Biochar as a soil amendment and habitat for microorganisms

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DECLARATIONS

I, Noraini Md Jaafar, declare that this thesis was composed by myself and the research detailed was conducted by myself, except for the instances detailed and quoted in the text and acknowledgments.

Noraini Md Jaafar

I, Noraini Md Jaafar, declare that Chapter 7 was concurrently conducted with Ms Sanjutha Shanmugam. We jointly designed Experiment 7.2, and the plant growth data are common to both of our studies.

Noraini Md Jaafar

I hereby declared that this thesis contains published work and/or work prepared for publication, some of which has been coauthored. The bibliographical details of the work and where it appears in the thesis are outlined below.


   Journal Paper – 3 authors
   Sharing of authorship as: Noraini Md Jaafar (70%); Peta Clode(20%); Lynette K Abbott (10%) for this manuscript.


   Book Chapter - sole author
ABSTRACT

This research sought to determine the role of biochar as a soil amendment and potential habitat for soil microorganisms, incorporating the use of high resolution microscopy techniques and determining the subsequent interaction of biochars with several soils, soil amendments (organic matter and P fertiliser) and their effects on soil microbial properties and plant growth. Biochar as a habitat has been suggested as one of the mechanisms which may help promote the microbial status of soil, including activity of arbuscular mycorrhizas. Procedures for observation and quantification of the habitat preferences on biochar were based on use of various microscopy imaging techniques (Chapter 3). Micrographs obtained from scanning electron microscopy (SEM) were used to characterise morphological characteristics of biochars. Woody biochars are potential habitat for soil microorganisms due to their high porosity and wide range of pore size based on pore distribution in microscopic images. SEM observations demonstrated differences in pore and surface properties of the biochar. Biochar particles were compared in soilless media and after deposition in soil. Fungal staining with fluorescent stains and preparation techniques for preserving and preparing biochar and/or biochar colonised by fungi were studied with both fluorescence and electron microscopy to determine the nature of fungal colonisation in or on biochar surfaces and pores in both soilless and soil systems. Biochar retrieved from soil and observed using fluorescence microscopy exhibited distinct hyphal networks on external biochar surfaces. The hyphal colonisation of biochar incubated in soil was much less than for biochar artificially inoculated with fungi in soilless medium.

Further soil microbial properties in response to biochar sources and biochar particle sizes were studied under laboratory conditions in soil from Moora (Chapter 4). Three biochar particle sizes (0.5–1 mm, 1-2 mm, 2-4 mm) from three sources (Wundowie, Saligna, Simcoa) were incubated in soil at 25°C for 56 days to observe soil microbial biomass and activity. Initial microscopy observations (Chapter 3) showed all three woody biochars provided potential habitat for soil microorganisms, and this was supported with BET characterisation of biochar (Chapter 4). Saligna biochar contained the highest density of pores and most uniform pore structure, followed by Simcoa and Wundowie biochars. Heterogeneity of pore and surface structures was found within a single biochar source and among the biochars studied. After 56 days incubation in soil, hyphal colonisation was observed on biochar surfaces and in larger biochar pores. Soil clumping occurred around biochar particles, cementing and covering biochar pores and
surfaces. This may have influenced surface area and pore availability for fungal colonisation. Increased particle size for each biochar source had little effect on soil microbial biomass carbon and phosphorus after 56 incubation days, which could probably be the result of soil clumping. However, a biochar particle size effect on soil microbial biomass carbon and phosphorus was significant after 28 days of incubation.

Soil management that includes biochar application could lead to complex interactions with organic matter. Interactions between two sources of woody biochars and two types of crop residue (canola and wheat) applied at 2% (v/v) were examined in soil from Cunderdin in a 112 day incubation study (Chapter 5). After 112 days incubation in soil, biochar application alone had no effect on soil microbial biomass phosphorus and carbon, or on soil respiration throughout 112 days in the absence of the two crop residues. Hyphal colonisation observed under fluorescence microscope and SEM on either Simcoa or Oil Mallee biochar in the presence of crop residues were similar to that found on either Simcoa or Oil Mallee biochar without crop residue addition to soil. The combination of Oil Mallee biochar and either of these two crop residues increased soil microbial biomass C after 112 days.

Investigation of the effects of biochar interaction with phosphorus (P) fertilisation on indigenous arbuscular mycorrhizal (AM) colonisation of roots of subterranean clover and wheat was conducted in two glasshouse studies (Chapter 6). First, AM fungal colonisation in subterranean clover was assessed when Simcoa biochar at different amounts (0, 5, 10, 25 and 50 t/ha) was applied to Cunderdin soil over 12 weeks without any P fertilisation. In the second experiment, mycorrhizal colonisation in wheat roots grown for 8 weeks was compared when two biochar sources (chicken manure biochar (CMB) and wheat chaff biochar (WCB)) at varying amounts (0, 2.5, 5 and 7 t/ha) with or without diammonium phosphate (DAP) fertiliser added to Minginew soil. Increasing the amount of Simcoa biochar did not affect the microbial biomass P, but it did affect mycorrhizal colonisation, especially at the later harvests (weeks 9 and 12) for subterranean clover. The woody Simcoa biochar applied at a high amount increased mycorrhizal colonisation in subterranean clover but CMB or WCB applied at a much lower application amount also stimulated AM colonisation in wheat. The varying response of mycorrhizal colonisation with biochar addition in both experiments could be related to different soil, plant and biochar sources tested.

Biochar amendments in previous experiments (Chapter 3-6) were conducted in different soil backgrounds. Further evaluation of woody biochar and its potential for
ameliorating soil properties including soil microbial biomass and mycorrhizal colonisation in the presence or absence of a lime-clay-biosolids product (LaBC®) was assessed in four additional soils in the final experiment (Chapter 7). Biochar sources (pyrolysed biochars and activated biochar) applied to these four soils were first investigated in an incubation study without addition of LABC®. Micrographs from SEM observations on Simcoa biochar after 28 days incubation in each of the four soils showed that the clayey soil had fewer sand grains attached to biochar particles and maintained a higher trend of microbial biomass than in the loamy sand soils. Activated biochar increased microbial respiration compared to the other pyrolysed biochars. In the corresponding glasshouse experiment with subterranean clover, the effect of applying a woody *Eucalyptus* (Simcoa) biochar to the four soils with or without addition of the lime-clay-biosolids product (LaBC®) was investigated. The combination of biochar and LaBC® was expected to improve soil microbial biomass and mycorrhizal colonisation. Soil microbial biomass, root length colonised by mycorrhizal fungi, and plant grown in the biochar-amended soil was lower than for the combination biochar+LaBC® or for LaBC® alone after eight weeks (56 days) in the three loamy sand soils. In contrast, microbial biomass and mycorrhizal root length colonisation and root growth did not respond to these treatments in the clayey soil.

In conclusion, mycorrhizal colonisation varied with biochar source and other soil amendments (P and the lime-clay-biosolid product). Microscopy evidence demonstrated that biochar could provide a habitat for soil fungi. However, the minimal response of soil microbial biomass to biochar in most experiments provided little evidence of biomass stimulating microbial activity at soil-biochar-plant-microbe interfaces even in the presence of recently added organic matter from different crops. Mycorrhizal responses after biochar addition to soil varied with soil type, host plant, biochar factors (source, amount, placement method) and interactions with soil amendments.
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4.2 Scanning electron micrographs (a-c) and fluorescent micrographs (d-f) of incubated and colonised Simcoa biochar particles with soil particles and fungal network on biochar external surfaces of Simcoa biochars incubated in soil taken from Moora, WA for 56 days.

4.3 Scanning electron micrographs of incubated and colonised Simcoa biochar particles with soil particles cementing the surfaces and pores.

5.1 SEM of heterogeneous pores exhibited in woody biochars prior to addition to soil in this incubation experiment for a) Simcoa biochar, and b) Oil Mallee biochar.

5.2 Fluorescence staining of microorganisms on Oil Mallee biochar amended in soil with (a), wheat residues, (b) canola residues and the (c) control and for Simcoa biochar particles incubated in soil amended with (d), wheat residues (e), canola residues and control (f) after 112 days.

5.3 Scanning electron micrographs showing fungal hyphae on Oil Mallee biochar incubated in soil for 112 days with wheat residues (a, b, c) and canola residues (d, e, f).

6.1 Fluorescence micrographs (a-d) and scanning electron micrographs (e-f) of fungal networks on external surfaces of Simcoa biochar particles retrieved from 25t/ha (a, b) and 50 t/ha (c, d) (Experiment 6.1), prepared and observed using Method 4 as in Table 3.2.

6.2 Scanning electron micrographs on characterisation of chicken manure biochar with wood chips (a-b) and wheat chaff biochar (c), prepared and observed using Method 1 as in Table 3.2.

7.1 Scanning electron micrographs of Simcoa biochar particles incubated in Soil 1 (a, b, c) and Soil 2 (d, e, f) for 4 weeks (28 days)(Experiment 7.1), prepared and observed using Method 4 as in Table 3.2.

7.2 Scanning electron micrographs of Simcoa biochar with soil particles incubated in Soil 3 (a, b, c) and Soil 4 (d, e, f) for 4 weeks (28days) (Experiment 7.1)prepared and observed using Method 4 as in Table 3.2.
Soil conditioners, including organic matter, can overcome soil-related problems and may be used to increase the fertility of soils in agricultural systems (Gosling et al., 2006). Carbonised, charred or pyrolysed materials have received considerable attention as soil amendments due to their distinct porous features and longevity in soil compared to other forms of organic matter (Lehmann et al., 2003; Lehmann, 2007; Lehmann and Joseph, 2009; Lehmann et al., 2011). Forms of carbonised materials available for use as soil amendments in agricultural soils include charcoal, activated carbon, materials derived from hydrothermal carbonisation and various biochars such as fast- and slow-pyrolysis biochars. Characteristics of these carbonised materials are generally dependent on the process by which they are produced, particularly pyrolysis temperature or other production parameters (Rillig et al., 2010).

Biochar is generally produced during pyrolysis of (waste) organic material (Lehmann and Joseph, 2009). Some forms of biochar have been shown to improve plant growth when applied to soil and biochar has been claimed to be a soil conditioner with a significant role in improving soil properties (Glaser, 2007; Rondon et al., 2007; Blackwell et al., 2010; Bruun et al., 2011; Jones et al., 2012).

Biochar addition to soil has been shown to both increase and decrease soil microbial biomass and soil fauna such as earthworms (Pietikainen et al., 2010; Beesley and Dickinson, 2011; Lehmann et al., 2011; Dempster et al., 2012a). The application of biochar is normally aimed at improving the development of soil beneficial microorganisms associated with nutrient cycling such as nitrogen and phosphorus. However, Dempster et al. (2012a) found that biochar (derived from *Eucalyptus* spp.) addition to soil decreased soil microbial biomass and associated N mineralisation. In relation to phosphorus cycling, biochar effects may be mediated through interactions with arbuscular mycorrhizal (AM) fungi, and both positive and negative responses have been recorded (Saito, 1990; Gaur and Adholeya, 2000; Solaiman et al., 2010). Solaiman...
et al. (2010) showed that woody biochar incorporation into soil increased AM colonisation (measured as percent root colonisation and root length colonised), but did not contribute to greater phosphorus uptake at early stages of plant growth.

Both direct and indirect mechanisms for biochar interactions with soil microorganisms have been proposed which could be associated with changes in soil conditions such as soil water status and soil pH (Ezawa et al., 2002; Gundale and DeLuca, 2006). They are similar to the proposed mechanisms underlying interactions between biochar and AM fungi (Warnock et al., 2007) which encompass (i) alterations in soil physico-chemical properties, (ii) interactions between AM fungi and other soil microorganisms, (iii) production of stimulative signalling compounds, and (iv) protection from predators. Among these mechanisms, the suggestion that biochar provides habitat and other favourable conditions for soil microorganisms is poorly understood and requires experimental verification. Furthermore, complications in understanding the response of soil microorganisms to biochar addition have been confounded by the use of biochars of different organic origin and pyrolysis condition with diverse physical and chemical properties (Thies and Rillig, 2009; Rillig et al., 2010; Anderson et al., 2011; Bruun et al., 2012).

Heterogeneous biochar characteristics including pore size, surface area and chemical composition must be considered when evaluating microbial effects in relation to habitat provision and other beneficial contributions of the biochar micro-environment. This includes knowledge of source materials (feedstocks) used to make the biochar, and potential interactions between biochar, fertiliser and plant residues, and whether or not there will be interactions with the soil. Without this information, it may be difficult to generalise about biochar amelioration of soil or habitat provision for soil microorganisms. The purpose of this research was to investigate interactions between biochar and soil microorganisms (Table 1.1).

Contradictory reports and reviews of biochar application to soil as soil amendments as well as the interactions between biochar and soil microorganisms including AM fungi
Table 1.1 Summary of experimental work on the application of biochar to soil.

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Objective</th>
<th>Soil Origin</th>
<th>Biochar Source</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 3</td>
<td>Methodology and Microscopy Objective: identifying biochar as a potential habitat for fungal hyphae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chapter 4 (Incubation)</td>
<td>Effects of woody biochars and particle sizes on microbial biomass and fungal colonisation</td>
<td>Moora (sandy soil)</td>
<td>(i) woody biochar</td>
<td>Biochar type and particle sizes</td>
</tr>
<tr>
<td>Chapter 5 (Incubation)</td>
<td>Effects of woody biochar with crop residues on soil microbial biomass, colonisation and soil phosphorus</td>
<td>Cunderdin (sandy soil)</td>
<td>(i) woody biochars</td>
<td>Biochar type and crop residues</td>
</tr>
<tr>
<td>Chapter 6 (Pot experiments)</td>
<td>Effects of application amount of woody biochar on AM fungi and plant growth Effects of biochar type and application amount with diammonium-phosphate fertiliser on AM fungi and plant growth</td>
<td>Cunderdin</td>
<td>(i) woody biochars (ii) chicken manure biochar, (iii) wheat chaff biochar</td>
<td>Biochar amount Biochar amount and diammonium-phosphate fertiliser</td>
</tr>
<tr>
<td>Chapter 7 (Incubation and pot experiments)</td>
<td>Effects of biochar on 4 different soils Effects of biochar and biosolid product containing lime and clay, LaBC® on AM fungi and plant growth</td>
<td>4 soils Chittering area (loamy sand and clayey soil)</td>
<td>(i) woody biochars</td>
<td>Biochar type Biochar and LaBC®</td>
</tr>
</tbody>
</table>

have previously highlight the need to consider the type of biochar, the soil conditions, and the agricultural system in which it is investigated (Sohi et al., 2009; Verheijen et al., 2009; Blackwell et al., 2010; Solaiman et al., 2010; Sohi et al., 2010; Lehmann et al., 2011).

Therefore, the topics addressed in this study were: (i) the role of biochar added to soil as a potential habitat for soil microorganisms especially fungi and (ii) the interactions
between biochar management (type, particle size, amount) and soil management practices (inorganic amendment such as fertiliser and organic amendment including organic residues and biosolids amendment) and their impact on soil microorganisms, including AM fungi. Biochar properties were studied in relation to their suitability as a habitat for soil microorganisms using microscopy techniques supported by soil chemical and biological assays. Incubation and glasshouse experiments were conducted to determine fungal colonisation in/onto biochar particles and the role of biochar as a microbial habitat in soil.

This was extended further to determine whether potential interactions of biochar management differed among soils of different background and histories of agricultural management practices. The related changes of biochar, mechanisms and interactions with soil by which biochar might alter the growth, survival and activities of soil microorganisms especially soil fungi are relevant to further evaluate the effects of biochar application in soil. An outline of the experiments is presented in Figure 1.1.

The aims of the research were:

1. to assess the habitat potential of biochar for soil microorganisms especially fungal colonisation (Chapter 3).
2. to determine the effects of biochar management (biochar source, particle size and biochar application amount) (Chapters 4 and 6) either with and without crop residues (Chapter 5), or diammonium-phosphate fertiliser (Chapter 6), or biosolid product containing lime and clay, LaBC® (Chapter 7) on soil microbial biomass and/or fungal colonisation in/onto biochar in south-western Australia soils.
3. to determine the effects of biochar on AM fungi with different biochar application amounts, with and without diammonium-phosphate fertiliser (Chapter 6), and with a biosolid product containing lime and clay, LaBC® (Chapter 7) in south-western Australia soils with different management histories.
Figure 1.1 Conceptual flow of experimental designs of biochar management and soil amendments
CHAPTER 2
LITERATURE REVIEW

2.0 Introduction

Biochar, the pyrolised product from pyrolysis of ‘waste’ organic material has been widely proposed as a soil ameliorant for improving soil properties (Lehmann, 2007; Rondon et al., 2007; Lehmann et al., 2011). However, biochar incorporation into soil can have both positive and negative effects on beneficial soil microorganisms, including arbuscular mycorrhizal (AM) fungi (Warnock et al., 2007). Both direct and indirect effects of biochar may be involved (Lehmann et al., 2011).

Direct and indirect mechanisms underlying interactions between AM fungi and biochar include the possibilities that (i) biochar provides a suitable habitat or shelter for soil microorganisms, protecting them from predators, (ii) soil conditions and plant growth can be influenced by mycorrhizas after biochar addition through changes in soil physico-chemical properties such as soil pH and water, and (iii) AM fungi interactions with soil microorganisms which may stimulate production of signalling compounds or alleviate production of detrimental compounds (Warnock et al., 2007).

Other mechanisms linking biochar to changes in the abundance or functioning of mycorrhizas include potential interference in plant-fungus signalling and detoxification of allelochemicals on biochar (Warnock et al., 2007; 2010). Investigations of how biochar might affect soil microorganisms have mostly focused on microbial attachment, microbial community shift and enzyme activities (Atkinson et al., 2010; Joseph et al., 2010; Sohi et al., 2010; Lehmann et al., 2011).

Two main areas of research on biochar and soil microorganisms require clarification. First, generalisations about responses to biochar application need to be considered in relation to the specific characteristics of the biochar product used. Second, experimental evidence is required to clarify mechanisms by which biochar influences microorganisms in soil. Biochar may also exhibit different interactions over time after its application to soil but this is not often studied or considered with regards to soil biota (Lehmann et al.
2011). There is a range of factors that could influence effectiveness of biochar as a soil amendment (Figure 2.1). Effects of biochar on soil microbial components need to be considered in the context of different biochar and soil backgrounds as well as soil management practices.

As soil microorganisms are sensitive to soil management, knowing the background of soil and biochar is important when managing soils with biochar, especially the amount applied when in combination with fertiliser and organic materials for optimal mycorrhizal symbiosis. This review focuses on biochar properties in relation to the factors controlling its variability, function and management leading to how biochar might alter the abundance and activity of soil microorganisms. Mechanisms by which, biochar might enhance the contribution of beneficial microorganisms in soil could also depend on other soil management. Based on potential similarities between mechanisms underlying interactions between soil microorganisms and biochar, this review focuses on AM fungi (Warnock et al., 2007) as a case study for considering biological influences of biochar on soil microorganisms especially fungi in soil.
2.1 Biochar as a soil amendment: overview of AM fungi

There is a general consensus that the incorporation of biochar into soil could be beneficial to soil microorganisms. However, biochars are heterogeneous, with a range in porosity and surface area and pH although they are commonly alkaline. Biological properties of biochar are often overlooked. The biochar beneficial impacts to soil have also been speculated based on observations of the pyrogenic soil containing burned plant and animal materials generally known as Terra Preta soil as well as dark earth soil. Arbuscular mycorrhizal (AM) fungi, used as a biofertiliser, have been considered in combination with biochar for their influence on soil properties such as nutrient retention, availability and uptake by plants (Warnock et al., 2007). However, the value of biochar as a general soil conditioner remains speculative because both positive and negative responses of soil microbial and mycorrhizal communities to biochar have been reported (Atkinson et al., 2010; Blackwell et al., 2010; Joseph et al., 2010; Moskal-del Hoyo et al., 2010; Sohi et al., 2010; Solaiman et al., 2010).

Some of the observed discrepancies of biochar influences on soil biological properties, including AM fungi, may result from conclusions based on experiments using biochars with different organic origin and diversity in physical and chemical properties (Rillig et al., 2010). Biochars derived from a range of plant and biomass sources have been studied in experiments that include both naturally occurring and inoculated AM fungi (Table 2.1). Research related to biochar and its effects on AM fungi emphasises incorporation of plant-derived biochar compared to animal manure-derived biochar. Generally, there is inadequate detail of experimental comparisons made on this feedstock to the physical nature of biochar and its impact on AM fungi.

The type and source of biochar is central to estimating the role of biochar as a microbial habitat and its benefit to soil. For example, in Japan, locally available rice husk biochar increased the proportion of AM roots colonised through soil pH modification and absorption of toxic substances and agrochemicals which inhibit root growth and microbial activity (Ishii and Kadoya, 1994). In Australia, locally available *Eucalyptus*
### Table 2.1 Examples of experiments on the application of biochar to soil and its effects on AM fungi (after Warnock et al., 2007; Lehmann et al., 2011).

<table>
<thead>
<tr>
<th>Biochar Feedstock</th>
<th>AM fungus</th>
<th>Soil Type</th>
<th>Main Effects on AM colonisation</th>
<th>Proposed Mechanisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10 mm charcoal from rice husk 1:50 (w/w) - rice husk: western spine bark</td>
<td><em>Glomus fasciculatum</em></td>
<td>River sand</td>
<td>Increased % mycorrhizal colonisation (52%)</td>
<td>Changes in soil pH, absorption of toxins</td>
<td>Ishii and Kadoya (1994)</td>
</tr>
<tr>
<td>Carbonised chaff (1mm): coconut charcoal 1:9 (v/v, 10% plot)</td>
<td><em>Gigaspora margarita</em>; <em>Glomus</em> sp. Inoculation of <em>Glomus</em> sp.</td>
<td>Volcanic devastation area</td>
<td>Increased % mycorrhizal colonisation (10-30%)</td>
<td>Habitat provision</td>
<td>Matsubara et al. (2002)</td>
</tr>
<tr>
<td><em>Eucalptus</em> biochar</td>
<td>Indigenous AM fungi</td>
<td>Clay loam</td>
<td>Reduced % mycorrhizal colonisation (20-38%) (+16%)</td>
<td>Not mentioned</td>
<td>Rondon et al. (2007)</td>
</tr>
<tr>
<td>Lodgepine, mango, peanut shell</td>
<td>Indigenous AM fungi</td>
<td>Low organic matter</td>
<td>Reduced % mycorrhizal colonisation (73%)</td>
<td>Not mentioned; Mechanisms other than changes in soil pH; inhibitory compounds in biochar</td>
<td>Warnock et al. (2010)</td>
</tr>
<tr>
<td><em>Leucaena</em> wood</td>
<td><em>Glomus aggregatum</em></td>
<td>Mansand and soil</td>
<td>No effect</td>
<td>Finer particles and surface area possible effect</td>
<td>Habte and Antal (2010)</td>
</tr>
<tr>
<td>Woody biochar</td>
<td>Indigenous AM</td>
<td>Sand, sandy clay loam, clay loam</td>
<td>Increased % mycorrhizal colonisation (30-70%)</td>
<td>Water uptake</td>
<td>Blackwell et al. (2010)</td>
</tr>
<tr>
<td>Woody biochar</td>
<td>Indigenous AM</td>
<td>Sandy clay loam</td>
<td>Increased % mycorrhizal colonisation (+45%)</td>
<td>Water uptake</td>
<td>Solaiman et al. (2010)</td>
</tr>
<tr>
<td>Hardwood dust</td>
<td><em>Glomus</em> sp. and <em>Gigaspora</em> sp.</td>
<td>Sandy loam</td>
<td>Increased % mycorrhizal colonisation (4.4-14%)</td>
<td>Changes to soil structure and soil pH.</td>
<td>Elmer and Pignatello (2011)</td>
</tr>
</tbody>
</table>
biochar had a similar positive effect on the percent mycorrhizal colonisation, possibly related to water uptake (Solaiman et al., 2010; Table 2.1). In other cases, there was no effect of woody *Eucalyptus* biochar (e.g. Rondon et al., 2007) or woody *Leucaena* biochar (e.g. Habte and Antal, 2010).

