</td>
<td>G↓CG↑C</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Taq1</td>
<td>24 h at 65°C</td>
<td>T↓CG↑A</td>
</tr>
</tbody>
</table>

5.2.4. ITS-PCR RFLP profiling and analysis

qPCR products were digested with two endonucleases (Hha1 and Taq1) (Fermentas Progen, Australia) which were four base-pair cutters (Table 5.1). Each reaction contained 10 U enzyme, 5 µL ITS-PCR product, 1 µL10× buffer and 10 µL sterile MilliQ® water. The solution was mixed by pipetting and incubated overnight at different temperatures (Table 5.1). Restriction digests were separated and visualised on 1.2% agarose gel containing 50 ng/L ethidium bromide and images captured for analysis as before. Restriction sizes were determined by reference to a GeneRuler™ as before. Only cleaved fragments totalling the uncleaved amplified product of ~600bp were included in the final analysis. Each fragment was scored as a binary value (presence or absence) and results were pooled to generate a score plot using principal components analysis in Minitab version 16. Isolates that were further apart were considered as genetically diverse for fire treatment (pre-burn and post-burn).
and season of fire (autumn, spring, summer and winter) compared with pre-burn and unburnt controls.

5.3. Results

5.3.1. DNA extraction and amplification

DNA extraction was successful for pre-burn and post-burn fungi from *G. major* and *P. revoluta* but unsuccessful for all 30 *T. pauciflora* isolates (Table 5.2). Fungi from *T. pauciflora* were regrown and DNA extraction was repeated but did not yield DNA and this was not pursued further due to time restrictions.

The optimum primer concentration for qPCR was 2 µM and the optimum temperature melt curve increments were 0.02°C and so these were used in subsequent amplifications. Amplification of DNA from the fungi isolated from *P. revoluta* and *G. major* produced a single PCR product of approximately 650 base pairs (bp) (Figure 5.1) for most isolates (Table 5.2). Differences in melting temperature and RFLP profiles were compared between pre-burn and post-burn isolates.
The Effect of Seasonal Burns on Australian Native Orchids

Figure 5. 1 Representative amplification of fungal isolates from G. major producing a single band at ~650 bp and quantified against GeneRuler ladder (lane 1)

Table 5. 2 DNA extraction, amplification and profiling of fungal isolates from three plant host species pre-burn and post-burn. NA=fungal extraction was unsuccessful.

<table>
<thead>
<tr>
<th>Step</th>
<th>G. major</th>
<th>P. revoluta</th>
<th>T. pauciflora*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-burn</td>
<td>Post-burn</td>
<td>Pre-burn</td>
</tr>
<tr>
<td>Extraction</td>
<td>30/30</td>
<td>24/30</td>
<td>28/30</td>
</tr>
<tr>
<td>Amplification</td>
<td>29/30</td>
<td>24/30</td>
<td>25/30</td>
</tr>
<tr>
<td>qPCR</td>
<td>29/30</td>
<td>24/30</td>
<td>26/30</td>
</tr>
<tr>
<td>RFLP Taq1</td>
<td>30/30</td>
<td>24/30</td>
<td>28/30</td>
</tr>
<tr>
<td>RFLP Hin61</td>
<td>30/30</td>
<td>24/30</td>
<td>28/30</td>
</tr>
</tbody>
</table>
5.3.2. qPCR peak melt temperature analysis of G. major isolates

There were three melting temperature peaks for pre-burn G. major isolates at 85.5, 86.5 and 87°C (Figure 5.2A) and two peaks for post-burn isolates at 86.5 and 87°C (Figure 5.2B). A principal components score plot based on peak melting temperatures resulted in three clusters (Figure 5.2C), with pre-burn autumn burn isolates the most different and represented in all three clusters.

The two largest clusters contained post-burn isolates from spring, summer and autumn burn plots that were the same as pre-burn and unburnt controls. The largest cluster (red box) contained a mixture of pre-burn (all five plots) and post-burn (control, spring, summer and autumn) but not winter post-burn as no plants emerged. Fungi from both pre-burn and post-burn plots, including controls, were present in this cluster.

The second largest cluster (green box) contained pre-burn isolates from all plots (control, autumn, winter and summer) except spring. Post-burn isolates in this cluster were only from autumn, spring and summer burn plots with no isolates from the control plot and no winter burn plants emerging.

The smallest cluster (black box) comprised two isolates from only the pre-burn autumn plot and these isolates were not represented in any other pre-burn or post-burn sites (control, spring or summer).
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Figure 5.2 qPCR melting temperature profiles of *G. major* fungal isolates in pre-burn (A) and post-burn (B) sites that were plotted using principal components analysis (C). No plants emerged in winter post-burn plots. Numbers indicate main peak patterns. Fungal isolates labelled as follows: 1. Orchid (g=*G. major*), 2. Treatment (u=pre-burn, b=post-burn), 3. Year of burn (1=2011, 2=2012), 4. Season of burn (c=control, a=autumn, sp=spring, su=summer, w=winter), 5. Plant replicate (1=plant 1, 2=plant 2, 3=plant 3), 6. Isolate identification (a-z).
5.3.3. RFLP analysis of G. major isolates

Digestion of amplified ITS-PCR products with Taq1 and Hin61 restriction enzymes produced different banding patterns for pre-burn and post-burn isolates (Figure 5.3 A-D). Multivariate analysis of the fragments produced score plots containing three main clusters, two groups and two closely similar isolates (Figure 5.4).