Key measurements on the AM fungal symbiosis and development in previous studies are limited. Most studies have examined the effects of biochar on mycorrhizal colonisation and sporulation (e.g. Ishii and Kadoya, 1994; Matsubara et al., 2002; Elmer and Pignatello, 2011) while measurements of phosphorus availability in plant and soil are used as indirect indicators of AM fungal effectiveness (Solaiman et al., 2010; Blackwell et al., 2010; Table 2.1). It is notable that soil background and mycorrhizal inoculation methods (inoculation or indigenous) also vary among studies which could have influenced the responses. Understanding of the mechanisms involved in how biochar may affect the hyphae of AM fungi, spore germination and sporulation, enzymatic activities and carbon or phosphorus economy interchange with its host plants are not well known.

### 2.2 Mechanisms of interactions between biochar and AM fungi

Several reviews have addressed on how biochar might affect soil biota and AM fungi (Sohi et al., 2010; Warnock et al., 2010; Lehmann et al., 2011). Direct and indirect mechanisms underlying interactions between AM fungi and biochar include the possibilities that (i) biochar provides a suitable habitat or shelter for soil microorganisms, protecting them from predators, (ii) soil conditions and plant growth can be influenced by mycorrhizas after biochar addition through changes in soil physico-chemical properties such as soil pH and water, and (iii) AM fungal interactions with soil microorganisms which may stimulate production of signalling compounds or alleviate production of detrimental compounds (Warnock et al., 2007). Other mechanisms linking biochar to changes in the abundance or functioning of mycorrhizas abundance and/or functioning include potential interference in plant-fungus signalling and detoxification of allelochemicals on biochar (Warnock et al., 2010). In line with the mechanisms suggested for interactions between AM fungi and biochar, similar
mechanisms associated with interactions between other groups of soil microorganisms and biochar have been proposed (Table 2.2).

For example, there is evidence that biochar can influence soil microbial biomass, growth and activity (Kolb et al., 2009; Kuzyakov et al., 2009; Liang et al., 2010; Table 2.2). In addition to gross effects of biochar on microbial biomass and soil respiration, biochar via changes in carbon fluxes in soil can alter microbial community structure and function (Steiner et al., 2008; Anderson et al., 2011). Yeast-derived biochar strongly increased the proportion of fungi whilst glucose-derived biochar preferentially built up soil bacterial biomass in two soils (Steinbeiss et al., 2009).

The porous nature of one biochar could have played a role in changing the microbial community in the soil and microbial colonisation associated with the biochar. Woody biochar from *Pinus radiata* was able to increase fungal and bacterial abundance and promote P solubilising bacteria (Anderson et al., 2011). In other studies, fungi, especially saprophytic fungi, were estimated to highly colonise biochar particles due to their association in decomposing fibrous organic matter (Ascough et al., 2010b; Moskal-del Hoyo et al., 2010).

Generally, there are inadequate details of experimental comparisons made on this feedstock to the physical nature of biochar and its impact on AM fungi. Mechanisms proposed by Warnock et al. (2007) for AM fungi may also apply to other soil microorganisms. Overall, factors such as biochar and soil management may also play important role in determining the effects and mechanisms of biochar interaction in soil.

### 2.3 Factors influencing biochar-soil microbe interactions: biochar

Since the review of Warnock et al. (2007) discussing potential mechanisms of biochar-AM fungi interactions, including the potential of biochar as a habitat for soil microorganisms, other studies of the potential impact of biochar-AM interaction have not focused on the mechanisms involved. The nature or physical characteristics of biochar in providing the habitat and protection for AM fungi is emphasised in this chapter because it is one of the main mechanisms that may involve direct biochar-mycorrhizal interactions.
<table>
<thead>
<tr>
<th>Biochar Feedstock</th>
<th>Soil Type</th>
<th>Main Effects on Soil Microorganisms</th>
<th>Proposed Mechanisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry</td>
<td>Alfisol/Chromosol</td>
<td>Increase in MBC</td>
<td>Improvement in soil physical properties</td>
<td>Chan et al. (2008)</td>
</tr>
<tr>
<td>Bull manure (dairy) and pine (<em>Pinus</em> spp.)</td>
<td>Mollisol, Entisol, Spodosol and Alfisol</td>
<td>Both SIR and BR increased with increasing charcoal amount</td>
<td>1. Substrate and nutrient availability from biochar 2. Altered soil properties benefiting microbial biomass and activity.</td>
<td>Kolb et al. (2009)</td>
</tr>
<tr>
<td>Papermill waste</td>
<td>Ferrosol, Calcarosol</td>
<td>Increased microbial activity (in ferrosol) but decreased microbial activity in calcarosol with wheat only Decline in microbial activity when unfertilised</td>
<td>None suggested</td>
<td>Van Zwieten et al. (2010a)</td>
</tr>
<tr>
<td><em>Pinus radiata</em></td>
<td>Silt loam soil</td>
<td>Increased fungal and bacterial abundance; promotion of P solubilising bacteria</td>
<td>C fluxes altered in soil</td>
<td>Anderson et al. (2011)</td>
</tr>
<tr>
<td>Wood (Eucalyptus)</td>
<td>Coarse sand</td>
<td>Decrease in microbial biomass C</td>
<td>Decrease mineralisation</td>
<td>Dempster et al. (2012a)</td>
</tr>
</tbody>
</table>
The heterogeneous properties of biochar from various materials and pyrolysis processes vary in their ameliorative effects on soil microbial colonisation, growth and benefit to plant and soil (Chan et al., 2007, 2008; Kuzyakov et al, 2009; Thies and Rillig, 2009; Blackwell et al., 2010; Rillig et al., 2010). The following biochar factors and effects will be further discussed in relevant to AM fungi in optimising both biochar and AM symbiotic benefit towards improving soil properties and plant growth.

### 2.3.1 Sources of biochar

Generalisations about the practical application of biochar have proven difficult due to heterogeneity among biochars and interactions with the soil environment into which biochar is applied. The heterogeneous properties of biochar can result from diversity of the original material used in the pyrolysis process (Blackwell et al., 2010). Furthermore, for practical purposes, an appropriate range of biochar particle size, amount and methods of application, especially in the field, needs to be considered for different biochar sources (Blackwell et al., 2009; Downie et al., 2009).

**Biochar characteristics**

The heterogeneity in both physical and chemical properties of biochar is associated with feedstock and pyrolysis parameters (Gundale and DeLuca, 2006; 2007; Downie et al., 2009). Mycorrhizal interactions with biochar have been compared using different types of biochar in a range of soil environments. Most biochars used have been plant-derived and include rice husk, pine and other woody materials (Warnock et al., 2007; Table 2.1). Experimental comparisons of the effect of the incorporation of biochars of plant and animal origin into soil on AM fungi are limited (Saito, 1990; Warnock et al., 2007). Therefore, the effects of biochar heterogeneity produced from various sources of organic materials, pyrolysis temperature, or biochar particles sizes and application amount on AM fungal growth, symbiosis and functions are not well understood.

**Biochar as a habitat in soil**

Biochar creates a micro-environment within the bulk soil upon its application (Thies and Rillig, 2009; Ogawa and Okimori, 2010; Lehmann et al., 2011). Within the biochar
micro-environment, biochar surfaces and pores can be colonised by bacteria, fungi and soil micro-fauna (Table 2.3).

**Table 2.3 Examples of microscopic observations of biochar as a habitat for soil microorganisms.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Methodology</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparison of fungal colonisation in biochar feedstocks before and after burning (pyrolysis)</td>
<td>Wood and charcoal fragments were manually broken, observed under reflected light microscope followed by SEM observation on transverse (T.S.), longitudinal tangential (L.T.S.), and longitudinal radial (L.R.S.) sections</td>
<td>Fungal hyphae observed, some fungal infestation and features of decay were preserved after burning</td>
<td>Moskal-del Hoyo et al. (2010)</td>
</tr>
<tr>
<td>Saprophitic white rot fungal colonisation (from laboratory trial on media) on biochar blocks</td>
<td>Blocks were lyophilised, split open, observed using SEM.</td>
<td>Distinct fungal growth found on charcoal, hyphal penetration through cracks.</td>
<td>Ascough et al. (2010b)</td>
</tr>
<tr>
<td>Characterisation of microbial life colonising biochar and biochar-amended soils (Fresh corn biochar colonised by microorganisms)</td>
<td>SEM, method not available</td>
<td>Fresh corn biochar with microorganisms in pores</td>
<td>Jin (2010) cited by Lehmann et al. (2011)</td>
</tr>
<tr>
<td>Fungal hyphae colonisation in fresh biochar pores</td>
<td>Method not available</td>
<td>Fungal hyphae found in fresh biochar</td>
<td>Lehmann and Joseph (2009) cited by Lehmann et al. (2011)</td>
</tr>
<tr>
<td>Changes in charcoal particle morphology of 100-year-old char</td>
<td>SEM observation on cross sections, inner and outer parts of biochar, EDX spectroscopy</td>
<td>Filamentous fungi infiltrated charcoal through larger pores and patches of mineral coating were found</td>
<td>Hockaday et al. (2007)</td>
</tr>
<tr>
<td>Ecological study of different ages wood charcoal from forest humus profiles</td>
<td>SEM observation on transverse and longitudinal plane of biochar</td>
<td>Senescent fungal hyphae were found in biochar</td>
<td>Zackrisson et al. (1996)</td>
</tr>
</tbody>
</table>
Previous studies of microbial colonisation on biochar surfaces included laboratory experiments using biochar retrieved from soil (Ascough et al., 2010a, 2010b; Moskal-del Hoyo et al., 2010) but there has been little characterisation of microbial colonisation of the internal structure of biochar compared to the external surface (Table 2.3). Laboratory studies of fungal colonisation of biochar showed massive fungal colonisation on surfaces and along cracks (Ascough et al., 2010b). However, there has been little discussion of experimental conditions and methodologies associated with observations of microorganisms in the biochar micro-environment. Biochar pores may be structurally stacked, and they may be altered by the presence of soil particles.

It is expected that microorganisms are preferentially attracted to biochar surfaces rather than to pores (Lehmann et al., 2011). Surface features are important for substrate recognition and attachment by soil microorganisms (Lehmann et al., 2011). Biochar surfaces could provide substrates that are important for biological activity (Thies and Rillig, 2009). Furthermore, surface attachment can protect microorganisms and increase the opportunity for synergistic interactions between biochar and soil microorganisms. Biochar pH is usually neutral to alkaline and may contain some phosphorus (Gundale and DeLuca, 2006; Yamato et al., 2006) which may be available for microbial uptake.

Fungal hyphae, such as those of AM fungi, have potential to dominate biochar surfaces due to their extensive hyphal networks (Lehmann et al., 2011). Hyphae could colonise both the external and internal surfaces of biochar, with differences in growth forms observed on external and internal surfaces of charcoal (Ascough et al., 2010b). Although the surface of biochar has been associated with slow degradation by soil microbial and chemical processes, it can become coated with organic material (Joseph et al., 2010; Lehmann et al., 2011) which contributes to a microbial habitat. Forms of biochar have been shown to retain moisture and adsorb cations (Liang et al., 2006; Blackwell et al., 2010; Solaiman et al., 2010) and this may indirectly influence soil microbial activity on biochar surfaces. A greater number of functional groups and oxidised sites on biochar surfaces could further facilitate microbial oxidation (Hockaday et al., 2007). Higher bacterial growth rates in association with biochar (Pietikainen et
al., 2000) indicated that attachment and physical protection may be enhanced by the surface chemistry, including hydrophobicity.

Variation in porosity is expected to alter the suitability of biochar as a habitat for soil microorganisms. Pore size and surface characteristics are likely to influence microbial attachment and presumably the ability of the microorganisms to enter and/or penetrate into the biochar (Lehmann et al., 2011). Biochar includes meso (<2μm), micro (2μm – 50 μm) and macro (>50μm) pore sizes (Downie et al., 2009) which may create micro-environments. Larger biochar pores may offer a new microhabitat to fungi, but no direct experimental evidence of the extent of pore colonisation by either soil bacteria or fungi is available. Furthermore, connectivity of pore spaces within biochar particles could influence important resources for microorganisms such as air and water diffusion through biochar and facilitate colonisation by soil microorganisms. Pores with diameters of 1 to 4μm and 2 to 64μm will be accessible to soil bacteria and fungal hyphae respectively (Swift et al., 1979), including hyphae of AM fungi (Saito, 1990). However, no studies so far have qualitatively and quantitatively demonstrated preferential colonisation by fungi and bacteria in biochar pores or on surfaces.

Chemical and physical changes in biochar can occur after it is incorporated into soil (Downie et al., 2009). Interactions between soil particles, especially clay, and biochar have been found (Joseph et al., 2010). Quantification of changes in biochar after interaction with soil however, has not been a focus in investigations of the consequences of microbial colonisation of biochar but it requires knowledge of the characteristics of biochar pores and surfaces of any biochar applied (Lehmann and Joseph, 2009). As soil particles become cemented and the surface area covered, soil may enter the biochar pores and alter their porosity and surface area. This could either limit or enhance the habitable spaces of biochar to soil microorganisms depending on the nature of the modification and the soil type. Lehmann et al. (2011) discussed various modes of microbial attachment to biochar, but the role of soil particles in influencing microbial attachment has not been clarified. Biochar surfaces could become cemented
by soil and soil could enter biochar pores, but it is not known whether this might have either positive or negative effects on microbial colonisation of biochar.

Among sources of feedstock, woody biochar has potential as a habitat because it has higher porosity compared the other sources of biochar such as chicken manure (Downie et al., 2009). Pores of 2–80μm diameter are known to occur in wood-derived biochars and may benefit activity of mycorrhizal fungi (Theis and Rillig, 2009). Woody biochar from *Pinus radiata* (Anderson et al., 2011) for example, was able to increase fungal and bacterial abundance and promote P solubilising bacteria. Fungi, especially saprophytic fungi, were estimated to highly colonise biochar particles due to their association in decomposing fibrous organic matter (Ascough et al., 2010b; Moskal-del Hoyo et al., 2010).

If there is a benefit from provision of habitat, biochar could protect AM fungal hyphae and spores or even stimulate hyphal development. It has been demonstrated that fungal hyphal penetrate pores of inert material such as vermiculite used for preparation of AM fungal inocula (Douds et al., 2005). Similarly, AM fungi were found sporulating inside the cavities of expanded clay and on the surface of clay material particles (Norris et al., 1992). Saito (1990) stated that the highly porosity of charcoal is not an effective substrate for saprophytes but it can favour AM fungi but the reason for this is not known. Perhaps hyphae of AM fungi extend into charcoal buried in soil and sporulate preferentially in such particles (Ogawa and Yamabe, 1986; Baltruschat, 1987). However, much of the scholarly discussion is speculation and there is little qualitative or quantitative evidence of preferential colonisation by fungi and bacteria in biochar pores or on surfaces compared with soil particles. Furthermore, details of experimental techniques and biochar handling regarding microbial colonisation inside or on biochar surfaces are often lacking.

Whilst limited reports on fungi for biochar and soil microorganisms interactions are available, in contrast, mechanisms related to biochar effects on bacteria and other biota have been widely studied (Pietikainen et al., 2000; Yin et al., 2000; Liang et al., 2010). Steiner et al. (2008) showed that biochar can stimulate microbial activity and
abundance, and they attributed this stimulatory effect to the mineralizable fractions of biochar. Anaerobics and cellulose hydrolizing bacteria are abundant as well the bacterial reproduction rate improved in biochar-amended soils (Kumar et al., 1987; Steiner et al., 2004). However, the carbon and nutrient status in soil amended with biochar can also adversely affect properties of microbial biomass and groups of organisms (Rondon et al., 2007; Cheng et al., 2008b). The influence of pH was also identified as a regulating factor for microbial abundance and activity with biochar addition to soil (Steiner et al., 2004; Rillig et al., 2010).

In relation to habitat provision of biochar to other microbial groups other than fungi, adhesion of *E. coli* has been reported (George and Davies, 1988) as well other bacteria (Jin, 2010). However, the level of bacterial adhesion to biochar pores and surfaces may vary according to the size of the biochar pore curvature, pore size, precipitates and electric current forming on biochar surfaces (George and Davies, 1988; Samonin and Elivoka, 2004; Cheng et al., 2008b). Smaller pore size and large curvature limit the bacterial adhesion. However, the shape of the biochar surfaces is not usually well defined.

### 2.3.2 Method of biochar application to soil

The method of placement of biochar in soil (either as a distinct layer (banded) or mixed through the surface layer) may influence the extent to which biochar affects soil microorganisms. Biochar banded in soils was shown to increase AM fungal colonisation measured as percentage of roots colonised (Blackwell et al., 2010; Solaiman et al., 2010). Banding biochar into a layer in field soil is normal practice compared to surface application due to the wind problems (Blackwell et al., 2009; 2010). Banding of biochar was effective for both AM fungi and plant growth in a field study at several sites (Blackwell et al., 2010) although this was not compared with any other method of biochar placement.

Banding and surface application of biochar are practical for field conditions whereas mixing biochar with soils, banding and surface application have been used in pot trials.
Chapter 2: Literature Review

(Blackwell et al., 2009). However, no experimental comparison of these methods is available for AM fungi, unlike the pot trial on ectomycorrhizal fungi, where responses to different methods of biochar application to soil have been investigated (Makoto et al., 2010). Biochar applied in a layer with ectomycorrhizal inoculum promoted larch plant growth when compared with mixing biochar with soil. This was attributed to the frequency of root contact with biochar enabling effective phosphate utilisation.

Banding biochar in the crop zone ensures biochar placement in the zone directly in contact with plant roots at the earliest growth stages (Blackwell et al., 2009). Biochar applied in bands also reduces biochar and topsoil loss caused by wind erosion and surface disturbance. The improvement in precision of sowing and fertilising machinery provide chances for crops to be sown in, or adjacent to, bands of incorporated biochar. In addition, the appropriate time in applying biochar to soil needs to be considered. Rutto and Mizutani (2006) proposed that biochar is best added once mycorrhizal symbiosis established. This was based on their conclusion that biochar (or the activated charcoal used in their study) could delay mycorrhizal associations through exudate absorption which adversely affects the fungus signalling process hence the symbiosis establishment.

2.3.3 Amount of biochar

The need to use the appropriate amount of biochar applied to soil is crucial to restore and maintain soil fertility and clearly critical for the effects on mycorrhizas, crop growth and nutrition (Ishii and Kadoya, 1994; Solaiman et al., 2010). Provision of soil conditions favourable for growth and activities of AM fungi needs to be taken into account when managing biochar and mycorrhizas in agricultural soils (Gazey et al., 2004). Biochar from vastly different parent materials and pyrolysis conditions may exert different chemical properties including nutrient concentrations which also determine suitable application amount of biochar used in soil management strategies. This application amount may vary among soil types, and land use histories which could constrain generalisations about the effects of biochar applied to soil (Schmidt and Noack, 2000).
Several studies have investigated the amount of biochar applied to soil on soil microorganisms (e.g. Kolb et al., 2009; Blackwell et al., 2010; Solaiman et al., 2010). While there is a need to use an appropriate amount of biochar for agronomic reasons (Blackwell et al. 2010; Solaiman et al. 2010), it is expected that there are also changes in the microbial environment, including that of AM fungi, which contribute to plant growth responses (Glaser et al., 2002; Kolb et al., 2009; Cross and Sohi, 2011). In terms of soil microbial biomass, Chan et al. (2008) found an increase in soil microbial biomass C depended on the type of biochar and N fertiliser addition. Microbial biomass C at higher application levels (25 and 50 t/ha) was significantly greater than that in the unamended control (Chan et al., 2008).

Selections of suitable amounts of biochar for application to soil for enhancing colonisation by AM fungi are expected to differ with soil and biochar source (Blackwell et al., 2010; Elmer and Pignatello, 2011). Inhibition of growth of AM fungi could result from higher than optimum amounts of biochar. The abundance of AM fungi in roots increased when hydrothermal carbonised biochar was added at 20% w/w, and higher concentrations resulted in reduced mycorrhiza formation. Inoculum dilution at excessive levels of biochar application or an adverse affect on host plants limiting C supply to the AM fungi have been proposed (Rillig et al., 2010). Furthermore, the most appropriate amount of biochar application may depend on the fertility of soil and its management, which could include organic matter management and other soil amendments such as fertiliser and lime (Blackwell et al., 2010). Biochar applied in optimal amounts and forms is expected to increase microhabitat availability in topsoils with low clay content (Solaiman et al., 2010). This may deliver mycorrhizal benefits (e.g. improve P acquisition by plants). Degraded soils may require higher amounts of biochar, but this requirement would vary with organic matter or nutrient status (Liang et al., 2006; Chan et al., 2007, 2008; Steiner et al., 2008; Kolb et al., 2009). Most studies involving different amounts of biochar applied to soil concluded that levels of that are acceptable for one type of soil and plant may not be suitable in another situation (Kolb et al., 2009; Blackwell et al., 2010).
2.3.4 Biochar particle size

There have been few studies of the impact of biochar particle size on microbial responses in soil. Different pyrolysis processes and feedstocks (organic origin) create biochar with different chemical, physical and size fractions (Keech et al., 2005; Gundale and DeLuca, 2006; Downie et al., 2009; Verheijen et al., 2009). Some biochars resemble the original cellular structure of the feedstock, in which large fragments correspond with woody plant material (Downie et al., 2009). Biochars also occur as large (> 4mm) through to fine particles (< 20 μm) (Glaser et al., 2001, 2002). Commonly, biochar contains a mixture of particle sizes (Downie et al. 2009) or it is ground after production into smaller fractions (Sohi et al., 2010). Larger particles of biochar may be less practical for agricultural purposes due to their bulky characteristics compared with smaller particle sizes.

The dust portion of biochar has the greatest surface area but may not be the most effective soil amendment due to wind erosion and practicality (Blackwell et al., 2009). Biochar surfaces can gradually oxidize in response to exposure to air, activities of soil microorganisms or roots and this may increase the cation exchange capacity (Joseph et al., 2010). Changes to the surface of biochar after exposure to the soil environment may also alter water and nutrient retention properties of the biochar (Joseph et al., 2010). The size of the charcoal pieces amended to soil is not expected to greatly affect nutrient uptake but may alter surface properties which influence microbial attachment (Verheijen et al., 2009).