Pre-burn fungi (represented in green letters in Figure 5.4) were the most diverse with representative isolates in each category as individuals, groups and clusters. Post-burn fungi were restricted to only the two larger clusters (red and green boxes) and only autumn post-burn samples produced the same isolates as pre-burn and post-burn controls (green box).

The three main clusters contained different fungi based on fire treatment, with the smallest cluster (black box) containing only pre-burn isolates from autumn whilst the larger clusters (red and green boxes) contained mixtures of pre-burn and post-burn isolates. Post-burn isolates were exclusively in different clusters based on the season of burn, e.g. the red box contained all the spring and summer post-burn isolates and the green box contained all post-burn isolates from the autumn burn.

The same fungi were present in control plots pre-burn and post-burn (green box) and were similar for seasonal post-burn plots, e.g. autumn isolates. Isolates from the seasonal pre-burn plot were predominantly in the same cluster (green box) with controls. In contrast, post-burn isolates were different (red box) and clustered together for spring and summer post-burn plots.
Figure 5.3 RFLP patterns of G. major isolates from pre-burn (A) and post-burn (B) plots cleaved with Taq1 enzyme and pre-burn (C) and post-burn (D) plots cleaved with Hin61 enzyme. Fragments were measured against GeneRuler™ ladder (first lanes) with a negative control (last lane). Pre-burn isolates were control (C1D, C1J, C2C, C2E, C3A, C3D), spring (SP1A, SP1C, SP2E, SP2H, SP3I, SP3K), summer (SU1C, SU1E, SU2D, SU2G, SU3F, SU3H), autumn (A1L, A1N, A2A, A2D, A3C, A3X) and winter (W1D, W1S, W2A, W2L, W3P, W3T). Post-burn isolates were control (C1A, C1E, C2D, C2J, C3C, C3H), spring (SP1A, SP1C, SP2C, SP2E, SP3A, SP3H), summer (SU1E, SU1K, SU2S, SU2P, SU3A, SU3H) and autumn unburnt (A1C, A1X, A2G, A2H, A3A, A3C).
Figure 5.4 Multiple component analysis of RFLP patterns of *G. major* isolates from pre-burn and post-burn plots. Fungal isolates labelled as follows: 1. Orchid (g=*G. major*), 2. Treatment (u=pre-burn, b=post-burn), 3. Year of burn (1=2011, 2=2012), 4. Season of burn (c=control, a=autumn, sp=spring, su=summer, w=winter), 5. Plant replicate (1=plant 1, 2=plant 2, 3=plant 3), 6. Isolate identification (a-z).
5.3.4. qPCR melt temperature analysis of *P. revoluta* isolates

At the time of collection, only spring and summer sites had post-burn data and so autumn and winter isolates were considered as unburnt instead of post-burn (blue text). A total of 45 isolates from pre-burn and unburnt autumn and winter plots, and 12 post-burn spring and summer isolates was included for analysis. Peak melting temperatures ranged from 82 to 87°C for pre-burn and post-burn *P. revoluta* isolates (Figure 5.5 A-B). Both pre-burn and post-burn isolates had two main peaks but did not overlap in melt temperature with post-burn isolates, which melted at greater temperatures.

Principal components analysis of the peak temperatures produced four clusters (Figure 5.5C). Two contained mixtures of pre-burn and post-burn isolates (blue and yellow boxes), whilst the other clusters contained only pre-burn isolates (purple and red boxes). Fungal diversity was greater for pre-burn plots, with fungi represented as more variants (red box). Both spring and summer post-burn plots produced isolates that were similar to post-burn controls (yellow box) and were devoid of any pre-burn isolates.

The fungi present in pre-burn plots were absent in post-burn plots for autumn, spring and summer. Only the red box contained isolates from both years but these originated from different plots. Isolates in the purple clusters were all from 2011 while isolates from blue and gold clusters were all from 2012 irrespective of the burn season and also included post-burn controls. No cluster contained the same fungi from pre-burn and post-burn plots.
Figure 5.5 qPCR melting temperature profiles of *P. revoluta* fungal isolates from pre-burn (A) and post-burn (B) sites that were plotted using principal components analysis (C). Post-burn plots were incomplete: spring and summer plots were burnt, autumn and winter plots were unburnt at the time of collection (blue text). Fungal isolates labelled as follows: 1. Orchid (*p*=*P. revoluta*), 2. Treatment (*u*=pre-burn, *b*=post-burn), 3. Year of burn (*1*=2011, *2*=2012), 4. Season of burn (*c*=control, *a*=autumn, *sp*=spring, *su*=summer, *w*=winter), 5. Plant replicate (*1*=plant 1, *2*=plant 2, *3*=plant 3), 6. Isolate identification (a-z).
5.3.5. RFLP analysis of *P. revoluta* isolates

RFLP fragments were produced from both *Taq*1 and *Hin61* restriction enzymes (Figure 5.6) and principal components analysis produced three main clusters, five groups and twelve individuals (Figure 5.7). Fungal diversity was the greatest from pre-burn sites, with ten individual isolates compared with only two isolates from unburnt sites. There were no obvious patterns for individual isolates, with random distances from each other irrespective of treatment (unburnt vs. burnt) or season of fire.

The green cluster contained fungi from all pre-burn plots (control, autumn, spring, summer and winter) while the other two clusters contained combinations of both pre-burn and post-burn isolates. Pre-burn isolates were represented in the green and red clusters and post-burn isolates were represented in the black and red clusters. Spring post-burn isolates in the red cluster were the same as control post-burn isolates.