Habte and Antal (2010) found that mycorrhizal colonisation of *Leucaena* roots was reduced when the growth medium was amended with fine (<0.3 mm in diameter) compared to coarse (<2.00 mm) charcoal. Lower levels of colonisation were associated with girdling of stems where the fine charcoal tended to accumulate. The large surface area could also enable greater absorption of toxic compound in soils (Antal and Gronli, 2003; Habte and Antal, 2010).
Practicality of biochar use as a soil amendment

The selection of biochar for use in agricultural soils needs to be based on physical characteristics and chemical composition to achieve success in soil amelioration. Theoretically, biochar with higher porosity, a greater density of larger pores, or large quantities of smaller particle sizes may benefit soil microorganisms, including mycorrhizal fungi. However, possible subsequent interactions between biochar and soil need to be taken into account. Furthermore, as the amount of biochar applied to soil can influence microbial processes, mixing biochar in the soil may also influence the distribution of microbial microsites in the soil in a different way to banding (Makoto et al., 2010). The application amount and method (mixing or banding) would normally be taken into account when incorporating biochar alone or with other amendments such as fertiliser for optimisation of nutrient capture (Blackwell et al. 2010). This could also change the interactions between biochar and soil microorganisms when organic matter or fertiliser is included.

2.4 Factors influencing biochar-microbe interactions: soil

It has been suggested that the efficacy of biochar-AM fungal interactions may be reduced in more fertile soils (Lehmann et al., 2011). Incorporating biochar in farming systems that use other soil amendments and practices could be beneficial, but biochar has a longer residence time in soil compared to other sources of carbon. As a carbon rich material, biochar is affected by soil processes but the changes in biochar occur slowly in soil and the effect on soil nutrients is not well understood (Lehmann, 2007; Lehmann and Joseph, 2009; Joseph et al., 2010). Application of fertiliser and labile organic matter had been used with biochar to optimise the benefits of these soil amendments (Blackwell et al., 2010; Graber et al., 2010).

2.4.1 Factors influencing biochar-microbe interactions: soil amendments

Dual incorporation of biochar with organic matter is normally associated with the goal of improving soil fertility. As a carbon source, biochar when added to soil could contribute to increasing the organic content in soil due to its recalcitrant nature. Addition of biochar as a nutrient source has been suggested (Rajkovich et al., 2012) and
biochar may also contain small amount of volatiles, substrate for microbial degradation and activities, and nutrients (Downie et al., 2009). Transformation of organic matter which contains phytotoxic compounds by pyrolysis could be used as a soil amendment to avoid a detrimental effect on plant and soil properties (Ishii and Kadoya, 1994), but most of the nutrients may be lost during pyrolysis. The availability of nutrients from soil organic matter may not necessarily be improved by biochar addition (Dempster et al., 2012b, 2012c).

There is a potential role of biochar in improving the microbial status of soil amended with other forms of organic matter. For example, Zackrisson et al. (1996) suggested that microbial activity played part in reactivating charcoal by decomposing attached materials to the charcoal and provide nutrient sources for microbial activity. Organic materials and minerals can be bound to biochar particles and it is important to note this when managing biochar and other sources of organic matter (Joseph et al., 2010). The structural nature of biochar could facilitate microbial development and indirectly accelerate adsorption and degradation of phenolic compounds (Keech et al., 2005). However, negative implications for soil microorganisms are also possible in certain cases involving organic substances through their interaction with biochar. In a study by Rutto and Mizutani (2006), application of activated charcoal slightly alleviated the negative detrimental effect of root bark extract but reduced the benefits derived from mycorrhizas for plant growth. The large surface area of materials such as activated charcoal enhances its ability to absorb organic compounds for soil detoxification purposes (Uchimiya et al., 2010).

Biochar can contribute slightly to soil nutrient status through provision of small amounts of nutrients or impurities in biochar (Lehmann and Joseph, 2009). This has been shown by Graber et al. (2010) whereby tar and labile compounds trapped in pores after pyrolysis could provide substrate for microorganisms. Furthermore, biochar application can alter soil phosphorus availability through modification to carbon, nutrient and pH in soil (Glaser et al., 2002; Matsubara et al., 2002). Charcoal may improve the growth and spread of AM fungi in roots by neutralising soil acidity (Ishii
and Kadoya, 1994). In contrast, addition of carbonised materials to soil can cause a decline in AM fungal colonisation and growth (Gaur and Adholeya, 2000).

The ability of biochar to retain nutrients and heavy metals is dependent on the sorption characteristics of biochars which are controlled by the relative carbonised and non-carbonised fractions and their surface and bulk properties (Uchimiya et al., 2010, 2011a, 2011b). However, there are concerns regarding the ability of AM fungi to develop in biochar amended soil with high levels of phosphorus. Biochar pH is usually neutral to alkaline and may contain some phosphorus (Gundale and DeLuca, 2006; Yamato et al., 2006) which may be available for microbial uptake. Mycorrhizal fungal effectiveness is affected by environmental and biological factors including P availability and mycorrhizal inoculum potential (Smith et al., 1992; Maeder et al., 2002). Thus, AM fungal development and function in soil amended with biochar would depend on biochar characteristics and soil nutrient status.

Wood-based biochar had the capacity to absorb measurable quantities of phosphate ions from a soil-free solution (Verheijen et al., 2009). The sorption of phosphorus to biochar may adversely affect how AM fungal hyphae inhabit the micro-environment of biochar. Mycorrhizal development responded positively to biochar at lower amounts of fertiliser applied to an agricultural soil (Blackwell et al., 2010). In this study, percentage in AM fungal colonisation increased when biochar was applied at 3 t/ha when the low level of phosphorus fertiliser was applied compared than the ‘full’ fertiliser application. In contrast, Yamato et al. (2006) observed that colonisation by AM fungi (%) was highest for bark charcoal application without phosphorus fertiliser application. A large number of studies on the effect of charcoal application on the enhancement of AM fungal colonisation have been conducted (Ogawa and Yamate, 1986; Saito, 1990; Ishii and Kadoya, 1994; Ezawa et al., 2002; Ogawa and Okimori, 2010), when no fertilisers were incorporated in the soil. However, mycorrhizal-biochar interactions would be expected to depend on the phosphorus status of the soil whether or not phosphate fertiliser was applied (Blackwell et al., 2010; Solaiman et al., 2010).
The absence of fertiliser can be compromised by applying higher amount of biochar. Blackwell et al. (2010) observed that biochar when applied at 3 t/ha resulted in greater root colonisation at the nil or low fertiliser rate. The low-level P fertiliser application in conjunction with biochar seems to have provided better conditions for mycorrhizal colonisation than the unfertilised soil or full fertiliser application. Biochar sorption of labile organic C could serve as a mechanism for decreased soil organic matter decomposition and concurrent P mineralization, could result in decreased P availability as suggested by Kuzyakov et al. (2009). Overall, biochar has received little attention with respect to the P status of soil compared to carbon, nitrogen and greenhouse emissions.

2.5 Conclusion

Biochar application to soil involves complex interactions with soil and soil management practices. Biochar is heterogeneous in nature, especially in pore and surface structure associated with pyrolysis processes and feedstock source. These physical features were proposed to be associated with the abundance and development of microorganisms, but quantification on the biochar pores and its effect size is inconclusive, making it difficult to support the claim of biochar as a significant habitat for soil microorganisms compared to the soil itself. In summary, soil background characteristics, including pH and P status, may lead to different interactions between soil and biochar (Table 2.4). As biochar is normally applied with fertiliser and commonly discussed in terms of a priming effect with labile organic matter addition, further investigations of interactions with soil microorganisms, including AM fungi are warranted.

Overall, there are significant effects of the type of biochar used, which largely influences the amount of each biochar applied to soil that could lead to beneficial effects on AM fungi. Hyphae of AM fungi have mainly been assessed within roots, not in the biochar micro-environment. The optimum amount of biochar application would need to be identified due to potential detrimental effects of higher biochar application levels on soil microorganisms or plant growth. The significance of biochar particle size has rarely been considered in relation to plant benefit or soil changes, but may influence
attachment of soil microorganisms to biochar surfaces. When biochar is applied with organic amendments, the mineralisation of biochar could be enhanced, but other concurrent effects of biochar on organic matter could also be important. Although significant interactions between biochar and fertiliser have been shown, the optimal amount of biochar when interacting with fertilisers may vary with biochar type.

Table 2.4 Factors (soil, management and biochar) likely to influence the role of biochar as a potential habitat for soil organisms and as an effective soil amendment.

<table>
<thead>
<tr>
<th>Biochar application aspect</th>
<th>Effects on soil microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochar types</td>
<td>Positive, negative and no effect on microbial respiration, microbial C, arbuscular mycorrhizas (only root colonisation)</td>
</tr>
<tr>
<td>Application amount</td>
<td>Lower amount must be accompanied with other nutrient source (e.g. fertiliser or labile organic matter) for microbial activities, arbuscular mycorrhizal colonisation</td>
</tr>
<tr>
<td>Particle size</td>
<td>Effect to soil microorganisms in bulk soil is not known, more on microbial attachment</td>
</tr>
<tr>
<td>Method of application</td>
<td>Effect to soil microbial colonisation in deposited versus bulk soil biochar dilution effect in soil and biochar contact with plant roots are not known</td>
</tr>
<tr>
<td>Organic amendment with biochar</td>
<td>Previously focused on biochar priming effect; organic matter helped biochar mineralisation rate, more on arbuscular mycorrhizal colonisation</td>
</tr>
<tr>
<td>Inorganic amendment with biochar</td>
<td>Limited studies on arbuscular mycorrhizal colonisation</td>
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</tbody>
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CHAPTER 3

METHODOLOGY: MICROSCOPIC OBSERVATION OF HABITABLE SPACES IN BIOCHARS AND COLONISATION BY FUNGAL HYphae

3.0 Abstract

Biochar is a potential micro-environment for soil microorganisms but evidence to support this suggestion is limited. Microscopy techniques offer an aid to visualise and/or quantify the potential role of biochar as a habitat for soil microorganisms. Therefore, a range of sample preparations and 2D microscopy imaging was explored to structurally characterise biochar in terms of a habitat for fungi in soil and to visualise, quantify and to understand the nature of fungal hyphae colonisation in/on biochar surfaces and pores under soilless and soil system. Scanning electron microscopy (SEM) observations of biochar pores and surface showed heterogeneity within and between biochar feedstocks. Energy dispersive X-ray (EDS) analysis validated variability of element and mineral properties of biochars obtained from bulk X-ray diffraction analysis. Fluorescence and electron microscopy enabled observations of fungal hyphae in/on biochar particles prior or after incubation in soilless media and soil. Fluorescent stains specifically allowed staining of the fungal hyphae and confirmed their presence. Staining of fungal hyphae associated with incubated biochar with fluorescent brightener SR 2200 was more effective than with Calcofluor White. Visualisation of hyphal networks associated with the biochar demonstrated under fluorescence microscopy was validated using SEM. However, observation and quantification of microorganisms in/on biochar and its surfaces is limited by (i) two dimensional (2D) imaging of 3D objects (ii) methods for preparation of biochar, and (iii) difficulties in making observations inside pores. Visualisation of microorganisms inhabiting biochars retrieved from soil was more difficult than was observation of soil-free biochar. The effects of soil entering the biochar pore remain to be studied. The role of biochar as a habitat appeared to be minimal after incubation in this agricultural soil for 56 days.
3.1 Introduction

Biochar is the product of biological materials that have undergone pyrolysis to create stable, carbonised organic matter that is heterogeneous and porous in nature. Due to its potential as a soil amendment, biochar has been extensively studied using a range of microscopy techniques (Bird et al., 2008; Chia et al., 2010; Joseph et al., 2010; Moskal-del Hoyo et al., 2010). The pore and surface structures of biochars potentially provide suitable habitats for soil microorganisms (Warnock et al., 2007; Downie et al., 2009). However, the extent of fungal colonisation of these potential habitats is not well understood. There are relatively few microscopic evaluations of biochar interactions with soil conducted under controlled conditions (Joseph et al., 2010; Lehmann et al. 2011; Table 2.3 in Chapter 2). Furthermore, changes in biochar structural characteristics over time and microbial colonisation of biochar pores after incubation in soil have been inadequately investigated (Hockaday et al., 2007; Bird et al., 2008; Ascough et al., 2010b).

Some studies concluded that biochar has potential as a habitat for soil microorganisms based on observations of fresh and pyrogenic biochars (Hockaday et al., 2007; Lehmann et al., 2011). Speculation on the possible role of biochar as a soil microbial habitat linked to enhancement of soil health has mainly been based on indirect measurements such as quantification of soil microbial biomass and other microbial indicators (Zackrisson et al., 1996; Pietikainen et al., 2000; Hockaday et al., 2007; Lehmann et al., 2011). Fungal growth on biochar surfaces and decomposition of biochar evaluated in laboratory Petri dish studies (Ascough et al., 2010b) showed biochar surfaces could be colonised by fungal hyphae. Light microscopy and scanning electron microscopy (SEM) showed that fungi colonised both the surface and interior of charcoals prepared at 300°C and 400°C over three months (Ascough et al., 2010b). However, laboratory inoculation of biochar may not be representative of soil conditions. Examination of pyrogenic biochar materials retrieved from forest fire sites clearly demonstrated evidence of biochar changes and fungal colonisation (Hockaday et al., 2007; Ascough et al., 2010a).
Hyphal colonisation on biochar surfaces and along cracks has been reported (Ascough et al., 2010b). Cracks in biochar particles may facilitate microbial colonisation (Ascough et al., 2010b; Verheijen et al., 2009). Pore connectivity and pore size are also likely to influence microbial colonisation of biochar but this has received little attention. Both the interior and external surfaces of pyrogenic biochar have been shown to be colonised by fungi (Hockaday et al., 2007). However, the preferential nature of fungal colonisation of biochar pores is unclear.

Colonisation of habitable biochar pore spaces by soil microorganisms may depend on microbial community composition as well as on changes that occur to biochar particles in soil over time (Ascough et al., 2010b, 2010b; Jindo et al., 2012a, 2012b). Once biochar interacts with soil, modification of physical and chemical characteristics of biochar can occur and attachment of soil particles to biochar surfaces may alter habitat suitability and microbial activity (Thies and Rillig, 2009; Lehmann et al., 2011). Further investigation of the fate of biochar particles once deposited in soil is needed to clarify the extent to which biochar pores, structural cracks, pore connectivity and surfaces contribute to biochar as a habitat for soil microorganisms (Thies and Rillig, 2009; Lehmann et al., 2011). For example, biochar as a habitat may be either over- or under-estimated in laboratory studies and may not be representative of soil conditions. Soil type and duration of biochar exposure to the soil may also influence the suitability of biochar as a microbial habitat.

This study explored several imaging techniques to visualise and quantify biochar habitat available to soil microorganisms. Microscopy techniques were used to estimate habitable space within biochar prior to and after its deposition in soil as well as challenges associated with visualisation of hyphae inhabiting pores and surfaces of biochar retrieved from soil (Figure 3.1). The aim was to assess a range of methodologies for biochar sample preparation and microscopy techniques for visualizing and quantifying the potential habitat properties of biochar.
Figure 3.1 Conceptual flow of experimental designs of biochar and soil amendments in Chapter 3 correlated to Chapter 4 (highlighted in blue box)
The objectives were:

1. to characterise (physical structure) potential habitable spaces in biochar suitable for colonisation by fungal hyphae through visualisation of biochar surfaces and quantification of pore size from biochar stocks (without soil contact),
2. to characterise (elemental and composition analysis) biochar stocks (without soil contact) using XRD and validation through SEM-EDS,
3. to optimise methods for visualising hyphae associated with biochar surfaces and inside biochar particles using optical fluorescence and scanning electron microscopy, and
4. to characterise the nature of fungal hyphae colonisation of biochar in the absence of soil and after its incubation in an agricultural soil.
3.2 Materials and Methods

3.2.1 Characterisation (physical structure) of biochar stocks through visualisation of biochar surface and pores from biochar (without soil contact)

One gram of each of the biochars obtained from Simcoa Ltd. (Bunbury, WA) (Blackwell et al., 2010) (Table 3.1) was air-dried to remove the majority of moisture prior to sample preparation. The sources of woody biochars were obtained from Simcoa Ltd, Bunbury, Western Australia and Dr Paul Blackwell, Department of Agriculture and Food, Western Australia. The biochars are named as ‘Wundowie’, ‘Simcoa’ and ‘Saligna’ hereafter.

Table 3.1 Biochar source and pyrolysis condition for ‘model biochar’ in this methodology section.

<table>
<thead>
<tr>
<th>Biochar ID</th>
<th>Biochar origin</th>
<th>Pyrolysis conditions</th>
<th>Parent material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simcoa</td>
<td><em>Eucalyptus</em> sp.</td>
<td>600°C</td>
<td>Plant</td>
</tr>
<tr>
<td>Wundowie</td>
<td>Mixture of <em>Eucalyptus</em> spp.</td>
<td>650°C</td>
<td>Plant</td>
</tr>
<tr>
<td>Saligna</td>
<td><em>Acacia saligna</em></td>
<td>400°C</td>
<td>Plant</td>
</tr>
</tbody>
</table>

Simcoa biochar was made from jarrah wood (*Eucalyptus marginata*) in 2008 by Simcoa Ltd. Wundowie biochar was collected from a 35-year-old stockpile of metallurgical charcoal made with a Lambiotte carbonisation reactor from jarrah (*Eucalyptus marginata*) and wandoo (*Eucalyptus wandoo*) at Wundowie Foundry (Wundowie, WA) (Blackwell et al., 2010). Both Wundowie and Simcoa biochar were produced at 600-650°C for 24 hours. Saligna biochar was produced from *Acacia saligna* branches at 400°C for 2 hours using a laboratory scale pyrolysis unit at Department of Agriculture and Food Western Australia. The biochars were sieved through a series of sieves (4 mm, 2 mm, 1 mm, 0.5 mm) into three categories (0.5-1 mm, 1-2 mm and 4 mm sizes). Only biochar particles between 0.5 mm and 4 mm were selected and applied to soil for easier retrieval after incubation for microscopy and analytical purposes.
3.2.2 Preparation and visualisation of biochar surface and pores

Two methods of preparation were used for observation of biochar surfaces and pores: a) whole biochar mounted on stubs, and b) resin embedded and cross-sectioned biochar (Figure 3.2). Mounting biochar on carbon stubs was used to observe the surface and pores structures of biochar. Some particles were manually cracked to observe the internal pores. The porous structure of biochar was also observed using cross-sections prepared of the resin-embedded biochar.

![Diagram of sample preparation and microscopy techniques used for characterisation and observation of the physical structure of biochar (prior to application in soil).](image)

**Figure 3.2** Steps in sample preparation and microscopy techniques used for characterisation and observation of the physical structure of biochar (prior to application in soil).
**SEM of whole and fractured biochar**

**Biochar mounted on carbon stubs for SEM observation**

For SEM, characterisation of biochar surfaces and pores, 5 to 10 particles of each biochar sized 0.5-1 mm were randomly selected, mounted on a carbon stub and coated with 5-10 nm Au. Observation by SEM was performed using a Phillips XL 30 ESEM or a Zeiss 55 VP-FESEM. Images of surface structure and pore structure of biochars were captured from 5 points on each particle at 3 consistent magnifications at 5-15 kV accelerating voltage (depending upon SEM used and its capability).

**SEM of embedded biochar**

**Resin embedded biochar particles for SEM observation**

Biochar particles were dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70% and 90% ethanol, dry 100% ethanol). Biochar particles of more than 2 mm were treated differently as the time required for dehydration process was longer and each step was repeated. After the dehydration, biochar particles were infiltrated in Procure Araldite resin and acetone mixture at a ratio of 2:1, 1:1 and 1:2 for 2 hours consecutively. Fresh Procure araldite resin was prepared once the biochar particles were ready to polymerise in mount. The biochar particles in resin were left to cure in an oven (60°C) under vacuum overnight. Coarse polishing was done with a series of sandpapers (100, 240, 400, 1000, 2000, 4000 grade of grit). These polished resin embedded biochars provided oriented cross sections through the biochar particles allowing visualisation of pore connectivity.

For imaging, polished sections were coated with 20 nm carbon. SEM was performed either by Phillips XL 30 ESEM or a Zeiss 55 VP-FESEM. Surface structure and pore structure of biochars were captured from 5 points on each particle at 3 consistent magnifications at 5-15 kV accelerating voltage. The NIH freeware package Image J software was used to measure the diameter of all visible pores in the SEM images. Comparisons were made to compare the pore size distribution between scanning electron micrographs of both whole biochar mounted on stubs and cross sectioned resin.
3.2.3 Characterisation (elemental and composition analysis) of biochar stocks (not incubated) using XRD through SEM-EDS

X-Ray diffraction (XRD) analysis of 1 gram biochar samples were carried out using conventional XRD analysis using a Philips PW 3020 diffractometer with a graphite diffracted beam monochromator (CuKα, 50kV, 20 mA) and scans from 4 to 70° 20. For Energy-dispersive Spectroscopy (EDS), ten biochar particles, were randomly selected, air dried, crushed into very fine particles and mounted on a stub before coating with 5-10 nm carbon. Basic imaging and element analysis of biochar samples was examined by SEM and qualitative energy dispersive X-ray using a JEOL 6400 SEM at 15 kV accelerating voltage.

3.2.4 Fluorescence imaging of biochar colonisation

Examination of microorganisms associated in or on biochar using optical fluorescence microscopy on colonised incubated biochar

Preparation of biochar for examining microbial colonisation was performed on both laboratory-based Petri dish (soilless) study and from a soil-based incubation experiment using Simcoa biochar as the model biochar.

Preparation and staining of microorganisms associated with biochar:

Sclerotinia sclerotiorum colonisation on biochar particles (Petri dish experiment)

For the Petri dish (soilless) study, twenty ‘Simcoa’ biochar particles from stock with 1-4 mm sizes were randomly selected, spread and placed on potato dextrose agar (PDA) media (Sigma-Aldrich) and inoculated with Sclerotinia sclerotiorum culture. Sclerotinia sclerotiorum was chosen as a representative fungus because it could grow in culture (unlike AM fungi). Each Petri dish (n=3) was inoculated with Sclerotinia sclerotiorum. After 3 days incubation, the biochar particles were retrieved, immediately preserved in 2.5% glutaraldehyde in phosphate buffered saline (PBS) solution (pH 7.2), and stored at 4°C.
Soil microorganisms associated with soil incubated biochar particles (incubation in soil as in Chapter 4)

For soil-based incubation experiment, ten ‘Simcoa’ biochar particles (1-4 mm) were randomly selected after 56 days incubation in an agricultural soil (loamy sand) with a history of wheat production, from Moora, Western Australia. This incubation experiment involved three particle size ranges (0.5-1mm, 1-2 mm, 2-4 mm) for each of three biochars (Saligna, Wundowie and Simcoa; Table 3.1 in Chapter 3) incubated in the soil for 56 days. Biochars were added to soil at an amount equivalent to 50 ton/ha and thoroughly incorporated. The amount of biochar applied was selected to optimise the response of soil microorganisms and was twice the amount used by Dempster et al. (2012a). The soil and biochar mixtures were incubated at 25°C aerobically in individual jars for 56 days. Biochar particles were collected after 56 days incubation. The biochar particles were preserved in 2.5% glutaraldehyde in PBS solution and stored at 4°C.