Summer post-burn isolates had the greatest diversity, with three isolates separated as individuals and three isolates in the black cluster. However, pre-burn isolates were different from those post-burn even though they were in the same cluster.
Figure 5.6 Patterns of *P. revoluta* isolates from pre-burn (A) and post-burn (B) plots cleaved with Taq1 enzyme and pre-burn (C) and post-burn (D) plots cleaved with Hin61 enzyme. Fragments were measured against GeneRuler™ ladder (first lanes). Pre-burn isolates were control (C1A, C1S, C2B, C2D, C3F, C2G), spring (SP1A, SP1J, SP2D, SP2K, SP3A, SP3B), summer (S1C, S1D, S2A, S2E, S3H, S3K), autumn (A1A, A1C, A2H, A3B) and winter (W1A, W1B, W2A, W2C, W3D). Post-burn isolates were control (C1K, C1L, C2D, C2J, C3D, C3F), spring (SP1A, SP1D, SP2C, SP2K, SP3N, SP3Y), summer (S1G, S1H, S2E, S2H, S3A, S3E), autumn unburnt (A1A, A1D, A2C, A2B, A3G, A3F) and winter unburnt (W1B, W1L, W2A, W2E, W3A, W3D).
Figure 5.7 Multiple component analysis of RFLP patterns of *P. revoluta* isolates from pre-burn and post-burn plots. Post-burn isolates from autumn and winter were unburnt and labelled as blue text. Fungal isolates labelled as follows: 1. Orchid (*p*=*P. revoluta*), 2. Treatment (*u*=pre-burn, *b*=post-burn), 3. Year of burn (1=2011, 2=2012), 4. Season of burn (*c*=control, *a*=autumn, *sp*=spring, *su*=summer, *w*=winter), 5. Plant replicate (1=plant 1, 2=plant 2, 3=plant 3), 6. Isolate identification (a-z).
5.4. Discussion

This is the first study to investigate the effect of seasonal burning on orchid mycorrhizal fungi compared with a field control as an internal standard. Fire changed the range of genotypes found and reduced fungal diversity compared with controls for both spring-flowering *G. major* and autumn-flowering *P. revoluta*. Despite the reduced fungal diversity, the least damaging season for a burn for fungi isolated from *G. major* and *P. revoluta* was based on two factors: greatest diversity and/or containing the same isolates as in the control. Results from this study suggest that the least damaging burn season was autumn for *G. major* and *P. revoluta*, when both were in leafing stages because both of these burn seasons produced post-burn fungi that were the same as from the controls.

5.4.1 Differences between *G. major* and *P. revoluta*

Fungal diversity was greater in isolates from *P. revoluta* than *G. major*, with more isolates outside clusters as individuals, and may reflect a greater degree of polygamous mycorrhizal relationships when fungi are not abundant or available. Since fires reduce fungal diversity (Wright & Tarrant, 1957; Vázquez *et al.*, 1993; Smith *et al.*, 2005) by sterilisation of the rhizosphere (Bruns *et al.*, 2002), particularly in frequent fire-prone habitats (Brundrett, 2007), orchids such as *P. revoluta* that exploit different genotypes of fungi may be able to (re-)establish in disturbed habitats and so expand their ranges and the size of their populations much more than those that have use fewer genotypes, such as *G. major*.

5.4.2 Effect of burns on fungal diversity

Fungal diversity was changed and reduced by fires, with pre-burn fungi from both *G. major* and *P. revoluta* having more clusters, groups and scattered individuals than post-burn fungi. Similar changes in fungal type, density and survival, particularly after high intensity fires, have been observed by others in ectomycorrhizal fungi, with up to 8-fold reductions in fungus biomass (Stendell *et al.*, 1999) and different pre-burn and post-burn fungi (Dahlberg, 2002). Smoke water contains large quantities of polyphenols and imidazole, which damage many components of fungal cells, including DNA. The new genotypes may be mutants of the original pre-burn fungi or
may be genotypes not isolated previously because of the sampling limited by the permit conditions. More extensive sampling may differentiate these possibilities.

The continued presence of the same fungi in successive years was only observed in *G. major* for pre-burn and post-burn controls and suggested persistence of the same fungus from year to year when undisturbed. The presence of the same fungi pre-burn and post-burn may indicate persistent reservoirs of mycorrhizal fungi in the soil or surrounding plants and lends support to the use of low-intensity fires to reduce fuel load but still maintain fungal dynamics to support native flora. The same fungi were also observed for autumn, spring and summer post-burn plots for *G. major*, but only in the qPCR melt temperature analysis that may not reflect single nucleotide mutations, not in the RFLP analysis, as discussed later.

Both orchids in this study associated with multiple fungal genotypes simultaneously but increased fidelity was noted post-burn, with both containing only two main clusters of fungi post-burn but *P. revoluta* having other scattered individual genotypes. The ability to switch fungal type according to what is available may be advantageous to *P. revoluta* as a strategy for increased distribution, population frequency and density. Other common orchids have also been noted as promiscuous, with many fungal partners (McCormick et al., 2006; Wright et al., 2010). The ability to exploit different fungi may result in the success of the plant host after fire, as observed with *P. revoluta*, which had more than four times greater plant numbers after fire than *G. major*. The differential survival of the pre-burn fungi and the appearance of new genotypes may be the key to the differing survival of their hosts after the burns.

### 5.4.2 Least damaging season for a burn

The season of least damage to fungal diversity coincided with host phenology for *G. major* (autumn) and *P. revoluta* (spring) respectively, when there was high soil moisture. The reason for this may be that low-intensity fires in wet seasons provide protection for seedlings, resulting in less damage than in senescent and dormant stages when moisture is low. The conservation of diversity is unlikely to be due to increased fungal activity (Sato et al., 2012) since fungi are active all year round with little correlation between mycorrhizal activity and leafing seasons (Rasmussen and
This is in contrast with AM fungi in grasses, which are host phenology-dependent and have optimal activity during seasons that favour the host growth (Bentivenga and Hetrick, 1992; Lugo and Cabello, 2002).

### 5.4.3 qPCR vs RFLP

Clustering was noted with fungi isolated from both orchids by both methods of analysis. qPCR and RFLP clustered most of the isolates similarly, with 70% agreement for isolates from *G. major* and 61% agreement for isolates from *P. revoluta*. The reasons for these discrepancies are the differences in how the methods work. RFLP analysis depends on differences in sequences whereas qPCR high resolution melt analysis depends on both changes in both sequence and length. Although the ITS size looked the same on gel electrophoresis, even a small difference in length would contribute a noticeable difference in melting temperature. Also, a change of one base to another of a similar type, e.g. the pyrimidines adenine and thymine, would change the RFLP type if it was within the target zone for an endonuclease but would not have a noticeable effect on the melting temperature. Sequencing of the ITS product would show which of these was responsible for the differences observed and was precluded by the time available, but should be performed in future. Also, the use of greater numbers of endonucleases may reveal and resolve the minority of discrepancies.

More importantly, both methods agreed in clustering most of the isolates and were feasible as complementary methods for analysing if there were genetic differences in fungal isolates before and after the burns, and so can be recommended for use in similar projects in future.
Chapter 6

Conclusion
The Orchidaceae is one of the most fascinating and species-rich of all plant families (Backhouse and Jeanes, 1995). Orchids depend on mycorrhizal fungi for their growth, nutrition and survival. This thesis studied the effects of seasonal burns on Australian native terrestrial orchids in order to find the least damaging season for a prescribed burn in two spring-flowered and one autumn-flowered species in common species of the genera Glossodia, Pterostylis and Thelymitra and their associated mycorrhizal fungi in the genera Sebacina, Ceratobasidium and Tulasnella respectively. This is because bush fires are an inevitable occurrence in Australia; about 50 million hectares of land are burned across Australia each year and fire is common in the seasonally dry parts of the continent. Prescribed burning is used to reduce the fire risk by reducing the fuel load. The use of prescribed burning is controversial and more information is needed to provide guidelines for effective fuel reduction for the conservation of ecosystems, especially of terrestrial orchids due to their endangered nature. This thesis aimed to contribute to this information and to test the reliability of common beliefs about the benefits of fire for orchids and their mycorrhizal fungi.

6.1. The effect of seasonal fire on plants response of G. major, T. pauciflora and P. revoluta

When considering plant responses, the best (least damaging) season for a burn varied considerably for each species. For the spring-flowered G. major it was spring and for T. pauciflora it was summer followed by spring, whereas for the autumn-flowered P. revoluta it was winter followed by spring. For all three species, with their different annual growth cycles, the best (least damaging) season for a burn was after seed dispersal (normally 2 months after flowering), when the plants are in the dormant stage with no above-ground parts present and when the maximum carbohydrate reserves are in the new fully formed underground tubers. The possibility of re-sprouting is related to carbohydrate reserves in the root (Smith 1965). The season of the burn affects carbohydrate reserves; therefore the timing of the burn is critical for plants to re-sprout (Butterly, Bentley & Plumb 1959, Jones and Laude 1960). Therefore, there was a distinct correlation between the season of the burn and the stage of development of individual orchid plants. It is frequently stated that late spring to summer burns are beneficial to spring-flowering orchids (TSU-
Threatened Species Unit, 2006; Keith, 1996; Kubiak, 2009) which agrees with this study’s results as the least damaging seasons.

The significant increase in *P. revoluta* plant number after winter, spring and summer seasonal burns may be due to the effects of smoke water rather than to the phenology stages reached. Smoke water has many stimulant effects, including seed germination, in many Australian species, e.g. Dixon et al. (1995) and McLean (1999), *Epacris impressa*, smoke water may have stimulated dormant tubers to emerge, seeds to germinate or protocorms to develop into leafy plants; this effect would depend on the amount of rainfall after the fire since fungi from *P. revoluta* were tolerant of smoke water, even at high concentrations.

6.2. The effect of seasonal fire on isolation of organisms from *G. major*, *T. pauciflora* and *P. revoluta*

The effect of fire on isolation of *Rhizoctonia*-like fungi was different for different genera, with decreases for fungi from *G. major* and *T. pauciflora* but increases for fungi from *P. revoluta* from spring and summer burns. For *G. major* and *T. pauciflora*, fire at all seasons (spring, summer, autumn and winter) significantly decreased the isolation of *Rhizoctonia*-like fungi from post-burn compared with pre-burn samples. This could be explained by other studies that have found overall reductions of the soil fungal population and growth (Renbuss et al., 1973) after a burn compared with unburnt soil irrespective of season and moisture levels (Saravanan et al., 2013b). For these reasons, post-burn mycorrhizal fungi re-infection may be reduced in the host orchids. By contrast, the isolation of *Rhizoctonia*-like fungi from *P. revoluta* flowering plants was significantly greater post-burn (spring and summer burns) compared with the unburnt control. Re-colonisation of orchid mycorrhizal fungi after a burn has not previously been documented in the literature. These results suggest that the fast-growing *Ceratobasidium* mycorrhizal fungi in *P. revoluta* colonised the site quickly from its reservoirs as the numbers of *Rhizoctonia*-like fungi isolated after spring and summer burns were greater than before the burns.