Staining

Ten sampled biochar particles colonised by microorganisms from the laboratory-based Petri dish and soil-based incubation experiments, which were preserved and stored, were first rinsed with PBS solution 3-5 times, each for 5 to 10 minutes to remove excess glutaraldehyde. To optimise staining, two fluorescent brighteners were investigated based on previous reports of crystallization problems with Calcofluor White M2R (Sigma Chemical, Poole). Fluorescent brightener Calcofluor White M2R, which binds to cellucose and chitin in cell walls, and SCRI Renaissance 2200 (FBA 220 liquid), supplied as stock aqueous at 20 % (Renaissance Chemicals), which binds to polysaccharides, were diluted to a 0.2% working dilution (Harris et al., 2002) and stored at 4°C until use in a covered bottle to prevent light transmission during storage. Biochar particles from the laboratory-based Petri dish study were used to compare the effectiveness of the fluorescent brighteners. Fluorescent brightener SR 2200 and Calcofluor White were each applied separately to each of five biochar particles for 5-10 minutes. They were rinsed 3 times using PBS each for 5 to 10 minutes to remove excess stain and observed using A Zeiss Axioplan 2 microscope via transmitted light and fluorescence, with objectives ranging from 2.5x – 100x magnifications. All images were
captured using a Zeiss Axiocam digital camera. Examination of fungal hyphae associated in or on biochar was made using scanning electron microscopy.

Only colonised biochar particles from Petri dish experiment were tested for comparison of the 2 fluorescent brighteners. A set of five colonised biochar particles were first stained by fluorescent brightener SR 2200 and another similar set was stained using fluorescent brightener Calcofluor White M2R for 5-10 minutes. They were rinsed 3 times using PBS to remove excess stain and imaged under fluorescence microscope. They are observed using a Zeiss Axioplan 2 microscope via transmitted light and fluorescence, with objectives ranging from 2.5x – 100x magnifications. All images were captured using a Zeiss Axiocam digital camera. Following the comparison of the two fluorescent brighteners, the colonised biochar from soil incubation experiments and following experiments hereafter were only stained with the most effective fluorescent brightener.

3.2.5 Correlative examination of fungal hyphae on biochar by fluorescence and SEM

Examination of fungal hyphae associated in/on biochar using SEM on colonised/ incubated biochar

Ten colonised biochar particles were selected at random from a soil incubation experiment (details further explained in Chapter 4) and stained with the most effective fluorescent brightener, SR 2200 as detailed above before being dehydrated in a series of increasing ethanol concentrations (30%, 50%, 70%). For biochar particles greater than 2 mm in diameter, the time required for the dehydration process needed to be longer, and each step was repeated. For initial fluorescence imaging, colonised biochar particles were submerged in 70% ethanol solution for observation under fluorescence microscope to observe fungal colonisation and to select the designated area or surface to be further examined under SEM. The biochar particles were then dehydrated completely to 100% ethanol critical point dried for approximately 2 hours. The intact biochar particles were carefully mounted on a carbon stub and coated with 5-10 nm Au for SEM imaging. The steps are summarised in Table 3. 2 and Figure 3.3.
Table 3.2 Summary of processes involved in observation of biochar as potential microbial soil habitat using several sample preparation and microscopy techniques.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Microscopy technique</th>
<th>Processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochar stock</td>
<td>SEM (Imaging)</td>
<td><strong>Observation : Surface properties</strong></td>
</tr>
<tr>
<td></td>
<td>(Method 1)</td>
<td>Intact/uncrushed biochar particles were air dried before put on stub and coated with Au.</td>
</tr>
<tr>
<td>Biochar stock</td>
<td>SEM-EDS (Analysis)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Method 2)</td>
<td>Crushed biochar particles were air dried before put on stub and coated with Ca</td>
</tr>
<tr>
<td>Resin embedded biochar</td>
<td>SEM</td>
<td><strong>Observation : Pores properties</strong></td>
</tr>
<tr>
<td>(Method 3)</td>
<td></td>
<td>Visualisation and Quantification of microbes inside the pores of polished biochar sections</td>
</tr>
<tr>
<td>Polished resin embedded biochar samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubated, colonised biochar</td>
<td>Fluorescence microscope</td>
<td><strong>Observation : Fungal colonisation</strong></td>
</tr>
<tr>
<td>(Method 4)</td>
<td></td>
<td>Visualisation of SR 2200 stained microbes on surface of biochar - the whole biochar particles at selected side (orientation/angles)</td>
</tr>
<tr>
<td>Fixed, stained, dehydrated and critical point dried biochar samples</td>
<td>SEM</td>
<td>Observation of microbes attached onto the surface of biochar</td>
</tr>
<tr>
<td>Air dried biochar, unstained</td>
<td>SEM</td>
<td><strong>Observation: Soil attachment</strong></td>
</tr>
<tr>
<td>(Method 5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3 Steps in sample preparation and microscopy techniques for observation of colonised biochar from laboratory-based incubation biochar and soil-based biochar experiments.
3.3 Results

3.3.1 Characterisation (physical structure) of initial biochar through visualisation of biochar surface and pores (not incubated)

Biochar surface and pore structures varied within a single source of a biochar that originated from *Eucalyptus* wood (Plates 3.1 to 3.2 a,b,c) or across biochar sources (further reported and discussed in Chapter 4). Some particles had rough surfaces while others were smooth. The crushing of particles into smaller particles may have contributed to heterogeneity in pore and surface structure, including cracks (see Chapter 4), in the biochar particles (Plate 3.2d). Exposed pores were visible but limited in surface observations (Plate 3.1), which were further enlarged (Plate 3.2 a,b,c). About 40 percent of the biochar particles had rough surfaces while others were smooth (Plate 3.1). Visible pores were not exposed in some particles while in others, pores were highly exposed.

![Plate 3.1](image)

Plate 3.1 Scanning electron micrograph of Simcoa biochar showing extensive variation in particle size range (0.5-1mm) and heterogeneity within a single biochar source. Biochar particles were treated according to Method 1 in Table 3.2. Scale bar = 1 mm.

3.3.2 Quantification of pore size distribution

Biochar derived from Simcoa woody feedstock varied in pore size distribution for samples examined in cross-section and as whole particles (Plate 3.2e and f). Cross-sections of biochar prepared using the resin-embedded method (Plate 3.2e)
Plate 3.2 Scanning electron micrographs showing heterogeneity in surface and pore structure of Simcoa biochar (pyrolyzed from *Eucalyptus* wood) for 0.5-1 mm particles (a-c, d and f) of biochar mounted on stubs (prepared according to Method 1) and (e) cross sectioned resin embedded (prepared according to Method 3). Rough surfaces (a, c), exposed pores (b, f) and cracks (d, arrow) were visible. Scale bar = 100 µm.
demonstrated the internal porous architecture and pore networking, whereas whole biochar fragments mounted on stubs enabled visualisation of cracks (Plate 3.2d) and the structure of surface pores (Plate 3.2f).

SEM micrographs were used to obtain pore size diameter which was measured using NIH freeware package Image J. Biochar cross sections prepared using the resin embedded method was used to characterise the porous architecture and pore networking within the biochar (Plate 3.2e), whereas with whole biochar mounted on stubs (Plate 3.2f) provided better structure of biochar pores and also enable observation of cracks in biochar (Plate 3.2d). Variability of surfaces and pores were also found in Wundowie biochar (Plate 3.3).

Based on Plate 3.2e and Plate 3.2f, cross-sections of biochar prepared using the resin-embedded method (Plate 3.2e) demonstrating the internal porous architecture and pore networking were compared to the whole biochar fragments mounted on stubs (Plate 3.2d,f). Estimates of the porosity distribution were similar when estimated using either the cross-sections of resin-embedded biochar or biochar mounted on stubs (Table 3.3).

Table 3.3 Comparison of the percentage pore-size distribution for Simcoa biochar determined by SEM imaging of whole fragments and cross-sections of biochar after resin-embedding. The minimum pore size measured was 4 µm, while the maximum pore size measured was 210 µm.

<table>
<thead>
<tr>
<th>Biochar</th>
<th>Numbers of pores per measured</th>
<th>Pore size &lt; 50 µm</th>
<th>Pore size &gt;50 µm – 100 µm</th>
<th>Pore size &gt; 100 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>500</td>
<td>86%</td>
<td>9%</td>
<td>5%</td>
</tr>
<tr>
<td>Cross sectioned</td>
<td>150</td>
<td>87%</td>
<td>7%</td>
<td>6%</td>
</tr>
</tbody>
</table>

3.3.3 Characterisation (elemental and composition analysis) of soil-free biochar using XRD and validation through SEM-EDS

Initial characterisation of elemental and mineralogical aspects of biochar was based on X-ray diffraction analysis, followed by SEM-EDS studies. Mineralogy of biochar from different particles sizes was distinctive for Wundowie biochar, comprising calcite, goethite and quartz (Table 3.4). Saligna biochar only contained carbon and calcite,
Plate 3.3 Scanning electron micrographs showing heterogeneity in surface and pore structure of whole Wundowie biochar particles (pyrolized from mixed species of *Eucalyptus* wood) for 0.5-1 mm particles. Some surfaces were rough (c, d) with unclear pore structure, while others (e, f) had clear pore structure and smooth surfaces. Scale bar = 200 µm.
While Simcoa biochar had mixtures of carbon, calcite and quartz. In comparison, Wundowie biochar comprised carbon, calcite and quartz but also had goethite, which was further validated in scanning electron microscopy and energy X-ray dispersive observation.

Elemental spectra obtained by SEM-EDS were consistent with XRD data. Both SEM and X-ray spectra indicated distribution of elements and mineral for Wundowie biochar. These included the occurrence of Ca, Al, Fe, Si and P elements. For example, the presence of Fe (Plate 3.4b, spectra C and E) was consistent with the occurrence of goethite minerals in Wundowie biochar. Minerals present in the biochar including calcite, quartz and goethite, were detected in X-ray spectra of particles shown in Plate 3.4a, b, d illustrating the diverse range of composition of this material. Apart from calcite crystals, most grains observed in the micrographs are Fe rich aggregates (Plate 3.4c, e) which demonstrated that biochar has a complex nature with a highly diverse composition.

Table 3.4 Mineral distribution from XRD analyses on 3 particles sizes of woody biochars.

<table>
<thead>
<tr>
<th>Biochar Source</th>
<th>Particle Size</th>
<th>Code</th>
<th>Carbon*</th>
<th>Calcite*</th>
<th>Quartz*</th>
<th>Goethite*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saligna</td>
<td>0.5-1mm</td>
<td>Sa1</td>
<td>√√√√√</td>
<td>√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-2 mm</td>
<td>Sa2</td>
<td>√√√</td>
<td>√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-4 mm</td>
<td>Sa4</td>
<td>√√√√√</td>
<td>√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simcoa</td>
<td>0.5-1mm</td>
<td>Sc1</td>
<td>√√√√</td>
<td>√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-2 mm</td>
<td>Sc2</td>
<td>√√√√√</td>
<td>√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-4 mm</td>
<td>Sc4</td>
<td>√√√√√</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wundowie</td>
<td>0.5-1mm</td>
<td>Wun1</td>
<td></td>
<td>√√√√√√√√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-2 mm</td>
<td>Wun2</td>
<td></td>
<td>√√√√√√√√√</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-4 mm</td>
<td>Wun4</td>
<td></td>
<td>√√√√√√√√√</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ticks represent relative degree of carbon and minerals distribution in each sample.
Plate 3.4 Elemental characterisation of Wundowie biochar. a) surface of Wundowie biochar (prepared and observed using Method 2 as in Table 3.2 and b) back scattered electron micrographs of elemental spectra (A, B, C, D, E, F) of Wundowie biochar at different intensity.
3.3.4 Examination of microorganisms associated in or on biochar using fluorescence microscopy on colonised/incubated biochar

*Inoculated and stained microorganisms associated with biochar (Petri dish)*

In the soilless Petri dish study, fluorescent stains Calcofluor White and SR 2200 confirmed the presence of hyphae on biochar particles, but SR 2200 stained the hyphae more clearly (Plate 3.5). There were some problems in using Calcofluor White as a fluorescent stain for hyphae (Plate 3.5a, b, c, d). Background staining was evident on biochar stained with Calcofluor White (became whitish instead of its original char blackish colour) making it difficult to observe the hyphae. Residues and traces of crystals formed by Calcofluor White were also observed (Plate 3.5a, c, d). In contrast, distinct and clear images of fungal hyphae without any background staining of biochar were improved when stained with RR 2000 (Plate 3.5 e, f). The crystallisation and background staining by calcoflour white made it difficult to distinguish and quantify microorganisms in or on the biochar. SR 2200 did not exhibit these characteristics and clear staining of fungal hyphae was observed (Plate 3.5 e, f), thus staining with fluorescent brightener, SR 2200 was the preferred method for staining fungal hyphae on biochar in the soil-based incubation studies and following experiments hereafter. In the Petri dish experiment, biochar particles incubated in soilless media were extensively colonised by hyphae but this was restricted to the biochar surface. There was little occurrence (estimated to be about 20-30%) of fungal penetration into pores within the biochar (Plate 3.5e); the fungal colonisation was confined primarily to biochar surfaces (Plate 3.5f).

*Soil microorganisms associated with soil incubated biochar particles (soil incubation experiment as in Chapter 4)*

Biochar retrieved from soil and observed using fluorescence microscopy exhibited distinct hyphal networking on external biochar surfaces (Plate 3.6 a, b). Fluorescence microscopy allowed observation of larger areas of fungal colonisation across the entire biochar particle (related to both the depth of field and larger fields of view). SEM confirmed fungal colonisation as observed earlier using fluorescence microscopy. The location of hyphae on biochar surface and within pore spaces was clear under SEM. Soil particles could also be clearly observed in smaller and larger biochar pores.
Plate 3.5 Fluorescence micrographs of biochar particles from soilless media stained with Calcofluor White (a-d); or SR 2200 (e and f) according to Method 4. Background staining and artifact crystallization is evident in Calcofluor White stained samples. Hyphae (arrows) colonising the surface of biochar particles were readily observed (c, e, f), but were generally found to not penetrate into the biochar as observed in fractured samples (e). Scale bars = 100 µm.
3.3.5 Examination of microorganisms associated in/on biochar using SEM on colonised/incubated biochar (soil incubation experiment as in Chapter 4)

Electron microscopy rendered a clearer image of and confirmed stained fungal hyphae colonising biochar. Fungal hyphae were found in larger biochar pores after incubation in soil (Plate 3.6d, e). Soil particles were also found in biochar smaller and larger pores (Plate 3.6f). SEM imaging provide confirmation of fungal colonisation earlier observed using fluorescence microscopy. The location of hyphae both upon the surface and within pore spaces was clearly observed using SEM, and allowed visualisation of biochar-hyphal interactions. Soil particles could also be clearly observed in smaller and larger biochar pores (Plate 3.6a,b). SEM provided further information on topology of biochar in relation to fungal hyphae (Plate 3.7).

3.3.6 Limitations associated with sample preparation and microscopy techniques

SEM imaging allowed visualisation of smaller areas in greater detail than did fluorescence microscopy. Fracturing biochar particles to observe internal regions via SEM enabled observation of fungal colonisation within the biochar. Detection and quantification of soil hyphae inside biochar pores was difficult due to soil interaction with biochar (Plate 3.6d, e, f). Thus, accurate quantification of hyphae was not possible through visualisation via either fluorescence or electron microscopy. Despite the difficulty of quantifying microorganisms in biochar pores, SEM observation provide location information and visualisation of fungal hyphae, as was found in larger pores.

The main obstacle to observation of hyphae was the presence of soil particles within the smaller pores. About 90 percent of the pores filled with soil particles were < 20 µm size. In summary, different approaches were used to investigate aspects of colonisation of biochar by fungal hyphae both within pores and on surfaces. Fluorescence microscopy was suitable for observing fungal colonisation across large areas of biochar surfaces. Preparation of biochar using critical point drying was suitable for observation of hyphae associated with biochar incubated in soil. The resin-embedded preparation of biochar was suitable for obtaining good cross sections for measuring pore size and connectivity within the biochar (Table 3.2, 3.3).
Plate 3.6 Fluorescence (a, b) (stained with SR 2200) and scanning electron (c-f) micrographs of colonised biochar particles. Soil fungal networks (arrows) in biochar pores, especially larger pores, and soil particles in pores (e, f) are seen in biochar incubated in an agricultural soil for 56 days, prepared according to Method 4. Scale bars = 100µm.
Plate 3.7 Fluorescence (a) and SEM (b) micrographs of biochar incubated in an agricultural soil for 12 weeks. Fungal hyphae were stained with SR 2200. Arrows highlight difference in depth of focus and difficulty in using fluorescence imaging for understanding hyphal-biochar surface interactions. Scale bars = 100 µm.
3.4 Discussion

Preparation procedures and observation of biochar using 2D microscopy techniques used in this study enabled visualisation of biochar pore and surface structure. The structural characteristics of biochar provide potential micro-habitats for soil organisms. In this study, woody biochars from *Eucalyptus* sp. and *Acacia* sp. which are native to Australia, have a potential role as a habitat based on initial characterisation of pore sizes distribution (further discussed in Chapter 4). This supports the earlier suggestion of biochar as a potential habitat by Warnock et al. (2007).

SEM imaging technique enabled visualisation of surface and pore structure of biochar following appropriate sample preparation. Considerable structural variability in biochar particles was observed. This was consistent with previous observations (Downie et al., 2009). The woody biochar used in this study has potential as a fungal habitat based on characterisation of biochar pore sizes (Downie et al., 2009; Thies and Rillig, 2009; Lehmann et al., 2011). Biochar includes meso (<2μm), micro (2μm – 50 μm) and macro (>50μm) pore sizes (Downie et al., 2009) which may create micro-environments. Larger biochar pores may offer a new microhabitat to fungi, but no direct experimental evidence of the extent of pore colonisation by either soil bacteria or fungi is available. Theoretically, pores with diameters of 1 to 4μm and 2 to 64μm should be accessible to soil bacteria and fungal hyphae respectively (Swift et al., 1979), including hyphae of AM fungi (Saito, 1990). In terms of biochar for initial characterisation, biochar particles mounted on stubs were easily prepared even when crushed, uncrushed or cut to observe the internal micro-environment. Most of the biochar particles were spilt open or cut for observation in other studies (Moskal-del Hoyo et al., 2010). Biochar particles are usually fractured or sectioned for observation (Moskal-del Hoyo et al., 2010). For the biochar used here, particles were either fractured or embedded in resin and polished in cross section to provide additional information on the pore connectivity, which is an important criterion for microbial colonisation. Indeed, this form of visualisation showed that limited connectivity of pores in this biochar could restrict colonisation by fungal hyphae, and confine it to surface layers. Although visualisation of pores was possible,
quantitative data on pore sizes were only obtained from SEM micrographs. In this work, quantification of biochar porosity was achieved from analysing scanning electron micrographs using Image J. Surface cracks in biochar particles may be important points of entry for fungal hyphae for colonisation of internal spaces within forms of biochar that have limited pore connectivity.

Compared to quantifying the pores from micrographs achieved from cracking biochar particles and mounted on stub, quantifying the pores and measuring them on polished slices of resin embedded biochar was easier due to the flat surface and correct orientation. Thus, resin embedded biochar preparation was appropriate for observing internal structures of biochar. However, this method had several limitations. Slicing and polishing the biochar particles, as the normal practices in microscopy preparation of solid particles, may not expose representative pore distribution for the whole particle due to their irregular shape and size. For example, in deeper sections of biochar particles, more pores could be discovered than in sections closer to the surface. Care and consistency should be taken regarding biochar thin section preparation so that both transverse and longitudinal cross-sections could contribute to pore architecture observation. These limitations of microscopy techniques also limit further information such as degree of connectivity between the pores, which can be important for microbial accessibility in each pore. Combination of both techniques (polished slices of resin embedded biochar and biochar mounted on stubs) could complimented each other and generate detailed information on physical nature of biochar.

Characterisation of element composition of various biochars was achieved using XRD and SEM-EDS. Mineralogy and element composition analysis using XRD is mainly representing the bulk analysis of biochar ash. Woody biochars in this study from XRD analysis showed that they were dominated by quartz and calcite which similar to most woody biochar (Grabert et al., 2010). SEM-EDS analysis in contrast, allowed identification of heterogeneity in individual particles within a biochar type. This was in line with characterisation of biochar using electron microscopy techniques and other method from previous studies in which biochar was heterogeneous in nature in terms of
mineralogy, elemental distribution and composition (Graber et al., 2010; Joseph et al., 2010; Verheijen et al., 2009, 2012). These variations had been shown to have various impacts on soil chemical amelioration by biochar (Spokas et al., 2009, 2011, 2012a; 2012b), but the impacts of these variations for microbial colonisation and preferential are not clear.

Heterogeneity among biochar from different parent material has been widely studied (Downie et al., 2009), but heterogeneity within a biochar source is often overlooked. These heterogeneities were observed in terms of element distribution, pore distribution and surface structure of a biochar (Lehmann and Joseph, 2009). The variability in the physical nature of biochar studied here corresponded with that reported for other biochar characterisations (Downie et al., 2009; Graber et al., 2010). Parent material and plant parts normally determine the porous structure of biochar (see Chapter 4). Heterogeneity of biochar especially within one source was further evaluated for Wundowie biochar. This was necessary because the initial XRD data on the minerals distribution varied in different particle sizes. Following to this, SEM-EDS further validated XRD analysis indicated the variability of minerals in every particle (sizes ranges of 0.5-1 mm, 1-2 mm, 2-4 mm) analysed, corresponding to XRD analysis.

Contamination of feedstock and interactions between the plant (feedstock) with soil could have contributed to variable mineralogy and elemental data found in the Wundowie biochar samples. Hence, it is crucial to characterise biochar prior to experimental use and studying the background of the feedstock in production of any biochar. In our case, Wundowie biochar was collected as from 35 year old stock pile where it was kept in storage (Blackwell et al., 2010). Both storage and handling of biochar need to be taken into consideration as biochar may contain moisture (Solaiman et al., 2010). Storage conditions may influence microbial colonisation of the biochar prior to application to soil.

As expected following Harris (2002) observations on crystallization problem by Calcofluor White, biochar particles stained by Calcofluor White also exhibited crystallisation and background staining. In contrast, SR 2200 met the expectation as the
better fluorescent brightener when used to stain microorganisms in and on biochar. Preservation, staining with efficient fluorescent brightener SR 2200 and preparation including careful handling and rinsing to avoid losing the fungal hyphae of colonised biochar particles were found crucial for optimum microscopy purposes. Visualisation of microorganisms associated with biochar was possible on biochar surfaces, which had been crushed and biochar fragments that had been left intact.