The best season for a fire to isolate fungi from *G. major* and *T. pauciflora* was autumn. The worst season was winter because there were few or no plants available after fire. Autumn was the leafing stage of *G. major* and *T. pauciflora*, which had a
greater proportion of *Rhizoctonia*-like fungi than the later developmental phases of flowering, fruiting, senescing and dormancy (Huynh *et al*., 2009). Fire at this stage might have reserved the active healthy fungi (intact) in its protected reservoirs of roots or tubers (5 cm below the soil). The best season to isolate fungi from *P. revoluta* was summer followed by spring (only spring and summer burns were conducted). Similarly, winter was the leafing stage for both orchids, when it had a large amount of *Rhizoctonia*-like fungi in its reservoirs. These results suggest that the relatively slow-growing *Sebacina* and *Tulasnella* mycorrhizal fungi in *G. major* and *T. pauciflora* respectively had only restricted survival and colonisation after fire.

The isolation of bacteria from *G. major* and *T. pauciflora* flowering plants increased after all seasonal burns (spring, summer, autumn and winter) compared with pre-burn. Similarly, some previous studies have indicated that fungal communities are more sensitive to fire than their bacterial counterparts (Raison, 1979; Vazquez *et al*., 1993; Baath *et al*., 1995; Bergner *et al*., 2004; Palese *et al*., 2004; Saravanan *et al*., 2013). By contrast, the isolation of bacteria from *P. revoluta* decreased post-burn (2012) compared with pre-burn (2011). This may be an artefact, either of the plant growth cycle or the weather. At the time of the pre-burn collections, *P. revoluta* was flowering whereas after the burn it was vegetative and is discussed in detail below. Also, during the post-burn sample collection period (January to March – 2012), the rainfall was reduced by more than half compared with 2011 and a wet or moist soil encourages bacteria growth (Wilkinson *et al*., 1989; Tsavkelova *et al*., 2003). Further research is needed to clarify if phenology or rainfall is responsible for this change.

A greater proportion of *Rhizoctonia*-like fungi was isolated from *P. revoluta* vegetative plants than flowering plants, suggesting that the orchid developmental phase affected the isolation of *Rhizoctonia*-like fungi. Similarly, larger proportions of *Rhizoctonia*-like fungi were isolated in leafing and budding stages than in later developmental phases of flowering, fruiting, senescing and dormancy in *Caladenia formosa* (Huynh *et al*., 2009). The decreased isolation of *Rhizoctonia*-like fungi from pelotons of flowering plants corresponded to large numbers of collapsed pelotons observed by SEM in flowering plants. This may be due to the interaction of orchid phenology, climate and mycorrhizal activity (Raleigh, 2005). The wet season in
Victoria and the leafing stage for *G. major* was autumn, where mycorrhizal activity is extensive in the orchid plant. For this reason, burning in autumn may have protected and reserved the fungi in underground organs of the orchid, suggesting that fungal patterns are related to host phenology and climate variation (Bentivenga and Hetrick, 1992, Rosendahl and Rosendahl, 1992, Sanders and Fitter 1992b, De Mars and Boerner 1995, Allen 1996, Lugo and Cabello 2002).

There was no effect of fire on the proportion of pelotons that failed to grow, except in *T. pauciflora* (autumn burn) and *P. revoluta* (summer burn). This lack of growth was possibly due to collapsed/dead fungi (Huynh *et al.*, 2004), a high proportion of which were seen by scanning electron microscopy (SEM) in flowering plants of *P. revoluta*. There were no other fungi isolated from pre-burn *G. major* and *T. pauciflora* but post-burn 2% of common soil genera such as *Fusarium* and *Trichoderma* were isolated. Further research is needed to elucidate if this is a common pattern after fire.

### 6.3. The effect of fire and burn season on the growth of orchid endophytic fungi

The effect of fire on orchid endophytic fungal growth varied depending on the orchid species and the burn season. For the spring-flowering *G. major*, the growth rate of endophytic fungi increased after the spring burn but decreased after the summer burn. By contrast, for fungi from the spring-flowered *T. pauciflora* and the autumn-flowered *P. revoluta*, there was no significant effect of a burn at any season on rate of growth. *P. revoluta* associates primarily with *Ceratobasidium*, which is able to utilize nitrate from ash after a burn and supply to the orchid via mycotrophy, which may explain the no significant effect of *P. revoluta* fungal growth from fire. The possible reason for the drastic effect of a summer burn on the growth of fungi from *G. major* may relate to the relative sensitivity of the orchid mycorrhizal fungi to compounds generated during the burn. In summer large amounts of dry and easily flammable litter accumulate on the ground and fire causes smoke that dissolves and produces smoke water in the soil. This is intensified when fire authorities use water to control the fire, so that smoke water spreads further and deeper into the ground. This may explain why the worst season for a prescribed burn on *G. major* fungal growth was summer and why it was more drastically affected than *P. revoluta*. 
The orchid endophytic fungi were sensitive to smoke water, as shown by the significant reduction in growth by the relatively small concentrations used here. This inhibition may be due to the phenolic and imidazole compounds present in smoke water, which damage the fungal cell wall and membrane. (Chumpookam et al., 2012; Sanglard et al., 1996; Jain et al., 2010).