Visualisation of fungal hyphae on biochar surfaces was successful using both fluorescence microscopy and SEM. It was easy to locate fungal hyphae on the surface of biochar as demonstrated in this soilless Petri dish study and in previous investigations (Ascough et al., 2010b; Moskal-del Hoyo et al., 2010). Furthermore, the level of fungal colonisation in soilless media was much greater than that which occurred on biochar incubated in soil. This was in line to previous work of Ascough et al. (2010b) in which observations were made based on a laboratory trial in the absence of soil (Ascough et al., 2010b). However, the extent of colonisation by fungal hyphae could be an overestimation because similar levels of colonisation were not observed when biochar was incubated in soil as part of the present study. Fungal hyphae in or on biochar particles retrieved after incubation in soil did not extensively colonise biochar particles compared with the soilless trial. This was confirmed in our study using a combination of fluorescence microscopy and SEM techniques. Fluorescence microscopy highlighted fungal colonisation while SEM provided greater detail into the structural relationship between the fungal hyphae and biochar surface.

There was added difficulty in quantification of fungal hyphae due to the presence of soil particles. Visualisation of the fungal network growing on biochar surfaces could be detected either using fluorescence microscopy or SEM. Fluorescent staining allowed confirmation of fungal hyphae using fluorescent brightener SR 2200 and this gave some indications of where fungal hyphae were located when observed under SEM. It is a normal procedure to first make observations under an optical microscope or fluorescence microscope before analysing further under SEM, as was found by Moskal-del Hoyo et al. (2010).
Soil particles were observed adhering to surfaces for biochar retrieved from soil. The impact of soil particles that enter biochar pores following incubation in soil is not known, but it would likely influence the capacity of biochar to act as a habitat for fungal hyphae. Some smaller pores were often covered by soil particles. Soil particles entering these pores include clay, silt and organic compounds (Joseph et al., 2010). Soil particles could either introduce microorganisms into the internal environment of biochar, or alternatively, limit their access. Predictions based on laboratory studies of microbial inoculated biochar in soilless media could overestimate the role of biochar as a habitat for soil fungi if soil particles limited access or if there is little connectivity between pores within the biochar.

Soil particles found adhered onto biochar surfaces and inside the smaller pores from biochar retrieved from soils could limit the quantification of fungi colonisation degree and preferences in various biochar pore sizes. The changes were expected and in line with in comparison to the biochar retrieved from soil (Brodowski et al., 2005). The influences of soils appeared in biochar pores is unclear. As biochar pores may be stacked (structurally), it would be expected that each biochar pore may change and influenced by soil particles and soil microorganisms. Soil clogging may be treated as 1) a mechanism for introduction of soil microorganisms to biochar internal microenvironment or 2) a mechanism for restricting air, water, space and movement of soil microorganisms in biochar pores.

Quantification of soil fungi in biochar pores was difficult to assess both in and on biochar particles. Careful consideration is necessary when observing and quantifying microorganisms in biochar pores. Fluorescence microscopy was used in this methodology as the first indication of distinctively stained fungi with other materials found on biochars. However, it has limitation in terms of magnification and resolution. Biochar observed under optical microscope for fluorescence microscopy was not flat and even. The biochar particles retrieved from the soil were normally > 0.5 mm size and could not be flattened or destructively prepared for observation under the microscope. This means the biochar could not be treated as are normal thin sections on slides when
observing fungi associated with biochar after incubation in soils. This limited the highest magnification to only 40x magnification to observe fungi on biochars due to its thickness and also resulted in a smaller depth of field. Observation on fluorescent bacterial colonies was not possible and difficult at this magnification. Other than that, observations were limited by the absence of smooth and even surfaces on the biochar, creating a further problem in focusing on the fungi.

Observation using 3D imaging techniques such as X-ray tomography and MicroCT would enable better physical characterisation of biochar (Bird et al., 2008). Non-destructive sample preparation for 3D imaging could overcome the problems associated with sample preparations for fluorescence microscopy and SEM in this study. Porosimetry data and 2D could be validated by 3D data (Rosenberg et al., 1999). It also applies when categorizing pores inhabited by fungi in which 3D visualisation, followed by modeling will improve understandings and fill the gap not possible by 2D visualisation. 3D observation such as MicroCT and X-ray tomography could overcome these limitations and can be accompanied by other supporting analyses such as qPCR on biochar particles to provide both visual evidence and quantification of microorganisms inhabiting the biochar and their activity. 3D imaging such as X-ray tomography could also provide insights into microbial colonisation and factors involved such as soil particles, water and pore connectivity in biochar microenvironment which influences the role of biochar as potential habitat for soil fungi under natural soil conditions.
3.5 Conclusion

Appropriate sample preparation and 2D microscopy analyses have enabled characterisation, visualisation and provided information on biochar structural characteristics and its micro-environment as a potential habitat for soil fungi. 2D imaging techniques such as SEM and SEM-EDS provided information on characterisation (physical and elemental) through visualisation of biochar surface and pore structure as well as providing micrographs for quantification of pore size distribution from biochar mounted on stubs and resin embedded biochar. Heterogeneity of biochar within and between feedstocks was observed. Energy dispersive X-ray (EDS) microscopy also enabled confirmations on variability of biochar elemental and mineral properties obtained from X-ray diffraction analysis. The appropriate sample preparation remain crucial especially in preserving and staining soil fungi with SR 2200 associated to biochar including fungal hyphae for 2D microscopy observations. The preparation and observation of the intact biochar particles colonised by fungal hyphae in soil posed a range of difficulties including obstruction by the presence of soil particles. Fluorescence and electron imagings of biochar retrieved after incubation in soilles and soil have successfully visualised fungal colonisation on biochar surfaces and pores. The extent of hyphal colonisation of biochar incubated in soil was much less than for biochar artificially inoculated with fungi in a soilless medium. Further observation of biochar surfaces and fractured biochar that exposed the internal biochar structures raised questions about the effects of interactions between soil particles and biochar for microbial colonisation. The role of biochar as a habitat appeared to be minimal after incubation in this agricultural soil for 56 days. Several limitations and difficulties occurred when using 2D imaging especially including uneven biochar surfaces, obstruction by the presence of soil particles and difficulties with observing microorganisms inside pores.
CHAPTER 4

SOIL MICROBIAL RESPONSES TO BIOCHAR OF VARIOUS PARTICLE SIZE, SURFACE AND PORE PROPERTIES

4.0 Abstract

Biochar is known for its heterogeneity, especially in pore and surface structure associated with pyrolysis processes and source of feedstock. The surface area of biochar is likely to be an important determinant of the extent of soil microbial attachment, whereas the porous structure of biochar is expected to provide protection for soil microorganisms. Potential interactions between biochar of different sources and particle size were investigated in relation to soil microbial properties in a short-term incubation study. Three particle size (sieved) fractions (0.5-1 mm, 1-2 mm, 2-4 mm) from three woody biochars (Simcoa, Wundowie and Saligna) were incubated in soil at 25°C for 56 days. Observation by SEM and characterisation of pore and surface area showed all three woody biochars provided potential habitats for soil microorganisms due to their high porosity and surface area. They were also found to be structurally heterogeneous, varying in porosity and surface structure both within and between the biochar sources. After 56 days incubation, hyphal colonisation was observed on biochar surfaces and in larger biochar pores. Soil clumping occurred around biochar particles, cementing and covering biochar pores exposed on surfaces. This may have influenced surface area and pore availability for fungal colonisation hence the biochar potential as habitat once biochar is deposited into soil. Increased particle size for each biochar source had little effect on soil microbial biomass carbon and phosphorus after 56 incubation days. However, particle size effects on soil microbial biomass carbon and phosphorus was significant after 28 days of incubation. The mechanisms associated with biochar changes and soil microbial biomass were unclear. Overall, Simcoa biochar had greater potential as a habitat than did Wundowie and Saligna due to its higher porosity and surface area, and showed the highest soil microbial biomass carbon after 28 days of incubation in soil. Transient changes in soil microbial biomass without a consistent trend were observed across biochars during the 56 days incubation.
4.1 Introduction

Feedstock characteristics and pyrolysis conditions contribute to biochar heterogeneity (Downie et al., 2009). Previous research on biochar as a soil amendment showed its potential to improve soil microbial properties (Glaser et al., 2002; Oguntunde et al., 2004; Yamato et al., 2006) and the occurrence of microorganisms in biochar or coal obtained after fire aging from 100 to 300 years (Hockaday et al., 2007; Zackrisson et al. 1996). Lack of consistency in experiments conducted on similar biochar and other contradictory outcomes of biochar research may be attributed to the heterogeneity of these pyrolysed materials. In general, woody biochars are porous with high surface areas which could provide habitat for soil microorganisms (Thies and Rillig, 2009; Graber et al., 2010). Although the effect of addition of woody biochar to soil on soil chemical properties has been studied (e.g. Dempster et al., 2012a), the influence of biochar heterogeneity, especially porosity and surface structure, on soil microbial communities is largely unknown. Several mechanisms have been proposed to explain how biochar may interact with the soil particles and influence soil microbial communities. This may be through creation of microhabitats (Zackrisson et al., 1996; Wardle et al., 1998), introduction of labile organic compounds for microbial growth (Graber et al., 2010) and activity or processes leading to nutrient retention (Cornelissen et al., 2004; Keech et al., 2005).

The high surface area of biochar is potentially a determinant of soil microbial attachment whereas the porous structure and particle size may affect microbial habitat provision and protection (Thies and Rillig, 2009; Lehmann et al., 2011). Surface attachment may offer protection to soil microorganisms and the opportunity for interactions with biochar. Surface-associated fungi and bacteria would able to degrade nutrients on biochar surfaces. In addition, the surface area of biochar particles may influence microbial requirement for water and nutrients (Atkinson et al., 2010; Sohi et al., 2010; Lehmann et al., 2011). It has also been claimed that biochar has a greater ability than other soil organic material to adsorb cations and organic matter (Liang et al., 2006). The potential to manipulating biochar roles in relation to the soil microbial preference as habitat and provision in soil depends on understanding the nature of
biochars which differ in pore and surface structure and the changes as well the processes involved which could influence soil biological properties.

There are few investigations of biochar particle size and microbial response in soil. Biochars occur as large (> 4mm) to fine particles (< 20 μm) (Glaser et al., 2000). Commonly, biochar contains a mixture of particle size (Downie et al., 2009) or it is ground after production into smaller fractions (Sohi et al., 2010). Woody biochars normally occur in large fragments (Blackwell et al., 2009; Downie et al., 2009) and may be less practical for use in agricultural (Blackwell et al., 2009). Biochar surfaces can gradually oxidise in with exposure to air, activities of soil microorganisms or roots and thereby increasing their cation exchange capacity (Joseph et al., 2010). Changes to the surface of biochar after exposure to the soil environment may also alter water and nutrient retention properties of the biochar (Joseph et al., 2010). The size of the biochar pieces applied to soil is not expected to greatly affect nutrient uptake but may alter surface properties which influence microbial attachment (Verheijen et al., 2009).

The aims of this study were: (i) to characterise three woody biochars varying in particle size and determine their potential as a microbial habitat in soil, (ii) to observe changes in biochar and fungal colonisation during a short-term (56 days) incubation through microscopy observation and, (iii) to monitor potential effects of biochar source and particle size on soil microbial biomass. Sample preparation and microscopy techniques described in Chapter 3 were applied for observation of biochar and associated microorganisms especially fungi (Figure 4.1). It was expected that the potential of biochar as a microbial habitat in soil would differ among biochar types or sources and particle sizes.

It was hypothesised that:

1. Woody biochars would be suitable as potential habitat for soil microorganisms based on their high porosity, pore size distribution and surface area, and
2. Biochar particles with higher porosity or smaller particle size would harbour more microbial biomass than those with smaller pores or larger particle size.
Chapter 4: Soil Microbial Responses to Biochar

BIOCHAR MANAGEMENT

CHARACTERISATION OF BIOCHAR (CHAPTER 3)

BIOCHAR SOURCE AND PARTICLE SIZE (CHAPTER 4)

BIOCHAR AND ORGANIC MATTER (CHAPTER 5)

Microscopic observations

Experiment 6.1

Experiment 6.2

BIOCHAR AMOUNT AND FERTILISER (CHAPTER 6)

Experiment 7.1

Experiment 7.2

Figure 4.1 Conceptual flow of experimental designs of biochar and soil amendments in Chapter 3 correlated to Chapter 4 (highlighted in blue box)
Chapter 4: Soil Microbial Responses to Biochar

4.2 Materials and Method

4.2.1 Experimental design

This incubation experiment involved three particles size ranges (0.5-1 mm, 1-2 mm, 2-4 mm) for each three biochars (Saligna, Wundowie and Simcoa (Table 3.1 in Chapter 3). Biochars were added to soil at an amount equivalent to 50 ton/ha, to optimise the response of soil microorganisms. The soil and biochar mixtures (incorporated and mixed by hand) were incubated aerobically in individual jars in a 25°C controlled room for 56 days. Soil/biochar mixtures were destructively collected for analysis on day 14, 28 and 56 after the start of incubation period. An equivalent set of soils was incubated in glass jars with a gas septum, water was added and adjusted to 45 percent water holding capacity and sealed to trap CO₂ for microbial respiration (Anderson, 1982).

4.2.2 Soil used for incubation

Soil (0-10 cm) was collected from Moora, WA, sieved (<2 mm) and kept at 4°C until used in the experiment. The soil samples were sent to CSBP Soil and Plant Laboratory, CSBP Ltd. (Kwinana, Western Australia) for basic soil chemical analysis and characterisation. The soil was acid (pH 4.3 (CaCl₂)) and contained 3% carbon, 12 mg/kg phosphorus, 65 mg/kg nitrate, and 1 mg/kg ammonium. All the analysis by CSBP Soil and Plant Laboratory was conducted following Rayment and Lyons (2011). Ammonium and nitrate were determined with 2M KCl-steam distillation and automated colour finishes, phosphorus using bicarbonate extractable phosphorus (Colwell, 1965), and organic carbon was measured by the method of Walkley and Black (1934). Soil pH was measured using a soil to solution ratio of 1:5 and textural analysis was assessed by particle distribution.

4.2.3 Biochar sources and size fractions

The three biochars (Saligna, Wundowie and Simcoa (Chapter 3; Table 3.1) were sieved through a series of sieves (4 mm, 2 mm, 1 mm, 0.5 mm) and collected as three particle size ranges (0.5-1 mm, 1-2 mm and 2-4 mm). Only biochar particles between 0.5 mm and 4 mm were selected and applied to soil for easier retrieval after incubation for microscopy and analytical purposes. The biochars were crushed and characterised by X-
ray diffraction analysis (described in Chapter 3). Carbonate analysis was conducted using the method of Rayments and Lyons (2011) and reported as carbonate equivalent.

4.2.4 Microscopy observations

For microscopy imaging, biochar pore and surface characteristics were observed using scanning electron microscopy (SEM) before (on the original biochar stocks) and 56 days after incubation in soil. Before addition to soil, the biochars were treated and prepared based on the methods described in Chapter 3 (Table 3.2). The pore size distribution of biochar was based on the examination of 10 particles of 0.5-1 mm sizes using SEM. Measurements and analyses were made from the micrographs using NIH freeware package Image J software. After 56 days, the incubated and colonised biochar particles were retrieved and fixed in glutaraldehyde until further use. Colonised, fixed Simcoa biochar particles retrieved from soil after 56 days incubation were then stained with fluorescent brightener SR 2200, before imaging using fluorescence microscopy. They were critical point dried, mounted on carbon tabs and examined with SEM as described in Chapter 3. Only Simcoa biochar particles were selectively examined as an example of the changes resulting from biochar-soil interactions.

4.2.5 Pore size distribution analyses and surface area measurement

SEM observations were performed on both intact and manually broken biochar particles (from the original biochar stocks) to verify the degree of porosity. Determination of biochar surface area was made using BET (Brunauer, Emmett, Teller) surface area analyser (Macrometics Gemini 2375 instrument with a Vac Prep 0612) (Braunauer et al., 1938). The biochar particles were maintained in their original sieved sizes (0.5-1 mm, 1-2 mm and 4 mm sizes), prepared in quantities of less than 0.1 g and outgassed at 300°C for 8 hours following the method described by Yu et al (2006). The surface area measurements were calculated based on 5 analysis points.

4.2.6 Soil microbial biomass and respiration

Soils samples collected at day 14, 28 and 56 after the start of incubation period were immediately analysed for microbial biomass carbon and phosphorus. Microbial biomass carbon (MBC) was determined using the fumigation extraction method (Vance et al., 1987). Fumigated and non-fumigated soil samples were placed in vials containing 20 g
soil dry weight equivalent, with 80 mL of 0.5M potassium sulfate and shaken for 1 hour. The dilution ratio of 1:7 was used to determine organic carbon using Oxidisable-C (Shimadzu TOC-5000a; Shimadzu Scientific instruments). The difference in organic carbon between fumigated and non-fumigated samples was calculated as MBC. A factor of 0.45 was applied to data for adjustment as recommended for agricultural soils (Wu et al., 1990; Joergensen, 1996; Joergensen and Mueller, 1996)

Microbial biomass phosphorus (MBP) was determined using the anion exchange membrane method (Kuono et al., 1995). Anion exchange membrane (AEM) strips were shaken with suspensions of 2 g dried soil in 30 ml distilled water with and without 1 ml hexanol addition for fumigation samples. After 16 hours shaking, the AEM strips were rinsed with 30 ml distilled water to remove soil. Phosphorus adsorbed by the AEM strips was then eluted by 0.5 M HCL, shaken for 2 hours, and determined using the colorimetric molybdenum blue method (Murphy and Riley, 1962). The amount of CHCl₃-released phosphorus was calculated from the difference between the amount of inorganic phosphorus adsorbed by the AEM in non-fumigated and fumigated soils.

Soil respiration (CO₂) in gas samples was run over 56 days, measured in an experimental assay in glass jars using the Analytical Development Co (ADC) CO₂ Gas Analyser series 225. Carbon dioxide absorbed infra red radiation and the relationship between CO₂ concentration and absorbance was set linear over a range of concentrations (Anderson, 1982). The 1 mL headspace gas was injected into the carrier gas stream (N₂) via a septum and through the detector. The response was measured on a chart recorder. Calibration of the instrument was done by introducing different volumes of a known concentration of carbon dioxide in an inert gas from 5.06% CO₂ standard (BOC Gases). The headspace was then released and allowed to adjust to atmospheric CO₂ concentration, and resealed for analysis. Atmospheric CO₂ concentration was adjusted based on values for a control treatment without soil.

4.2.7 Statistical analysis

Analysis of variance (ANOVA) was performed using Statistical Analysis System (SAS) software version 8.02 for Windows (SAS Institute Inc. (1992-98). Comparison of
treatment means (within each biochar) was made using the Duncan’s Multiple Range Test (DMRT) at 5% confidence level.

4.3 Results

4.3.1 Characterisation of biochars

The history of each woody biochar (i.e. pyrolysis conditions and parent materials) is summarised in Table 3.1 (Chapter 3). Simcoa biochar had the highest C content (73.8%) followed by Wundowie and Saligna biochars. Simcoa and Saligna biochars were alkaline, while Wundowie biochar had the lowest pH (Table 4.1).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Simcoa</th>
<th>Wundowie</th>
<th>Saligna</th>
</tr>
</thead>
<tbody>
<tr>
<td>C Content (%)</td>
<td>73.8</td>
<td>61.1</td>
<td>58.2</td>
</tr>
<tr>
<td>EC (mS/cm)</td>
<td>0.54</td>
<td>0.07</td>
<td>5.73</td>
</tr>
<tr>
<td>pH (H\textsubscript{2}O)</td>
<td>8.65</td>
<td>4.89</td>
<td>8.15</td>
</tr>
<tr>
<td>pH (CaCl\textsubscript{2})</td>
<td>7.62</td>
<td>3.74</td>
<td>7.42</td>
</tr>
<tr>
<td>Carbonate (mg/kg)</td>
<td>2.77</td>
<td>3.08</td>
<td>6.34</td>
</tr>
</tbody>
</table>

*EC: electrical conductivity, pH water: biochar in 1:5 biochar:water, pH CaCl\textsubscript{2}: biochar in 1:5 biochar: CaCl\textsubscript{2}*

Prior to application to soil, sieved fractions of each biochar revealed high variation in particle size fraction (Table 4.2). Simcoa, Wundowie and Saligna biochars had a similar proportion of particle size 1-2 mm. Wundowie biochar, had the smallest fraction in the 2-4 mm range. Saligna and Wundowie biochars had no particles greater than 4 mm.

**Biochar physical characteristics: porosity from microscopy observations**

Pore size distribution varied among the three woody biochars (Table 4.3). SEM micrographs showed that woody biochars had high variability in porosity and pore size as well as in surface area (Table 4.3, Table 4.4). All three biochars were made from wood and were macroporous. Saligna biochar made from the woody plant *Acacia saligna* had pores that were most uniform in size. The percentage of pores size less than
50 µm estimated from electron micrographs was highest in Saligna biochar, followed by Simcoa and Wundowie biochars (Table 4.3).

**Table 4.2 Particle size distribution of each biochar after sieving**

<table>
<thead>
<tr>
<th>Biochar</th>
<th>Size &gt;4 mm</th>
<th>2-4 mm</th>
<th>1-2mm</th>
<th>0.5-1mm</th>
<th>Finer than &lt;0.5mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simcoa</td>
<td>7</td>
<td>28</td>
<td>28</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Wundowie</td>
<td>-</td>
<td>8</td>
<td>29</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td>Saligna</td>
<td>-</td>
<td>23</td>
<td>30</td>
<td>28</td>
<td>19</td>
</tr>
</tbody>
</table>

**Table 4.3 Pore size distribution (percentage) determined from SEM micrographs using Image J software.**

<table>
<thead>
<tr>
<th>Biochar ID</th>
<th>Pore size &lt; 50 µm</th>
<th>Pore size 50 µm – 100 µm</th>
<th>Pore size &gt; 100 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simcoa</td>
<td>86</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Saligna</td>
<td>95</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Wundowie</td>
<td>5</td>
<td>85</td>
<td>10</td>
</tr>
</tbody>
</table>

*Biochar pore (diameter) size distributions were based on a mean of 10 particles

Characterisation of morphological heterogeneity in pore and surface structures using SEM demonstrated differences both within and among biochars. Examples of pore variation for each biochar are exhibited in Plate 4.1a, b,c). Simcoa biochar (Plate 4.1a) had fewer larger pores than did Wundowie biochar (Plate 4.1b). Saligna biochar had the least number of larger pores (Plate 4.1c). Plate 4.1 d shows unknown compounds (tar or condensed volatile) inside pores of Simcoa biochar. Plate 4.1e showed the presence of fungal hyphae growing inside pores of Simcoa biochar prior to application to soil.