This different strength of inhibition by different fungi suggests that they may differ qualitatively or quantitatively in enzymes (laccase) capable of metabolising compounds in smoke to toxic metabolites (Zagory and Parmeter, 1984). Some Rhizoctonia-like orchid mycorrhizal fungi, including S. vermifera, produce polyphenol oxidases, including laccase, and the activity may also vary with strain (Rasmussen, 1995; Zelmer et al., 1996). The different sensitivities of the fungi isolated from G. major at different seasons suggest that the activity of such enzymes varies seasonally and is greatest in summer. In summer, there may also be greater concentrations of smoke water left in the soil after a burn, which would be more inhibitory to S. vermifera growth. This may also explain the reduction in the G. major number of plants after the summer burn. If S. vermifera needs to re-invade the dormant tubers to activate growth and its growth is inhibited by smoke water, there may be insufficient growth to re-establish the orchid mycorrhiza and the orchid may have reduced emergence from dormancy. By contrast, the relative lack of sensitivity in fungi from P. revoluta may mean that it outgrows the competing inhibited fungi and may explain the increased emergence of P. revoluta after fire.

6.4. The effect of seasonal fire on the genetic diversity of Rhizoctonia-like fungi isolated from G. major, T. pauciflora and P. revoluta

Pterostylis revoluta plants were polygamous (simultaneously associated with multiple fungal strains with different genotypes) and were infected by different fungal strains in different seasons and after fire. In P. revoluta, fungal genotypes in 2011 were absent in 2012 irrespective of the post-burn season (spring and summer) or treatment (unburnt or burnt), and new fungal genotypes were introduced in 2012. After spring and summer burns, the dominant fungi pre-burn switched to genetically and phenotypically different types. These changes may have been caused by
damage to the fungi by the compounds in smoke water, such as damage to the cell walls, membranes and possibly also DNA (Shukla and Mishra, 2009; Bolhuis and Aldrich-Wright, 2014).

The diversity of G. major orchid endophytic fungi after the autumn burn was the same as in pre-burn plots but after summer burns the genetic diversity of Rhizoctonia-like fungi decreased. In G. major, there were both monogamous (fungal isolates had only one genotype) and polygamous plants, but the majority of the pre-burn plants (60%) and all the post-burn plants were monogamous suggesting that some fungi were persistent if undisturbed irrespective of season. This may have contributed to the greater effects of the burns on G. major than P. revoluta, as discussed below.

P. revoluta survived fire well by exploiting its promiscuous associations with multiple fungi and may contribute to its abundant population. By contrast, in G. major there were both monogamous (fungal isolates had only one genotype) and polygamous plants, but the majority of the pre-burn plants (60%) and all the post-burn plants were monogamous. If G. major is more restricted in its ability to form functional mycorrhizae with the fungi present, and those specific fungi are more drastically affected by fire and the resulting smoke water than those from other orchids, this reduces its chances of survival after fire. These results have important implications for plant responses to severe climatic events or to more gradual environmental changes such as global warming and the increased fire risk. Further research is needed to investigate the extent to which these results are common to other orchids.

6.5. Overall conclusion - choice of season for prescribed burning

This thesis has reported on the first critically planned experimentation on the effects of seasonal burning on three common terrestrial orchids in dry sclerophyll forest (open woodland) in Victoria and has shown the following for the first time:

1. Fire was not universally beneficial to orchid survival
2. Fire affected the ability to isolate orchid mycorrhizal fungi
3. Orchid mycorrhizal fungi differed in their ability to grow after fire
4. Orchid mycorrhizal fungi differed in their sensitivity to smoke water

5. The genetic diversity of orchid mycorrhizal fungi changed after the burn

6. The contrasting effects of fire on the orchid mycorrhizal fungi were similar to the contrasting effects of fire on the survival of their host orchids.

The main aim of this thesis was to find the best (least damaging) season for prescribed burning in this habitat, which is typical of those in which many Australian terrestrial orchids are found. Clearly a compromise is needed for conservation of all three orchids. According to plant responses, for the spring-flowered orchids, the best season to burn was late spring (\textit{G. major})-summer (\textit{T. pauciflora}), while the best season to burn for \textit{P. revoluta} was winter-spring (Table 6.1). According to the growth of fungi from \textit{G. major} and \textit{P. revoluta}, the least damaging season was spring and there was no effect of any seasonal burns on the growth of fungi from \textit{T. pauciflora} (Table 6.1). Therefore, late spring is the best compromise for a burn on a site inhabited by all three orchids.

\textbf{Table 6.1} Summary of least affected seasons for plant and fungal responses.

<table>
<thead>
<tr>
<th></th>
<th>\textit{G. major}</th>
<th>\textit{T. pauciflora}</th>
<th>\textit{P. revoluta}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant growth</td>
<td>Spring/Summer</td>
<td>Summer/Summer</td>
<td>Winter/Summer</td>
</tr>
<tr>
<td>Fungal isolation</td>
<td>Autumn/Spring</td>
<td>Autumn</td>
<td>Summer/Spring</td>
</tr>
<tr>
<td>Fungal growth</td>
<td>Spring</td>
<td>No effect</td>
<td>Summer/Spring</td>
</tr>
<tr>
<td>Fungal diversity</td>
<td>Autumn</td>
<td>Pre-burn</td>
<td>Pre-burn</td>
</tr>
</tbody>
</table>
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The Effect of Seasonal Burns on Australian Native Orchids