**Biochar physical characteristics: surface area from BET analysis**

Surface characteristics and pore volume were determined at five points in the biochar particles (Table 4.4). Biochars derived from wood were heterogeneous, and this heterogeneity was observed in all particles sizes. Problems related to degassing and determination of biochar surface area at multiple points were encountered. BET surface
### Table 4.4 Surface area, pore volume and pore diameter on various particle sizes and biochar.

<table>
<thead>
<tr>
<th>Biochar</th>
<th>Particle size (mm)</th>
<th>Surface Area, SA (m²/g)</th>
<th>Volume (cm³/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saligna</strong></td>
<td>0.5-1.0</td>
<td>-15.51</td>
<td>50.30</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>16.92</td>
<td>6.79</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>3.36</td>
<td>2.91</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Simcoa</strong></td>
<td>0.5-1.0</td>
<td>314.98</td>
<td>83.46</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>355.58</td>
<td>96.83</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>243.06</td>
<td>92.07</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Wundowie</strong></td>
<td>0.5-1.0</td>
<td>16.33</td>
<td>26.86</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>11.72</td>
<td>-6.40</td>
</tr>
<tr>
<td></td>
<td>2-4</td>
<td>7.72</td>
<td>18.27</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cum. Pore Volume = Cumulative pore volume; SA = surface area. The negative values in micropore area were obtained as output from BET (Brunauer, Emnett, Teller) surface area analyzer at 5 analysis points basis.*
Plate 4.1 SEM micrographs of pores in woody biochars: a) Simcoa biochar, b) Wundowie, c) Saligna biochar, d) Simcoa biochar pore filled with unknown material, e) fungal network (arrow) in larger pores (100 micron) of initial Simcoa biochar stock, f) soil particles adhering onto/into Simcoa biochar pores when incubated in soil taken from Moora, WA for 56 days. Scale bar= 200 µm.
area of biochar ranging from 5.32 m²/g to 452 m²/g was calculated for Wundowie and Simcoa biochars respectively. External surface area was highest in particle size 0.5-1 mm of Saligna and Wundowie biochars. However, for Simcoa biochar, the highest external surface area was generated by particles in the range of 1–2 mm. BET surface area decreased with an increase in particle size, as shown for Saligna biochar where a 6 fold decrease in BET surface area was calculated for particles 2-4 mm compared to 0.5-1 mm range. Most of the surface area was associated with biochar pores (micropore area). Saligna and Wundowie biochars, despite being derived from different feedstocks, both had a lower micropore area, external surface area and BET surface area with the highest surface area found in particles within the range of 0.5-1 mm than the 2-4 mm particle size fraction. In contrast, the highest surface area was found in particles 1-2 mm for Simcoa biochar.

4.3.2 Biochar interactions with soil: microscopy

After 56 days incubation, biochar particles retrieved from soil were separated into 0.5-1 mm, 1-2mm and 2-4 mm for the allocated treatments. Examples of comparison of Simcoa biochar, before (Plate 4.1a, d, e) and after (Plate 4.1f) application to soil showed that pore availability for microbial habitat could be affected by soil particles cementing and/or occurring within biochar pores. Some of the biochar particles had soil attached within pores (Plate 4.1f). The fungal hyphae found after biochar had been deposited in soil could not be confirmed to be similar to or different to the hyphae present in the biochar prior to application to soil. After 56 days incubation in soil, both smaller and larger pores of biochar were observed to be clogged by soil particles. The blockage of smaller pores by soil particles was greater than in larger pores (Plate 4.1f).

As shown in Plate 4.2 and Plate 4.3, soil particles were attached to the external surfaces of Simcoa biochars (Plate 4.2) after 56 days incubation in soil. Hyphal networks were easily observed via fluorescence and SEM techniques. However, quantification was not possible as this incubation was done under natural soil conditions. Problems (see Chapter 3) were encountered with focusing on and observing hyphae on some biochar particles due to their uneven surfaces when viewed using the fluorescence microscope (Plate 4.2e,f). Some fungal hyphae observed on surfaces extended into larger pores within the biochar particles (Plate 4.3).
Plate 4.2 Scanning electron micrographs (a-c) and fluorescent micrographs (d-f) of incubated and colonised Simcoa biochar particles with soil particles and fungal network (arrow) on biochar external surfaces of Simcoa biochars incubated in soil taken from Moora, WA for 56 days. Micrographs (e) and (f) taken from a similar spot of one biochar particle highlighted the problem associated with focusing and observing microorganisms on particular biochar particles and with uneven surfaces. Scale bar = 100 µm.
Plate 4.3 Scanning electron micrographs of incubated and colonised Simcoa biochar particles with soil particles cementing the surfaces and pores. Micrographs a, b, c show fungal hyphae (arrow) observed in pores of incubated Simcoa biochars while micrographs d, e, f show soil particles on biochar surfaces (d) and pores (e, f) of Simcoa biochars incubated in soil taken from Moora, WA for 56 days. Scale bar = 20 µm.
Several examples of fungal colonisation of larger pores were observed (Plate 4.3). Larger pores of some incubated Simcoa biochar particles had fewer attached soil particles and fungal hyphae were visible (Plate 4.3a-c). These hyphae were attached to the wall of larger pores (sized about 100 micron). In contrast, smaller pores (20 μm) were clogged by soil particles (Plate 4.3c-f) limiting observation of fungal hyphae. Soil aggregates of more than 20 μm were associated with biochar surfaces (Plate 4.3c, e, f).

4.3.3 Soil microbial biomass, respiration and soil pH
There was no significant effect of either biochar type or particle size on microbial biomass carbon or on microbial biomass phosphorus after 14 days (data not shown). After 28 days, the only effect on microbial biomass carbon was observed for Saligna biochar which increased in fractions greater than 1mm (Table 4.5). Microbial biomass phosphorus increased with increasing size fraction for all three biochars (Table 4.5). Little change was observed in either microbial biomass carbon or microbial biomass phosphorus after 56 days (data not shown). There was no effect of biochar type or biochar particle size fraction on soil respiration or soil pH at any measurement time during the incubation. No significant correlation was found among microbial biomass, respiration and soil pH for any of the three woody biochars.
### Table 4.5 Effects of biochar particle size on soil microbial biomass and soil pH at the 28th day incubation of Saligna, Wundowie, and Simcoa biochar in soil.

<table>
<thead>
<tr>
<th>Biochar</th>
<th>Particle size</th>
<th>MBC (mg C kg(^{-1}) dry soil)</th>
<th>MBP (mg P kg(^{-1}) dry soil)</th>
<th>Microbial (CO(_2)) respiration (μg g(^{-1}) CO(_2) dry soil day(^{-1}))</th>
<th>Soil pH (water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saligna</td>
<td>0.5 – 1</td>
<td>92.72 b</td>
<td>0.76 b</td>
<td>75.44 a</td>
<td>4.87 a</td>
</tr>
<tr>
<td></td>
<td>&gt;1.0 – 2.0</td>
<td>155.61 a</td>
<td>0.92 b</td>
<td>72.62 a</td>
<td>4.84 a</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0 – 4.0</td>
<td>182.26 a</td>
<td>1.83 a</td>
<td>79.58 a</td>
<td>4.78 a</td>
</tr>
<tr>
<td>Wundowie</td>
<td>0.5 – 1</td>
<td>156.18 a</td>
<td>0.65 b</td>
<td>79.81 a</td>
<td>4.65 a</td>
</tr>
<tr>
<td></td>
<td>&gt;1.0 – 2.0</td>
<td>152.73 a</td>
<td>1.39 a</td>
<td>68.78 a</td>
<td>4.65 a</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0 – 4.0</td>
<td>165.84 a</td>
<td>1.65 a</td>
<td>74.18 a</td>
<td>4.62 a</td>
</tr>
<tr>
<td>Simcoa</td>
<td>0.5 – 1</td>
<td>164.14 a</td>
<td>0.87 b</td>
<td>77.24 a</td>
<td>4.78 a</td>
</tr>
<tr>
<td></td>
<td>&gt;1.0 – 2.0</td>
<td>176.88 a</td>
<td>1.61 a</td>
<td>78.69 a</td>
<td>4.82 a</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0 – 4.0</td>
<td>197.85 a</td>
<td>1.33 a</td>
<td>83.79 a</td>
<td>4.77 a</td>
</tr>
</tbody>
</table>

DMRT of means at P < 0.05 and levels of significance for a two factor ANOVA:

<table>
<thead>
<tr>
<th>Factor</th>
<th>P-value</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochar type (B)</td>
<td>0.0404*</td>
<td>0.0001*</td>
</tr>
<tr>
<td>Particle size (P)</td>
<td>0.0117*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>B*P</td>
<td>0.1541 ns</td>
<td>0.7518 ns</td>
</tr>
</tbody>
</table>

Means followed by the same lower case letters within a biochar in a column are not significantly different at P < 0.05 by Duncan Multiple Range test (DMRT). * = significantly different at P <0.05, respectively and ns = not significant. MBC = Microbial biomass Carbon, MBP = Microbial biomass Phosphorus, CO\(_2\) emission = cumulative carbon dioxide.
4.4 Discussion

Characterisation of three woody biochars was made based on the hypothesis that woody biochar would provide potential habitats for soil microorganisms because of their high pore and surface area distribution. This hypothesis was supported by microscopic observation and surface area analyses. Biochars known to be highly porous, as shown by Saligna, Simcoa and Wundowie biochars, generated high surface area. This was in line with woody biochar made from *Eucalyptus* plant material (Blackwell et al., 2010). Blackwell et al. (2010) reported 0.49 m²/g of pores 2-5µm measured by porosimetry in the Simcoa biochar. The value of 86% of the pores sized less than 50 µm size in Simcoa biochar found in this study could also include similar pore size of 2-5 µm which generated the highest BET surface area. Previous observations for woody biochars were found to be highly porous compared to biochar produced from organic sources such as manures, leaves and other wastes (Bird et al., 2008; Downie et al., 2009).

Heterogeneity in pore size distribution and chemical composition across woody biochars observed in this study was expected to be due to be associated with the different feedstocks. Wundowie biochar was a mixture of two *Eucalyptus* spp. And it exhibited differences in pore distribution to that of Simcoa which was prepared from one *Eucalyptus* sp. and to Saligna biochar which originated from *Acacia saligna*. Saligna and Simcoa had high porosity with a lower percentage of larger pores whilst Wundowie biochar had low porosity but a high percentage of smaller pores. The physical heterogeneity of these woody biochars was in line with the finding that heterogeneity of biochar pore structure across sources was mostly attributed to pyrolysis conditions and parent material (Ozçimen and Ersoy-Meriçboyu, 2010). A pyrolysis temperature of 400°C was used to produce Saligna biochar and a higher temperature (>600°C) was used for Wundowie and Simcoa biochars. Similar effects of pyrolysis conditions and varying feedstocks on the final biochar outputs and ameliorative effects to soil have been observed by others (Chan et al., 2007, 2008b; Downie et al., 2009; Uchimiya et al., 2010).

While heterogeneity of pore and surface characteristics among Saligna, Simcoa and Wundowie biochars is well understood, factors contributing to the heterogeneity in pore
and surface characters within a biochar source are not clear. Various plant parts such as lateral branches and trunks which differ greatly in cellular and chemical structure could attribute to the heterogeneity in pore structure and surface area. The heterogeneities observed within and among biochar sources and particle size ranges could influence the suitability of biochar as a habitat for soil microorganisms.

Biochar with high porosity was expected to harbour greater fungal hyphae associated with greater surface area for attachment and habitable space. Simcoa biochar had more potential as a habitat for soil microorganisms based on its higher surface area than did Saligna biochar or the higher porosity Wundowie biochar. Although larger pores from Simcoa biochar were observed to be colonised by fungi, surface area and pore availability for fungal colonisation is visually restricted if only determined via 2D microscopy techniques as discussed in Chapter 3.

Simcoa biochar consistently revealed higher pore surface area than external surface area and showed three fold greater micropore surface area than external surface area for all particle size ranges examined. When incubated in soil for 28 days, Simcoa biochar consistently had higher and increased soil microbial biomass P than other biochars. Though these biochars had higher porosity or larger pores and were expected to accommodate soil microorganisms and improved soil microbial biomass in soil, this was not always the case throughout this short term 56 day incubation period.

After 56 days incubation, little and inconsistent effects of these three woody biochars on microbial respiration and microbial biomass were observed. Woody biochar was previously shown to increase microbial activity in soils by providing basic microbial requirements including habitat, water, labile source and nutrients (Lehmann et al., 2003; Wardle et al., 2008). Although the effects of soil particle accumulation on biochar after 56 days is not known, the duration of 56 days was justified to be sufficient to observe the biochar effects on soil biology and nutrients availability based on previous studies (Cheng et al. 2008b; Nelson et al., 2011). Cheng et al. (2008b) found that a small amount of wood derived biochar incubated for 56 days was mineralized within the 56 days (2 months) period.
Simcoa biochar which had higher porosity and higher BET surface area compared to Saligna or Wundowie biochar (Table 4.3 and 4.4), had the highest microbial biomass carbon after 28 days of incubation (Table 4.3 and 4.4). With more pores of <50 μm in diameter (Table 4.5), presumably, Simcoa biochar would able to accommodate more soil microorganisms, protecting them from predators, thereby improving their survival and leading to greater soil microbial biomass. Pores of 2–80 μm in diameter are known to occur in most wood-derived biochars and this pore size range could support activity of beneficial fungi (Thies and Rillig, 2009; Hammer et al., 2014). Many pores would be accessible and act as shelter for soil bacteria and fungal hyphae of 1 to 4μm and 2 to 64μm in diameter size (Swift et al., 1979). Recently, Hammer et al. (2014) found that mycorrhizal fungi colonizing biochar surfaces and microsites could improve phosphorus acquisition, supporting the suggestion that biochar from woody feedstocks with high porosity and surfaces could benefit fungal hyphae growth.

External surface area decreased with increasing particle size according to characterisation using the BET surface area machine (Table 4.4) highlighting the potential importance of smaller biochar particle size. Biochar applied to soil as smaller particle size fractions could have improved microbial biomass through available microbial surface attachment, habitable space and hence their activity and survival in biochar and/or in the soil. However, there was little effect of particle size within each biochar source on soil microorganisms measured as microbial biomass phosphorus and carbon after 56 incubation days, although clearer effect of particle size within each biochar source was exhibited at the 28th incubation day.

Larger particle size fractions (2-4 mm) of Simcoa, Wundowie and Saligna biochars resulted in increased microbial biomass phosphorus after 28 days incubated in soil. One of the reasons contributing to an effect of biochar particle size, pore or surface area could be related to soil attachment. The mechanisms which may have contributed to such observation were not clear, but it may be due to localised effects of larger sized particles in soils or to unknown soil interactions with biochar. Although biochar particle size may not strongly affect microbial biomass in soil in which it is being deposited, alterations in biochar surface properties after deposition in soil may influence microbial
attachment (Verheijen et al., 2009). The role of soil on/in biochar particles could overshadow the effect of variation in biochar particle size.

The role of soil particles in clogging biochar pores and in cementing biochar surfaces could overshadow effects of biochar pore and surface structure but this is yet to be understood in relation to the microbial responses and biomass in soil. Furthermore, the influence of soil attachment in affecting microbial activity needs to be demonstrated. However, soil attachment would play a role in introducing microorganisms, nutrients and water sources to the biochar micro-environment which could facilitate colonisation of internal structures of the biochar. Subsequent interactions of biochar with soil could also change biochar available porosity. This has been shown in previous studies in which adsorption of organic matter to biochar can decrease porosity by blocking pores (Kwon and Pignatello, 2005). Improvement in the soil nutrient retention capacity could be another contributing factor (Cornelisson and Gustafsson, 2003, 2004; Cornelisson et al., 2005; Keech et al., 2005). There is also a possibly that there is active use of some biochar components as a metabolic substrate for microorganisms.

There were no changes in soil pH attributable to the application of the three biochars used in this study. Graber et al. (2010) and Anderson et al. (2011) indicated that some other factors may be at play when biochar application via pH changes did not stimulate microbial changes in soil. Possible indications could be the presence of tars or labile compound in or on biochar that serve as substrate for microbial growth in biochar treated soil (Graber et al. 2010; Anderson et al. 2011). Further soil nutrient analyses were not investigated and pH differences in influencing microbial activity on biochar surfaces compared to the bulk soil was beyond the scope of this study. It is likely that some different processes are involved in soil near the biochar compared with those in the bulk soil.

Future examination on biochar as a habitat for soil microorganisms could include pre- and post- determination of changes in biochar surface area. However, technical limitations could pose a challenge. In addition to characterisation of biochar, adequate sampling and replication when characterising biochar is required for accuracy, especially for surface area measurement. Technical issues related to measurement of
surface area using the BET machine, as noted in this experiment, must be recognised to
improve better understanding relationships between biochar surface area and porosity
measurement. This includes concern associated with the outgassing processes for
determination of biochar porosity and surface area at multiple points as observed in
previous studies (Braida et al. 2002; Badalyan et al., 2003; Yu et al., 2006).

Methodological uncertainties include thermal transpiration and outgassing mass of
charred material highlighted associated with the measurement. Various methods of
degassing and the BET measurement heating temperature have been reported (Chun et
al., 2004; Yu et al., 2006). Chun et al. (2004) used approximately 0.2 g char sample
with overnight outgassing (more than 15 hour) at 105 °C but no information on particle
size of the biochar was mentioned. In contrast, outgassing of ground charcoal was
carried out at 300 °C for 8 hour by Yu et al. (2006). However, the biochars used in this
study were not ground, instead they were maintained in their respective particle sizes, so
that it would be possible to estimate external area of different particle sizes as well as
micropore area which generated BET surface area. Different degassing techniques and
analysis methods applied may vary for surface area determination for biochar samples,
either ground and un-ground, and this could be the underlying reason for the negative
values obtained for micropore area analysis in this study. This aspect remains to be
investigated.
4.5 Conclusion

All three sources of woody biochars used in this 56 days incubation experiment (Simcoa, Wundowie and Saligna) demonstrated potential as a habitat for soil microorganisms due to high porosity and surface area. These biochar were found to be heterogeneous, varying in porosity and surface structure both within and between the biochar sources. Fungal hyphae were observed in or on biochar both before and after biochar was deposited in soil. Hyphal colonisation in larger biochar pores could be further studied in relation to the microbial preferences, and factors that attract fungi incolonising larger pores. Once deposited in soil, soil clumping and attachment to the biochar particles was observed. Soil particles found on or in biochar would affect the surface area and blockage of smaller pores. The role of soil on/in biochar particles could overshadow the effect of biochar and their varying particle size. Smaller particle size could not necessarily improved microbial biomass in soil once cemented with soil clumps. Mechanisms and factors contributing to increased microbial biomass phosphorus for the largest particle size particle (2-4 mm) of Simcoa, Wundowie and Saligna after 28 days incubation in soil were not identified. Further study is needed to determine biochar-soil interactions that influence the role of biochar as a habitat for soil microorganisms. Increased particle size in each of the three biochar sources had little effect on soil microbial biomass carbon and phosphorus after 56 incubation days. Simcoa biochar had greater potential as a habitat than did the Wundowie and Saligna biochars, but was only associated with higher soil microbial biomass carbon at the 28 day assessment.
CHAPTER 5

BIOCHAR ADDITION TO SOIL WITH CROP RESIDUES

5.0 Abstract

Soil management that includes biochar application could lead to complex interactions with organic matter. Dual incorporation of biochar with organic matter may influence the soil microbial status through biochar interaction with organic compounds. Two *Eucalyptus* biochars that had been prepared using different pyrolysis conditions (Oil Mallee biochar pyrolysed at 450°C and Simcoa biochar pyrolysed at 650°C) were incubated with crop residues (canola and wheat) for 112 days to determine the effect of crop residues on fungal colonisation of biochar and whether an interaction between biochar and crop residues stimulated soil microbial biomass. Both crop residues and biochars were applied at 2% (v/v) and thoroughly mixed through 100g of an agricultural soil. The soil mixtures were incubated in 200 ml containers at 25°C in a constant temperature room. The hypothesis investigated was that biochar addition to soil in the presence of these crop residues would increase microbial biomass compared to soil amended with crop residues or *Eucalyptus* biochar alone. After 112 days incubation in soil, micrographs showed fungal hyphae colonisation of biochar was unaffected by the presence of the crop residues. Fungal colonisation of biochar incubated in soil for 112 days with either wheat residues or canola residues appeared to be similar to that of the biochars incubated in soil without residues. In the absence of biochar, soil incubated with only crop residues (wheat or canola) showed increased soil microbial biomass P and respiration at 28 days. Only canola residue maintained the increased soil microbial biomass P and respiration for 112 days. Biochar application alone had no effect on soil microbial biomass phosphorus and carbon, or respiration throughout 112 days in the absence of the two crop residues. The combination of Oil Mallee biochar and presence of either of the crop residues increased soil microbial biomass C after 112 days, while Simcoa biochar when combined with either crop residue had no effect on soil microbial biomass and respiration.
Chapter 5: Biochar and Crop Residues

5.1 Introduction

Dual incorporation of biochar with organic matter has also been increasingly studied for sustainable soil productivity (Beesley et al., 2010; Beesley and Dickinson, 2011; Cross and Sohi, 2011; Bolan et al., 2012). Biochar combined with humus or added organic materials has been investigated in both forest and agricultural soils (Pietikainen et al., 2000; Steinbeiss et al., 2009). Biochar has been applied with crop residues, compost and manure to improve soil fertility and crop production (Beesley et al., 2010; Blackwell et al., 2010; Solaiman et al., 2010). Steinbeiss et al. (2009) showed that biochar with or without N could stimulate the loss of soil organic C in both agricultural and forest soils. In other studies, incorporation of biochar with organic matter was recommended for accelerated decomposition in the composting process (Bruun and El-Zahery, 2012). These studies indicate the potential for incorporating biochar with organic residues as a combined soil amendment.

Potential benefits of biochar associated with changes in soil microbial activity and organic matter decomposition have been studied (Liang et al., 2010; Beesley et al., 2010; Bruun and El-Zehery, 2012). Accelerated decomposition of soil organic matter after biochar amendment is possible and shifts in the microbial community and microbial biomass are expected (Wardle et al., 1998; Pietikainen et al., 2000; Wardle et al., 2008). Liang et al. (2010) reported organic material stabilisation in soil which contained aged charcoal from a tropical environment. Acceleration in decomposition of soil humus was reported in a 10-year study of litter bags in the boreal zone (Wardle et al., 2008), with rapid humus loss when charcoal was added to soils. Pietikainen et al. (2000) provided evidence that humus, when combined with biochar, could promote soil microbial abundance. They suggested that the charcoal layer induced changes in the microbial community (Pietikainen et al., 2000).

The underlying mechanisms of biochar and organic matter interactions remain speculative. The structural nature of biochar could facilitate microbial growth and indirectly accelerate degradation of organic compounds, including phenolic compounds (Keech et al., 2005; Liang et al., 2006; Thies and Rillig, 2009; Rillig et al., 2010; Jindo
et al., 2012a, 2012b). Biochar, may offer a habitat and facilitate colonisation of soil fungal hyphae as shown in the previous chapter and elsewhere (Gundale and Deluca, 2006; Warnock et al., 2007; Lehmann et al., 2011).

Zackrisson et al. (1996) suggested that microbial activity is important in reactivating charcoal via provision of nutrients from decomposing materials that become attached to the charcoal. Biochar pores could protect microbes from predators, and the adsorption of organic matter to biochar could provide source of energy for microbial activity (Warnock et al., 2007). Organic materials and minerals have been observed to be bound to biochar particles (Joseph et al., 2010) and the large surface area of materials such as activated charcoal enhances its ability to absorb organic compounds for soil detoxification purposes (Uchimiya et al., 2010a, b). Previous studies have shown that biochar also can have labile fractions which could stimulate microbial growth and activity (Anderson et al., 2011; Graber et al., 2010).

Alteration in soil properties could arise from biochar, organic matter and soil microbial interactions. Addition of biochar and organic matter could also influence soil alkalinity and water status and indirectly affect nutrient availability and microbial access to carbon and other sources for microbial activity (Wardle et al., 1998; Pietikainen et al., 2000). Improvement in soil water status and pH due to biochar and/or organic matter amendment could benefit soil fungi involved in decomposition and AM fungi (Solaiman et al., 2010) Fungi associated with lignin degradation may also be influenced preferentially, but this is not known.

In contrast, negative influences on soil microorganisms involving organic substances could be associated with biochar (Kimetu and Lehmann, 2010). Examples of interactions between biochar and organic residues were shown to alleviate the detrimental effect of root bark extract but simultaneously reduced the benefits derived from mycorrhizas for plant growth (Rutto and Mizutani, 2006). Thus, biochar application as a soil amendment for soil management could lead to complex interaction with organic matter and this requires further investigation.
Potential interactions between biochar and crop residues which increase soil microbial biomass and nutrient availability is the major focus in this study (Figure 5.1). The hypothesis investigated was that the addition of two types of crop residues (canola and wheat) as additional carbon and substrate sources in the presence of two woody (Eucalyptus) biochars pyrolysed under different pyrolysis conditions can increase microbial activity, hyphal colonisation and soil P. Therefore, the aims were:

1. to observe fungal hyphae colonisation of two woody (Eucalyptus) biochars prepared using different pyrolysis conditions in soil amended with canola and wheat residues using fluorescence and electron microscopy,

2. to determine the effects of canola and wheat residues on soil microbial development (soil microbial biomass carbon and phosphorus, and soil respiration) in the presence of two woody (Eucalyptus) biochars prepared using different pyrolysis conditions, and

3. to determine the effects of canola and wheat residues on the phosphorus status of soil in the presence of two woody (Eucalyptus) biochars prepared using different pyrolysis conditions.
Figure 5.1 Conceptual flow of experimental designs of biochar and organic matter as soil amendments in Chapter 5 (highlighted in blue box)
5.2 Materials and Method

5.2.1 Experimental design
An incubation experiment was set up with two types of organic residues in combination with two biochars and maintained at 25°C for 112 days. Treatments comprised three crop residues treatments (control, canola residue and wheat residue) with three biochar treatments (no biochar, Simcoa biochar and Oil Mallee biochar). 100 g of soil was mixed with 2% (v/v) crop residues and 2% (v/v) biochar, incubated in 200 ml vials with moisture maintained at 45% water holding capacity. The incubation containers were arranged in a completely randomized design. There were four destructive sampling times with twice the incubation period used in Chapter 4 (28, 56, 84, and 112 days). There were three replicates for each treatment. One day prior to soil sampling, each container was tightly closed to entrap carbon dioxide for 24 hours.

5.2.2 Soil and crop residues used for incubation
Soil was taken from the long term no-till The Western Australian No-Tillage Farmers Association (WANTFA) site near Cunderdin, WA with 0.1% N, 1.2% C, C:N ratio of 11.31, pH 6.4 (CaCl$_2$), available Colwell P, 38 mg/kg; ammonium N, 2.5 mg/kg and conductivity, 0.125 dS/m). Crop residues were collected from treatments within the long-term non-till WANTFA site. Wheat residues had 0.82 % N, 41.30% C and 0.06% P while canola residues had 1.09% N, 40.19% C, and 0.08% P.

5.2.3 Biochar sources and size fractions
The two sources of woody biochars were obtained from Simcoa Ltd, Bunbury, Western Australia and from Best Energy, Australia. Simcoa biochar used in Chapters 3 and 4 was made from jarrah wood (*Eucalyptus marginata*) in 2008 by Simcoa Ltd. and characteristics were mentioned in those chapters. Oil Mallee (*Eucalyptus* sp.) biochar was produced by BEST Energies via a slow pyrolysis at 450°C contained 0.38% N, 56.38% C. Both biochars were chosen based on the criteria that they originated from *Eucalyptus* feedstock but varied in pyrolysis conditions. The biochar particles used in this experiment ranged from 0.5-4 mm in size.
5.2.4 Incubated biochar preparation and microscopy observation

Biochar characteristics were determined using scanning electron microscopy (SEM) before (on biochar original stocks) and after 112 days incubation in soil. The original source of biochar (prior to its addition to soil) was treated and prepared as described in Chapter 3 (Table 3.2 and Figure 3.2, 3.3). SEM observations were made on the biochar pores in order to verify and quantify the degree of porosity as described in Chapter 3. The incubated particles of biochar retrieved from the soil were prepared for fluorescence microscopy and SEM as detailed in Chapter 3. Ten biochar particles were selected for examination of the changes in biochar after interaction with soil.

5.2.5 Soil analysis

Fresh soil samples at each harvest day (28, 56, 84, and 112 days) were weighed separately for soil microbial biomass carbon, microbial biomass phosphorus and soil pH as determined and described in Chapter 4. Available soil P was determined using non-fumigated samples from microbial biomass P and measured by spectrometry using the molybdenum-blue method (Murphy and Riley, 1962). Organic P was measured using the ignition method of Saunders and Williams (1955), and spectrometry using the molybdenum-blue method (Murphy and Riley, 1962).

5.2.6 Statistical analysis

Analysis of variance (ANOVA) was performed using the Statistical Analysis System (SAS) program version 8.02 for Windows. Comparison of different treatment means was checked by the Duncan’s Multiple Range Test (DMRT) at 5% confidence level. All variables taken were analysed by factorial analysis by time of sampling. Interrelationships were analysed using SAS (SAS Institute Inc. (1992-98)).
5.3 Results

5.3.1 Biochar structure (SEM)

Both woody *Eucalyptus* biochars used in this study were highly porous (Plate 5.1). The pores varied from 10 µm to 200 µm in diameter. Simcoa biochar had fewer large pores in comparison with Oil Mallee biochar (Plate 5.1).

![Plate 5.1 SEM of heterogeneous pores exhibited in woody biochars prior to addition to soil in this incubation experiment for a) Simcoa biochar, and b) Oil Mallee biochar. Scale bar = 200 µm.](image)

5.3.2 Biochar colonisation by fungal hyphae

Simcoa biochar was observed to have some fungal hyphae attached even before it was incubated in the soil (Chapter 3). In contrast, no fungal hyphae were observed on Oil Mallee biochar prior to its addition to soil (Plate 5.1). After the biochars were incubated in soil for 112 days, fungal hyphae were observed using fluorescent staining in the absence of added crop residues (Plate 5.2c and f). Fungal hyphae were clearly observed colonising the surface of both Oil Mallee and Simcoa biochars in all treatments (Plate 5.2 a-c). Fungal colonisation of biochar was observed after incubated in soil for 112 days with either wheat residues (Plate 5.2a, d) or canola residues (Plate 5.2b, e) as well on the biochars incubated in control soil without residues (Plate 5.2). Since there was some fungal contamination of the Simcoa biochar prior to its application to soil, only Oil Mallee biochar was retrieved for further observation using SEM. Some fungal colonisation was observed on biochar surfaces (Plate 5.3a, b, c, d, e) and in pores (Plate 5.3f) for Oil Mallee biochar, particles were retrieved from both crop residues. The hyphal networks were interconnected (Plate 5.3a).
Plate 5.2 Fluorescence staining of microorganisms on Oil Mallee biochar amended in soil with (a) wheat residues, (b) canola residues, and (c) control and for Simcoa biochar particles incubated in soil amended with (d) wheat residues, (e) canola residues, and (f) control after 112 days. All samples were prepared and observed using Method 4 (Table 3.2). Scale bar = 50 µm.
Plate 5.3 Scanning electron micrographs showing fungal hyphae (arrow) on Oil Mallee biochar incubated in soil for 112 days with wheat residues (a, b, c) and canola residues (d, e, f). All samples were prepared and observed using Method 4 (Table 3.2). Scale bar = 50 µm.
5.3.3 Effects of crop residues and biochars on soil microbial biomass phosphorus, carbon and respiration

After 28 days of incubation of wheat (WH) and canola (CAN) residues in the soil without biochar, microbial biomass P increased (P<0.05; Figure 5.2a). This effect was maintained for 112 days in soil amended with wheat residues but not canola residues (Figure 5.2b). Addition of the biochars to soil did not affect microbial biomass P in the absence of crop residues at either day 28 or day 122 (P<0.05; Figure 5.2a, b). At 28 days, in comparison to Oil Mallee biochar alone (OM), addition of Oil Mallee biochar did not affect (P>0.05) microbial biomass P in the presence of canola residues (CAN+OM) but decreased it in the presence of wheat residues (WH+OM) (P<0.05; Figure 5.2a). There was no effect of adding Simcoa biochar (SIM) on microbial biomass P in the presence of either residue at 28 days (Figure 5.2a). At 112 days, addition of the biochar had no effect on microbial biomass P in the presence of crop residues (Figure 5.2b).

Soil microbial biomass C did not differ in soil amended with either crop residue alone, the control (CON) soil or soil with added biochar alone, after 28 days (P>0.05; Figure 5.2c). However, after 112 days, addition of canola residues alone (CAN) increased (P<0.05) microbial biomass C while wheat residues did not change microbial biomass C in the absence of biochar (Figure 5.2d). Biochar addition alone (treatments OM and SIM) to soil had no effect on microbial biomass C in the absence of crop residues for 112 days (P>0.05). In soils with crop residues, addition of both biochars to soil increased (P<0.05) microbial biomass C after 28 days in the combined presence of canola residues (treatments CAN+OM and CAN+SIM) but this was not the case for soils treated with wheat residues (treatments WH+OM and WH+SIM) after 28 days (Figure 5.2c). Addition of Oil Mallee biochar to soil increased in microbial biomass C after 112 days for both residues (treatments CAN+OM and WH+OM) (Figure 5.2d). Addition of Simcoa biochar to soil did not affect (P>0.05) microbial biomass C after 112 days when incubated with canola residues (CAN+SIM) but it increased (P<0.05) microbial biomass C when incubated with wheat residues (WH+SIM) (Figure 5.2d).
Chapter 5: Biochar and Crop Residues

Figure 5.2 Combinations of Simcoa and Oil Mallee biochar with canola residues and wheat residues on microbial biomass P (a, b) and microbial biomass C (c, d) at days 28 and 112. CON=Control, OM=Oil Mallee biochar, SIM=Simcoa biochar, CAN=Canola residues, WH=wheat residues, CAN+OM=Canola residues with Oil Mallee biochar, CAN+SIM = Canola residues with Simcoa biochar, WH+OM=wheat residues with Oil Mallee biochar, WH+SIM=wheat residues with Simcoa biochar. Means followed by the same lower case letters are not significantly different across treatments at P<0.05 by DMRT.
Soil microbial respiration increased (P<0.05) after 28 and 112 days of soil incubation with crop residues (wheat and canola) in the absence of biochar (treatments CAN and WH), compared to control soil (CON) (Figure 5.3a, b). In contrast, addition of biochar alone (treatments OM and SIM) did not change soil microbial respiration in the absence of crop residues at either day 28 or day 112 (P>0.05).

At 28 days, addition of Oil Mallee biochar did not affect soil microbial respiration in the presence of either canola residues (CAN+OM) or wheat residues (WH+OM) (Figure 5.3a). After 112 days, Oil Mallee biochar decreased soil microbial respiration in the presence of wheat residues (WH+OM) and had no effect in the presence of canola residues (CAN+OM) (Figure 5.3b). There was no effect of adding Simcoa biochar on microbial respiration in the presence of either residue at 28 days and 112 days (P>0.05).

Increased soil pH with addition of crop residues (canola and wheat) was found at 28 days (P<0.05; Figure 5.3c). However, only canola residues, maintained the increase in soil pH at 122 days (P>0.05; Figure 5.3d). Addition of biochar alone increased soil pH at both 28 and 122 days incubation.

Addition of Oil Mallee biochar to soil increased (P<0.05) soil pH after 28 days in the presence of canola residues (CAN+OM), but not in the presence of wheat residues (WH+OM) (Figure 5.3c). In contrast, after 112 days, addition of Oil Mallee biochar to soil in the presence of wheat residues (WH+OM) increased soil pH (P<0.05). There was no effect of application of Oil Mallee biochar to soil with added canola (CAN+OM) on soil pH after 112 days.

Addition of Simcoa biochar followed a similar trend to that of Oil Mallee biochar whereby soil pH increased (P<0.05) after 28 days in the presence of canola residues (CAN+SIM), but not in the presence of wheat residues (WH+SIM) (Figure 5.3c). There was no effect of Simcoa biochar when added to soil with crop residues on soil pH after 112 days.
Figure 5.3 Combinations of Simcoa and Oil Mallee biochar with canola residues and wheat residues on microbial carbon dioxide emission (a, b) and soil pH (c, d) at days 28 and 112. CON=Control, OM=Oil Mallee biochar, SIM=Simcoa biochar, CAN=Canola residues, WH=wheat residues, CAN+OM=Canola residues with Oil Mallee biochar, CAN+SIM = Canola residues with Simcoa biochar, WH+OM= wheat residues with Oil Mallee biochar, WH+SIM= wheat residues with Simcoa biochar. Means followed by the same lower case letters are not significantly different across treatments at P<0.05 by DMRT.
5.3.4 Effects of crop residues and biochars on soil phosphorus status

Soil available P did not change with addition of crop residues (canola and wheat) in the absence of biochar (treatments CAN and WH) at 28 days (P>0.05; Figure 5.4a). Addition of canola residues alone did not affect (P>0.05) available P after 112 days but addition of wheat residues increased (P<0.05) available P compared to control (Figure 5.4b). Addition of biochar alone to soil (treatments OM and SIM) had no effect on soil available P in the absence of crop residues at both 28 and 112 days (P>0.05; Figure 5.4a, b).

Addition of Oil Mallee biochar increased (P<0.05) soil available P in the presence of both crop residues (treatments CAN+OM and WH+OM) at 28 days and this was maintained 112 days for canola residues (P<0.05) but not for wheat residues (Figure 5.4a, b). Addition of Simcoa biochar with wheat residues (WH+SIM) did not affect soil available P compared to wheat residues alone (WH) at 28 days. At 112 days, an increase (P<0.05) in available P with Simcoa biochar was found when incubated with canola residues (CAN+SIM), but there was no effect (P>0.05) of Simcoa biochar with wheat residues (WH+SIM) (Figure 5.4b).

Addition of canola residues alone (CAN) reduced soil organic P at both 28 and 112 days while addition of wheat residues increased available P at 112 days when biochar was absent (P<0.05; Figure 5.4c, d). Addition of Oil Mallee biochar (OM) and Simcoa biochar alone (SIM) to soil had no effect on soil organic P in the absence of crop residues (P>0.05; Figure 5.4c, d).

Addition of Oil Mallee biochar did not affect soil organic P in the presence of either crop residue (CAN+OM and WH+OM) at 28 days and this was maintained to 112 days (P>0.05; Figure 5.4c, d). In contrast, addition of Simcoa biochar in the presence of canola (CAN+SIM) improved soil organic P compared to canola alone for both 28 and 112 days of incubation (P<0.05).
Figure 5.4 Combinations of Simcoa and Oil Mallee biochar with with canola residues and wheat residues on soil available P (a, b) and soil organic P (c, d) at day 28 and 112. CON=Control, OM=Oil Mallee biochar, SIM=Simcoa biochar, CAN=Canola residues, WH=wheat residues, CAN+OM=Canola residues with Oil Mallee biochar, CAN+SIM = Canola residues with Simcoa biochar, WH+OM= wheat residues with Oil Mallee biochar, WH+SIM= wheat residues with Simcoa biochar. Means followed by the same lower case letters are not significantly different across treatments at P<0.05 by DMRT.
5.4 Discussion

Biochar addition to soil in the presence of crop residue was compared to soil amended with crop residues or Eucalyptus biochar alone, with the hypothesis that biochar and crop residue would improve soil microbial biomass and stimulate fungal colonisation of biochar. However, microscopy observations showed that surfaces of woody Eucalyptus biochars became colonised by fungal hyphae regardless of the addition of either crop residue.

The presence of the crop residues alone increased microbial biomass P and soil respiration as observed as early as day 28 compared to biochar alone. In contrast to biochar, crop residues could be degraded and provide carbon sources and slowly release nutrients for microbial stimulation. There were no changes in soil microbial biomass P and C or microbial respiration when Eucalyptus biochars were added to soil in the absence of crop residues. These lack of response of soil microbial biomass P and C, and microbial respiration to biochar compared with the added organic matter corresponds with recalcitrant properties of biochar compared to labile organic matter (Kimetu and Lehmann, 2010). Previously, Kimetu and Lehmann (2010) also showed that neither Eucalyptus saligna biochar or leaf residues of T. diversifolia amended soils influenced cumulative microbial respiration compared to unamended soil.

When biochar was added with the crop residues, soil with biochar and canola residues had greater microbial biomass C (at either 28 or 112 days, depending on the biochar) than did soil with added crop residues or biochars alone, as expected. The application of crop residues with Eucalyptus biochars enhanced beneficial soil biological properties (e.g. soil microbial C) in previous investigations (Pietikainen et al., 2000; Liang et al., 2010).

There could be many contributing factors associated with differences in the extent to which different crop residues might improve microbial biomass and soil P status compared with biochar (Pietikainen et al., 2000; Liang et al., 2010). A difference in the quality of crop residues added (Fierer et al., 2001; Kuzyakov et al., 2000) may further explain these differences. The chemical characteristics of crop residues can positively influence microbial interactions with biochar associated with nutrient availability (Jindo
et al., 2012a; Ngo et al., 2013). In addition, retention of soil moisture by crop residue addition to soil could also stimulate microbial activity (measured as microbial respiration in this study). The reason for the limited effects associated with wheat residues compared with canola residues may be associated with C/N ratio. A longer incubation period or reduced amount of crop residue added to the soil could be used to explore this further. Increasing the amount of biochar added to soil for direct or indirect effect on lower C mineralization could also be a factor which influences biochar-organic matter interactions (Cheng et al., 2008; Liang et al., 2010).

Improved soil pH following application of crop residues with biochar, especially after 28 days, could be associated with the higher microbial biomass C observed in canola amended soils. Influences of biochar and organic matter on soil alkalinity, which indirectly affect nutrient availability and microbial access to carbon and other sources for microbial activity, have been reported (Wardle et al., 1998; Pietikainen et al., 2000; Solaiman et al., 2010). Higher available P, as found in crop residue amended soils in combination with biochar, could also result from improved soil pH and microbial activity. Soil pH, soil organic carbon and available phosphorus contents were found to be positively correlated as reported by Jing et al. (2013). A higher pH can increase organic C decomposition (Waschkies and Hüttl, 1999; Liang et al., 2010).

The potentially complex interactions of two biochar sources with two crop residues studied here, especially for MBP and available P, could be explained by the differences in biochar quality. Oil Mallee biochar in combination with either crop residues had higher available P than for crop residues alone, and the lower pyrolysis temperature compared with that used for Simcoa biochar may have contributed to the availability of P from its labile fractions. The increased soil organic P with addition of canola residue and biochar could be associated with P originating from either of these amendments.

The beneficial effect of wheat residues on MBP observed in the presence of Oil Mallee biochar but not with Simcoa biochar over 112 days although surface interactions with labile carbon including sorption capacity and stability could be involved (Singh and Cowie, 2014). Similarly, the lack of synergy between crop residues and biochar constrasts with that observed in related studies (Lentz and Ippolito, 2012).
5.5 Conclusion

When two woody (*Eucalyptus* spp.) biochars were mixed throughout an agricultural soil (with a history of no-tillage cropping) with either wheat or canola residues at 2% (v/v) and incubated for up to 112 days, fungal hyphae colonisation of biochar with either wheat residues or canola residues appeared to be similar to that of the biochars incubated in soil without residues. In the absence of biochar, soil incubated with only crop residues (wheat or canola) showed increased soil microbial biomass P and respiration at 28 days. Only canola residue maintained the increased soil microbial biomass P and respiration for 112 days. Biochar application alone had no effect on soil microbial biomass phosphorus and carbon, or respiration throughout 112 days in the absence of the two crop residues. The combination of Oil Mallee biochar and either crop residues increased soil microbial biomass C after 112 days, while Simcoa biochar when combined with either crop residues had no effect on soil microbial biomass and respiration.
CHAPTER 6
DEVELOPMENT OF ARBUSCULAR MYCORRHIZAL FUNGI
IN SOIL AMENDED WITH BIOCHAR

6.0 Abstract

Biochar management in soil may influence arbuscular mycorrhizal (AM) colonisation in several ways. This study aimed to investigate biochar interactions with indigenous AM colonisation in roots of subterranean clover and wheat grown in two agricultural soils. Two glasshouse experiments were conducted using different amounts of biochar(s) either amended or not amended with diammonium phosphate (DAP) fertiliser. It was hypothesised that AM colonisation would increase with increasing biochar but the optimum amount of biochar would differ with biochar source and phosphorus fertilisation. In the first experiment, growth of subterranean clover was assessed following application of Simcoa biochar, a woody biochar from *Eucalyptus* sp. applied at 0, 5, 10, 25 and 50 t/ha, over 12 weeks without any phosphorus fertiliser. In the second experiment, wheat was grown for 8 weeks in soil which had been amended with either chicken manure biochar (CMB) or wheat chaff biochar (WCB) applied at 0, 2.5, 5 and 7.5 t/ha with and without DAP fertiliser. The amount of biochar required to influence mycorrhizal colonisation varied with biochar source and host plant in these experiments. For subterranean clover, increasing the amount of Simcoa biochar did not affect the microbial biomass P, but it increased AM colonisation (%), particularly at later harvests (weeks 9 and 12). For wheat in the absence of added DAP, both CMB and WCB increased the proportion of mycorrhizal roots after 8 weeks but it was only decreased at the highest amount of WCB when DAP was added. Increases in the proportion of mycorrhizal root were more marked at depth irrespective of DAP application. The actual length of root colonised by AM fungi increased with application of WCB but not with CMB. Microscopy evidence showed that biochar particles were colonised by soil fungi and created soil micro-aggregates through hyphal networking.
Chapter 6: Development of Arbuscular Mycorrhizas in Soil Amended with Biochar

6.1 Introduction

Strategic use of organic matter may favour mycorrhizal development and improve its function (Gosling et al., 2006). Biochar (Lehmann et al., 2011) has potential to provide habitat for fungal hyphae within its pores and on surfaces as reported in Chapters 3, 4 and 5. As a source of organic matter, biochar can also influence AM fungi through changes in soil physio-chemical properties and microbial interactions (Warnock et al., 2007). It is generally assumed that biochar pores can facilitate growth of AM fungi. Thus, the addition of biochar to soil has potential to enhance the formation of mycorrhizas and associated plant benefits. However, previous microscopy observations of interactions between biochar and microorganisms, including AM fungi, provide limited experimental evidence to support this proposed mechanism (Ascough et al., 2010b; Jin, 2010; Lehmann et al., 2011). Although hyphae, including AM fungal hyphae have been shown in SEM micrographs colonising biochar (Lehmann and Joseph, 2009; Lehmann et al., 2011), experimental details, soil interactions and further microscopy evidence are generally limited. Furthermore, the potential benefit of biochar may be over-estimated due to factors such as biochar heterogeneity and soil background including soil management.