The Effect of Seasonal Burns on Australian Native Orchids


The Effect of Seasonal Burns on Australian Native Orchids


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Appendices
Appendix 2.1. Total number of *P. revoluta*, *G. major* and *T. pauciflora* plants in control, spring, summer, autumn and winter plots pre-burn (2011) and post-burn, (2012 / 2013), control plot with no burn.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-burn</th>
<th></th>
<th></th>
<th>Post-burn</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Pterostylis revoluta</em></td>
<td><em>Glossodia major</em></td>
<td><em>Thelymitra</em></td>
<td></td>
<td><em>Pterostylis revoluta</em></td>
<td><em>Glossodia major</em></td>
</tr>
<tr>
<td>Control A</td>
<td>20</td>
<td>58</td>
<td>4</td>
<td>23</td>
<td>39</td>
<td>4</td>
</tr>
<tr>
<td>Control B</td>
<td>21</td>
<td>47</td>
<td>3</td>
<td>28</td>
<td>41</td>
<td>3</td>
</tr>
<tr>
<td>Control C</td>
<td>20</td>
<td>45</td>
<td>4</td>
<td>10</td>
<td>29</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>150</td>
<td>11</td>
<td>61</td>
<td>109</td>
<td>10</td>
</tr>
<tr>
<td>Spring A</td>
<td>30</td>
<td>19</td>
<td>3</td>
<td>96</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Spring B</td>
<td>26</td>
<td>11</td>
<td>4</td>
<td>65</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Spring C</td>
<td>48</td>
<td>6</td>
<td>3</td>
<td>64</td>
<td>12</td>
<td>2</td>
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<td>225</td>
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<tr>
<td>Summer</td>
<td>27</td>
<td>6</td>
<td>3</td>
<td>50</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
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<td>11</td>
<td>4</td>
<td>30</td>
<td>8</td>
<td>4</td>
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<td>29</td>
<td>10</td>
<td>150</td>
<td>18</td>
<td>12</td>
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<td>Autumn A</td>
<td>17</td>
<td>36</td>
<td>6</td>
<td>23</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Autumn B</td>
<td>12</td>
<td>43</td>
<td>4</td>
<td>14</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Autumn C</td>
<td>21</td>
<td>33</td>
<td>4</td>
<td>20</td>
<td>8</td>
<td>2</td>
</tr>
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<td>112</td>
<td>14</td>
<td>57</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>Winter A</td>
<td>35</td>
<td>10</td>
<td>6</td>
<td>130</td>
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</tr>
<tr>
<td>Winter B</td>
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<td>8</td>
<td>188</td>
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<tr>
<td>Winter C</td>
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<td>4</td>
<td>145</td>
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<td>Total</td>
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<td>18</td>
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<tr>
<td>Grand</td>
<td>379</td>
<td>357</td>
<td>63</td>
<td>956</td>
<td>187</td>
<td>37</td>
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</table>
Appendix 2.2. Summary of ANOVA statistical analysis on the effect of fire pre-burn and post-burn for *G. major* from control, spring, summer, autumn and winter plots and plant number and plant measurements.

<table>
<thead>
<tr>
<th>Plot</th>
<th>Factor (pre-burn &amp; post-burn)</th>
<th>p value</th>
<th>Significance</th>
<th>Test</th>
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<tr>
<td>Control</td>
<td>Number of flowering plants</td>
<td>0.235</td>
<td>NS</td>
<td>T-test</td>
</tr>
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<td></td>
<td>Number of vegetative plants</td>
<td>0.243</td>
<td>NS</td>
<td>T-test</td>
</tr>
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<td>Number of flowering plants</td>
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<td>NS</td>
<td>T-test</td>
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<td></td>
<td>Number of vegetative plants</td>
<td>0.667</td>
<td>NS</td>
<td>T-test</td>
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<td>Summer</td>
<td>Number of flowering plants</td>
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<td>NS</td>
<td>T-test</td>
</tr>
<tr>
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<td>0.822</td>
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<td>Autumn</td>
<td>Number of flowering plants</td>
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<td>SD</td>
<td>T-test</td>
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<td></td>
<td>Number of vegetative plants</td>
<td>0.005</td>
<td>SD</td>
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<td>Winter</td>
<td>Number of flowering plants</td>
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<td>SD</td>
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<td>KW</td>
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<td>NS</td>
<td>T-test</td>
</tr>
<tr>
<td>Control</td>
<td>Width of stem (*× 50)</td>
<td>0.046</td>
<td>SD</td>
<td>KW</td>
</tr>
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<td>Width of leaf</td>
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<td>SD</td>
<td>T-test</td>
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<td>Length of leaf</td>
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<td>SD</td>
<td>T-test</td>
</tr>
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<td>Width of flower</td>
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<td>T-test</td>
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<tr>
<td>Summer</td>
<td>Plant height</td>
<td>0.061</td>
<td>NS</td>
<td>T-test</td>
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<td>Width of stem (*× 50)</td>
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<td>NS</td>
<td>T-test</td>
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<td>SD</td>
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<td>Plant height</td>
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</tr>
<tr>
<td>Winter</td>
<td>Width of stem (*× 50)</td>
<td>0.033</td>
<td>SD</td>
<td>T-test</td>
</tr>
<tr>
<td>Winter</td>
<td>Width of leaf</td>
<td>0.05</td>
<td>SD</td>
<td>KW</td>
</tr>
<tr>
<td>Winter</td>
<td>Length of leaf</td>
<td>0.05</td>
<td>SD</td>
<td>KW</td>
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<td>SD</td>
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</tr>
<tr>
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Appendix 2.3. Summary of statistical analysis on the effect of fire pre-burn and post-burn for *T. pauciflora* from control, spring, summer, autumn and winter plots and plant number and plant measurements.

<table>
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<tr>
<th>Factor (pre-burn &amp; post-burn)</th>
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<th>Test</th>
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</thead>
<tbody>
<tr>
<td>Control Number of flowering plants</td>
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</tr>
<tr>
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<td>SD</td>
<td>T-test</td>
</tr>
<tr>
<td>Winter Number of flowering plants</td>
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<td>T-test</td>
</tr>
<tr>
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<tr>
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</tr>
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<td>T-test</td>
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<td>Spring Width of stem (*× 50)</td>
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<td>Spring Length of flower</td>
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<td>T-test</td>
</tr>
<tr>
<td>Spring No. of buds</td>
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<td>T-test</td>
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<td>KW</td>
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**Appendix 2.4.** Summary of statistical analysis on the effect of fire pre-burn and post-burn for *P. revoluta* from control, spring, summer, autumn and winter plots and plant number and plant measurements.

<table>
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<th>Plot</th>
<th>Factor (pre-burn &amp; post-burn)</th>
<th>p value</th>
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<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
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<td>SD</td>
<td>T-test</td>
</tr>
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<td>Control</td>
<td>Number of vegetative plants</td>
<td>0.002</td>
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</tr>
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<td>T-test</td>
</tr>
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<td>Number of vegetative plants</td>
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<td>T-test</td>
</tr>
<tr>
<td>Summer</td>
<td>Number of flowering plants</td>
<td>0.015</td>
<td>SD</td>
<td>T-test</td>
</tr>
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<td>Summer</td>
<td>Number of vegetative plants</td>
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<td>KW</td>
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<td>0.037</td>
<td>SD</td>
<td>T-test</td>
</tr>
<tr>
<td>Autumn</td>
<td>Number of vegetative plants</td>
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<td>NS</td>
<td>T-test</td>
</tr>
<tr>
<td>Winter</td>
<td>Number of flowering plants</td>
<td>0.046</td>
<td>SD</td>
<td>T-test</td>
</tr>
<tr>
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<td>Number of vegetative plants</td>
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<td>Plant height</td>
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<td>Width of stem (<em>× 50</em>)</td>
<td>0.05</td>
<td>SD</td>
<td>KW</td>
</tr>
<tr>
<td>Control</td>
<td>Width of flower</td>
<td>0.037</td>
<td>SD</td>
<td>T-test</td>
</tr>
<tr>
<td>Control</td>
<td>Length of flower</td>
<td>0.011</td>
<td>SD</td>
<td>T-test</td>
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<td>Spring</td>
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<td>SD</td>
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<td>Width of stem (<em>× 50</em>)</td>
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<td>Plant height</td>
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<td>SD</td>
<td>KW</td>
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<td>Width of stem (<em>× 50</em>)</td>
<td>0.046</td>
<td>SD</td>
<td>KW</td>
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<tr>
<td>Summer</td>
<td>Width of flower</td>
<td>0.046</td>
<td>SD</td>
<td>KW</td>
</tr>
<tr>
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<td>Plant height</td>
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<tr>
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<td>Width of stem (<em>× 50</em>)</td>
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<td>SD</td>
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<td>SD</td>
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<td>KW</td>
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<td>KW</td>
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<td>Winter</td>
<td>Width of flower</td>
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<td>KW</td>
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<tr>
<td>Winter</td>
<td>Length of flower</td>
<td>0.037</td>
<td>SD</td>
<td>KW</td>
</tr>
</tbody>
</table>
Appendix 3.1: Preparation of Fungal Isolation Medium

NaNO₃  0.3g
KH₂PO₄  0.2g
MgSO₄.7H₂O  0.1g
KCl  0.1g
Yeast extract  0.1g
Sucrose  5.0g
Agar (Powder)  10g
Distilled water  1L

Adjust pH to 4.5-5.0 before adding agar. Then add agar and melt before autoclaving at 121 °C for 15 minutes.
**Appendix 5.1; Protocol for DNA extraction using Favor Prep™ Plant Genomic DNA Extraction Mini Kit (Favorgen, Taiwan).**

**Step 1 – Tissue Dissociation**

- Cut off 500 mg (up to 100 mg) of fresh or frozen plant tissue or 5 mg (up to 100 mg) of dried sample.
- Grind the sample under liquid nitrogen to a fine powder. Transfer it to a microcentrifuge tube.

**Step 2 – Lysis**

- Add 400 µl FAPG1 Buffer and 8 µl RNase A(50 mg/l) into the sample tube and mix by vortexing.
- Incubate at 65 °C for 10 minutes. During incubation, invert the tube every 5 minutes. At the same time preheat required elusion buffer (200 µl per sample) at 65 °C.
- Add 130 µl of FAG2 buffer and mix by vortexing.
- Incubate in ice for 5 minutes.
- Place a filter column in a 2 ml collection tube.
- Apply the mixture from previous step to the Filter Column. Centrifuge for 3 minutes at full speed (13,000 rpm)
- Discard the filter column and carefully transfer clarified supernatant in Collection tube to a new microcentrifuge tube.

**Step 3 – DNA binding**

- Add 1.5 volumes of FAPG3 buffer (ethanol added) to the cleared lysate and mix immediately by vortexing for 5 seconds. For example, add 750 µl FAPG3 Buffer to 500 µl lysate.
- Place a FAPG column in a 2ml collection tube.
- Apply 750 µl mixture from previous step to the FAPG column.
- Centrifuge at full speed (13,000 rpm) for 2 minutes.
- Discard flow through in the Collection tube.
Step 4 – Wash

- Add 500 µl of W1 Buffer (ethanol added) into to the column.
- Add 750 µl of Wash Buffer (ethanol added) into to the column.
- Centrifuge at full speed (13,000 rpm) for 3 minutes to dry the column matrix.

Step 5 – DNA elusion

- Transfer dried FAPG column into a clean 1.5 ml microcentrifuge tube.
- Add 50-200 µl of preheated Elusion Buffer into the centre of the column matrix.
- Stand for 3 minutes until Elusion Buffer absorbed by the matrix.
- Centrifuge at full speed (13,000 rpm) for 2 minutes to elute purified DNA.