Specific interactions between mycorrhizal fungi and forms of biochar with different characteristics are either poorly clarified or contradictory (Warnock et al., 2007; Table 1.1). Biochar pores may be physically changed on surfaces and with formation of cracks in biochar (Ascough et al., 2010b). They may also be covered with soil particles thereby altering pore access as observed in Chapter 3 and 4. Thus, there is a need to observe interactions between biochar and fungi in soil over time to better understand effects on fungal development. Visualisation of the biochar-soil-fungi continuum using microscopy could provide evidence as to whether biochar habitat provision (Lehmann et al., 2011) modifies mycorrhizal development either positively or negatively. Modification of potential biochar habitat, primarily pores and surfaces, could alter gas, moisture and microbe movement which could influence microbial abundance in soil (Lehmann et al., 2011). Variation in observations of interactions between mycorrhizal
fungi and biochar might be associated with the amount of biochar applied to soils where AM fungi have been studied. There are difficulties in making comparisons between experiments and details about indigenous AM fungi and are often not reported (Warnock et al., 2007). This limits understanding of how mycorrhizal symbioses could be optimised by applying biochar to soil. However, beneficial effects of adding various amounts of biochar to soil on AM fungi have been demonstrated (Ishii and Kadoya, 1994; Solaiman et al. 2010; Warnock et al., 2010; Elmer and Pignatello, 2011). Increased mycorrhizal colonisation was demonstrated following application of hydrothermal carbonised material at 20% w/w but higher concentrations were detrimental to mycorrhizal colonisation (Rillig et al., 2010).

Despite the wide range of biochar sources that have been compared, the effective range in quantity of applied biochar to soil is poorly understood in relation to biochar management and soil status. The optimum amount of biochar suitable for soil application is expected to vary according with its source and soil (Cross and Sohi, 2011; Warnock et al., 2010). Large quantities (2.0% and 4.0%, w/w) of a lodgepole pine biochar reduced the abundance of AM fungi in roots by 58% and 73% respectively (Warnock et al., 2010). Peanut shell biochar reduced mycorrhizal root colonisation by 74% and extraradical hyphal lengths by 95% and mango wood biochar applied at 23 and 116 t C ha$^{-1}$ decreased mycorrhizal abundance by 43% and 77% in similar work by Warnock et al. (2010). In several studies, the amount of biochar applied to agricultural soil differed when other soil amendments were added for various reasons such as when addressing the nutrient requirement for plant growth.

The impact of method of biochar application in combination with readily available nutrient sources to soil, including fertiliser, is not well understood. Although no experimental comparison of biochar application method (i.e. banding vs surface or mixing biochar into the soil) on AM fungi has been made, banding of biochar in soil increased colonisation by AM fungi (Blackwell et al., 2010). Blackwell et al. (2010) also showed that a low biochar application level (1 t/ha) may increase wheat yield and reduce fertiliser requirement when applied to soil. Similarly, Solaiman et al. (2010)
showed that mycorrhizal colonisation of wheat roots increased with amendment of Oil Mallee biochar applied with 100 kg/ha slow-release mineral fertiliser containing 7% phosphorus. They also demonstrated an increase in mycorrhizal colonisation for deep-banded biochar amendment in subterranean clover bioassays.

The consequences of increased mycorrhizal colonisation due to biochar addition may include improvement in nutrient and soil water availability (Solaiman et al., 2011). However, biochar addition to soil with increasing soil phosphorus status could reduce AM fungal development and efficacy. For example, high soil nutrient availability reduced mycorrhiza formation with biochar application (Gaur and Adholeya, 2000; Warnock et al., 2010) and when biochar was applied with abundant nutrient availability especially P (Gryndler et al., 2006). Mycorrhizal colonisation is known to be affected by agricultural soil conditions, especially inorganic or organic phosphorus fertilisation (Smith et al., 1992; Treseder and Allen, 2002). Mineral fertilisation for example, in the form of N, P₂O₅ and K₂O reduced the mycorrhiza development, particularly AM fungal hyphal length (Gryndler et al., 2006). In the absence of phosphorus fertiliser, Yamato et al. (2006) observed that colonisation by AM fungi (% root length colonised) was highest for bark charcoal application. In contrast, incorporation of either the mineral or soluble fertiliser to biochar improved mycorrhizal root length colonised (Solaiman et al., 2010).

Interactions among biochar type, amount, soil phosphorus and fertilisation with respect to their effects on AM fungi are not well understood for different types of biochar. It is unclear whether the biochar application method plays some part in influencing AM fungal colonisation of roots. While agricultural soils in south-western Australia are commonly sandy and infertile for agricultural plants in their natural state (Tennant et al., 1992; Moore, 2001), research on the addition of biochar with and without fertilisation could provide insight into interactions with indigenous AM fungi. Therefore, two glasshouse experiments (Figure 6.1) were conducted to investigate the response of naturally occurring AM fungi in two agricultural soils from south-western Australia varying in P status following amendment with biochar.
Figure 6.1 Conceptual flow of experimental designs of biochar and soil amendments in Chapter 6 (highlighted in blue box).
The agricultural soil used in each experiment varied in soil phosphorus. It was hypothesized that (i) mycorrhizal colonisation would increased with increasing biochar increment when phosphorus was not applied, and that (ii) an optimum amount of biochar for facilitating mycorrhizal colonisation would depend on P fertilisation and P status of the soil.

6.2 Materials and methods

6.2.1 Experiment 6.1 (Effects of Simcoa biochar amount on AM fungi)

Experimental Design

A factorial glasshouse experiment was conducted with application of five levels of application of biochar (0, 5, 10, 25, 50 t/ha) and four sampling times throughout a 12 weeks growth period. Subterranean clover (*Trifolium subterraneum* L. var. Seaton Park) was grown for 12 weeks.

Soil and biochar characteristics

The agricultural soil (top 10cm) was collected adjacent to the long-term non-till WANFTA site near Cunderdin, Western Australia. Simcoa biochar was used as in Chapter 3 (Table 3.1). There were three replicates of each treatment. This *Eucalyptus* biochar (0.60% N, <0.01%P, <0.001% K) was obtained from Simcoa Ltd (Bunbury, Western Australia) which had been made from Jarrah wood (*Eucalyptus marginata*) pyrolysed at 600°C. Soil was air dried and sieved to 2 mm prior to analysis by CSBP Soil and Plant Laboratory. Ammonium-N and Nitrate-N were determined using 2M KCl-steam distillation and automated colour finishes, phosphorus and potassium using bicarbonate extractable phosphorus (Colwell, 1965) and organic carbon by the Walkley and Black (1934) procedure. Soil pH was measured in a soil to solution ratio of 1:5, and textural analysis was assessed by particle size distribution and classed based on soil textural triangle. The soil had the following characteristics: pH 6.4 (CaCl$_2$); Colwell P, 38 mg/kg; organic C, 1.2%; ammonium N, 2.5 mg/kg and conductivity, 0.125 dS/m), as
analysed by CSBP (see Chapter 3). Simcoa biochar characteristics were similar to those described in Chapter 3.

**Biochar placement, plant maintenance and harvest**

Simcoa biochar (0, 5, 10, 25, 50 t/ha equivalent amounts) was thoroughly mixed in 2 kg soil per pot, respectively. The treatments were placed in a completely randomised block design in the glasshouse. Subterranean clover seeds were sown and soil was maintained at field capacity. Plants and soils were sampled 3, 6, 9 and 12 weeks after planting. At each harvest, shoot and root biomass were determined (fresh weight) then oven dried at 60°C to constant weight. Sub-samples of fresh roots were used for assessing mycorrhizal colonisation.

**Soil and plant analyses**

At each harvest, microbial biomass carbon (Vance et al., 1987) and microbial biomass phosphorus as determined by the anion exchange membrane method (Kuono et al., 1995) were extracted from fresh soil as described in Chapter 4. Soils were also analysed for pH and electrical conductivity.

**Mycorrhizal colonisation**

Approximately 0.3-0.5g of roots (fresh weight) were randomly sub-sampled and cut into 1-2 cm lengths. Root segments were placed in 20 mL 10% KOH for 5-7 days for root clearing and subsequent staining (Gazey et al., 1992). The stained roots segments were examined for the presence or absence of arbuscules, vesicles and hyphae under a dissecting microscope based on gridline intersect technique. The proportion of root colonised by AM fungi was calculated using the grid line intersect method (Giovanetti and Mosse, 1980). The total root length colonised was estimated by multiplying the total root length by the fractional colonisation based on Tennant’s equation (Tennant, 1975).

**Incubated biochar preparation and microscopy observation**

Fluorescence microscopy and scanning electron microscopy (SEM) were used to observe colonised incubated biochar retrieved from the soil after 12 weeks. The biochar
particles were prepared (fixed, stained, and critical point dried) as described in Chapter 3. They were examined from the two highest amounts of biochar application (25 and 50 t/ha) for assessment of potential biochar-soil interactions and fungal colonisation.

6.2.2 Experiment 6.2 (Effects of biochar type, amount and fertiliser on AM fungi)

Experimental design
The experimental design for this second experiment (Experiment 6.2) had two biochar treatments (chicken manure biochar and wheat chaff biochar), four biochar application treatments (0, 2.5, 5.0 and 7.5 t/ha; dry weight basis), and two phosphate fertiliser treatments. There were three replicates of each treatment. Biochar from sources other than wood biochar was chosen as to diversify the range of biochar used throughout this thesis. The lower amount of biochar application (0 - 7.5 t/ha) was based on the recommended amount of biochar application approximately 3 t/ha for WA soils (Blackwell et al., 2010)

Soil and biochar characteristics
Soil was collected from the top 10 cm of an agricultural soil near Minginew, WA with the following characteristics: pH 4.3 (CaCl$_2$), available Colwell P, 12 mg/kg; organic C, 2.59%; ammonium N, 1 mg/kg and conductivity, 0.125 dS/m. Soil was analysed as described for Experiment 6.1. The two sources of biochar used were from plant based and animal based biochar. Chicken manure biochar (CMB) comprised a mixture of chicken litter and wood chips (pH 7.31 (CaCl$_2$), 38.34 % C, 2.04% N) and wheat chaff biochar (WCB) (pH 8.27 (CaCl$_2$), 53.14 % C, 2.24% N) were both produced at 450°C by BEST Energies Agrichar (Source: GRDC Biochar Research Group). Biochar particle size ranged from <0.5 to 4 mm (unsieved).

Fertiliser application and plant maintenance
Pots contained two kg soil and a band of biochar was applied at 0, 2.5, 5 or 7.5 t/ha at a depth of 5cm below the soil surface (7 cm from bottom of each pot which was lined with a plastic bag). Diammonium phosphate fertiliser (DAP) (N:P:K 18:20:0) was applied as a band 2 cm above the biochar layer in each pot (Figure 6.2). Four wheat
Chapter 6: Development of Arbuscular Mycorrhizas in Soil Amended with Biochar

(Triticum aestivum L. var. Brookton) seeds were sown per pot at a depth of 3 cm and thinned to two plants, two weeks after sowing. Soil was maintained at field capacity by daily watering to weight. Pots were maintained on a glasshouse bench for eight weeks.

Soil sampling and harvesting
Tillers were counted one day before harvest. Shoots were cut at 1 cm above the soil surface and soil containing roots was separated into layer A (top 5 cm) and layer B (bottom 7 cm) (Figure 6.2). Shoots and roots were oven-dried at 60°C to constant weight and reported as g per pot. Fresh root samples were collected for determination of mycorrhizal root colonisation as in Experiment 6.1. Dried plant samples were ground and digested and plant tissue phosphorus was determined and was analysed as in Experiment 6.1.

Soil microbial biomass, mycorrhizal fungi and soil chemical analyses
In this experiment, microbial biomass C (Vance et al., 1987), microbial biomass P (Kuono et al., 1995), mycorrhizal colonisation, soil inorganic N and soil available P were analysed for the upper soil layer containing biochar (top layer) and the lower layer of soil (bottom layer) (Figure 6.2). Mycorrhizal colonisation as described earlier in 6.2.1 (Experiment 6.1) was determined from roots separated from both the top soil layer containing biochar and the bottom layer of soil. Ammonium N and nitrate N for inorganic N were measured from the non-fumigated samples used to estimate microbial biomass carbon as in Experiment 6.1. Available soil P was determined using non-fumigated samples from microbial biomass P and measured by spectrometry using the
molybdenum-blue method (Murphy and Riley, 1962). Only the top soil layer containing biochar was dried and analysed for pH and electrical conductivity.

**Biochar and incubated biochar preparation for microscopy observation**

Both chicken manure biochar and wheat chaff biochar stocks were characterised and observed using SEM prior to mixing with soil (week 0) and colonised biochar was retrieved from soil for observation after harvesting the plants (8 weeks). The biochar particles from both sources were retrieved from the highest level applied (7.5 t/ha) in fertilised top soil. The biochar stocks and colonised biochar were treated and prepared as described in Chapter 3 (Table 3.2, Figures 3.2 and 3.3). Colonised biochar retrieved from soil was observed using fluorescence microscopy and/or SEM.

**6.2.3 Statistical Analysis (both experiments)**

Analysis of variance (ANOVA) for top and bottom soil samples within the pots was performed using Statistical Analysis System (SAS) program version 8.02 for Windows. Mycorrhizal colonisation data were transformed using square root and log (x+1) for normalisation prior to statistical analysis. Comparisons of means between the different treatments (biochar amount) and within fertiliser and biochar sources were checked using Duncan’s Multiple Range Test (DMRT) at 5% confidence level. All variables were analysed by factorial analysis at the time of sampling.
Chapter 6: Development of Arbuscular Mycorrhizas in Soil Amended with Biochar

6.3 Results

6.3.1 Experiment 6.1

Effects of Simcoa biochar on subterranean clover growth

There were transient effects of biochar on shoot growth of subterranean clover. At 12 weeks, increasing the amount of biochar had no significant effect (P≥0.05) on subterranean clover shoot growth. Biochar applied at the highest amount (50 t/ha) reduced plant growth at weeks 6 and 9 (P<0.05; Table 6.1). At week 9, plant growth was greater (P<0.05) in soil amended with 10 t/ha of biochar than in untreated soil. The highest amount of biochar (50 t/ha) resulted in the lowest plant growth between 6 to 9 weeks after planting. The amount of biochar applied had no significant effect on root weight or root length (P≥0.05; data not shown).

<table>
<thead>
<tr>
<th>Biochar amount (t/ha)</th>
<th>Shoot biomass (g/pot) *</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Week 9</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.13 a</td>
<td>1.02 ab</td>
<td>3.76 b</td>
<td>8.80 a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.17 a</td>
<td>1.10 a</td>
<td>3.82 b</td>
<td>8.30 a</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.14 a</td>
<td>1.08 a</td>
<td>4.16 a</td>
<td>8.60 a</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.14 a</td>
<td>1.05 a</td>
<td>3.84 b</td>
<td>8.68 a</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.17 a</td>
<td>0.87 b</td>
<td>3.53 c</td>
<td>8.58 a</td>
<td></td>
</tr>
</tbody>
</table>

*Shoot biomass was weighed and reported for two plants per pot. Means in each column with the same letter(s) within each sampling week are not significantly different according to DMRT at 5% confidence level (P>0.05)

Effects of Simcoa biochar on mycorrhizal development and microbial biomass

Biochar amendment had an inconsistent effect on mycorrhizal colonisation (Table 6.2). At weeks 9 and 12, mycorrhizal colonisation (%) was increased compared with the no-biochar control at 25 and 50 t/ha biochar, with no effect of biochar on length of root colonised (data not shown). There was no effect of biochar application amount on microbial biomass carbon throughout the 12 weeks planting period (data not shown). Similarly, biochar addition did not affect microbial biomass phosphorus except in week 9 with the highest amount of biochar applied (data not shown).
Table 6.2 Interaction between Simcoa biochar amount and time on mycorrhizal colonisation in subterranean clover (Experiment 6.1).

<table>
<thead>
<tr>
<th>Biochar amount (t/ha)</th>
<th>Mycorrhizal Root Colonised (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 3</td>
</tr>
<tr>
<td>0</td>
<td>39 b</td>
</tr>
<tr>
<td>5</td>
<td>43 b</td>
</tr>
<tr>
<td>10</td>
<td>51 a</td>
</tr>
<tr>
<td>25</td>
<td>43 b</td>
</tr>
<tr>
<td>50</td>
<td>43 b</td>
</tr>
</tbody>
</table>

Means in each column with the same letter(s) within each sampling week are not significantly different according to DMRT at 5% confidence level (P>0.05)

Microscopy observations of Simcoa biochar

Based on the observation of an increase in mycorrhizal colonisation (%) at week 12 with at the highest two levels of biochar application, biochar particles were retrieved from soil amended with 25 and 50 t/ha Simcoa biochar and observed using fluorescence microscopy. Biochar particles from the 25 t/ha treatment (Plate 6.1a, b) were colonised by networks of fungal hyphae as were biochar particles in the 50 t/ha treatment (Plate 6.1 c, d). Hyphal networks were observed attached to soil particles and micro-aggregates on the biochar in the 25 t/ha treatment (Plate 6.1a). Biochar particles retrieved from the 50 t/ha treatment was further observed with SEM (Plate 6.1). Hyphal networks were also noted in the 50 t/ha biochar treatments under SEM (Plate 6.1e, f).

Effects of Eucalyptus Simcoa biochar on soil properties

Soil pH was significantly increased by biochar application at the highest level in week 3, but there was no effect of biochar on soil pH thereafter (data not shown). Soil EC was not affected by any biochar amount applied at weeks 3, 6, 9 or 12 after planting (data not shown).
Plate 6.1 Fluorescence micrographs (a-d) and scanning electron micrographs (e-f) of fungal networks on external surfaces of Simcoa biochar particles retrieved from 25 t/ha (a, b) and 50 t/ha (c, d) (Experiment 6.1), prepared and observed using Method 4 as in Table 3.2. SEM micrograph (e-f) of biochar with soil particles and fungal network (arrow) on biochar external surfaces of Simcoa biochar retrieved in 50 t/ha treatment mixture with soil from Cunderdin, WA for 12 weeks, prepared and observed using Method 4 as in Table 3.2. Micrographs a,b = biochar retrieved from 25 t/ha Simcoa biochar. Micrographs c,d,e,f = biochar retrieved from 50 t/ha Simcoa biochar. Scale bar = 50 µm.
6.3.2 Experiment 6.2

Effects of biochars (WCB and CMB) on wheat

When no DAP fertiliser was applied, application of 2.5 to 7.5 t/ha wheat chaff biochar (WCB) increased wheat shoot biomass (P<0.05), but there was no effect of chicken manure biochar (CMB) on wheat shoot biomass (P≥0.05) (Table 6.3). However, when DAP fertiliser was added, shoot biomass increased (P<0.05) with application of 7.5 t/ha of CMB and with all levels of application of WCB (Table 6.3). Similarly, root growth (cumulative for top and bottom soil layers in the pots) increased with application of biochar, irrespective of the amount applied, for both CMB and WCB when DAP fertiliser was applied (P<0.05). Neither of the biochars affected either cumulative root biomass, or root biomass in the top and bottom of the pot when DAP was not applied (Table 6.3). Root biomass increased with application of both biochars when DAP fertiliser was added (P<0.05) except for the lowest level of CMB (P≥0.05).

There was no significant interaction between biochar type, amount and DAP fertiliser on tiller number (data not shown).

Effects of biochars on mycorrhizal fungi

In both the top and bottom soil layers, mycorrhizal colonisation (% root length colonised) increased with application of both CMB and WCB in the absence of DAP fertiliser (P<0.05; Table 6.4). When DAP was added with CMB, there was no effect on % root length colonised in the upper soil layer, but in the lower soil layer, mycorrhizal colonisation increased irrespective of the level of CMB applied (P<0.05). When DAP was added with WCB, there was a decrease in % root length colonised in the upper soil layer for 7.5 t/ha but no changes for the other levels of biochar application (P<0.05). In contrast, in the lower soil layer, mycorrhizal colonisation (%) increased with application of 5 t/ha WCB (P<0.05) and there were no changes with other WCB application levels (P≥0.05; Table 6.4). In further contrast to the absence of an effect of CMB on the actual length of root colonised in the upper soil layer, WCB application at all levels increased the length of root colonised in the absence of DAP, and for 7.5 t/ha in the lower soil.
layer (P<0.05; Table 6.4). WCB application with DAP increased the length of root colonised for the 2.5 and 5 t/ha treatments in the upper soil layer (P<0.05; Table 6.4) and for all levels of application in the lower soil layer (P<0.05; Table 6.4).

Table 6.3 Effects of two sources of biochar on overall plant and root growth of wheat plants after 8 weeks at varying amount with and without fertiliser application (Experiment 6.2).

<table>
<thead>
<tr>
<th>Biochar</th>
<th>DAP</th>
<th>Amount (t/ha)</th>
<th>Plant biomass (g)</th>
<th>Root biomass (g)</th>
<th>Plant P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(total)</td>
<td>(top)</td>
<td>(bottom)</td>
</tr>
<tr>
<td>CMB</td>
<td>nil</td>
<td>0</td>
<td>1.68 a</td>
<td>1.51 a</td>
<td>0.76 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>1.67 a</td>
<td>1.54 a</td>
<td>0.84 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1.75 a</td>
<td>1.53 a</td>
<td>0.77 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>1.99 a</td>
<td>1.60 a</td>
<td>0.91 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>2.69 b</td>
<td>0.58 b</td>
<td>0.34 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>3.26 b</td>
<td>1.04 a</td>
<td>0.54 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>3.37 ab</td>
<td>1.11 a</td>
<td>0.67 a</td>
</tr>
<tr>
<td></td>
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<td>5</td>
<td>3.86 a</td>
<td>1.23 a</td>
<td>0.70 a</td>
</tr>
<tr>
<td>WCB</td>
<td>nil</td>
<td>0</td>
<td>1.60 b</td>
<td>1.34 a</td>
<td>0.61 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>1.80 a</td>
<td>1.29 a</td>
<td>0.79 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>1.86 a</td>
<td>1.47 a</td>
<td>0.81 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>1.94 a</td>
<td>1.37 a</td>
<td>0.71 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>2.19 b</td>
<td>0.54 b</td>
<td>0.28 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>3.41 a</td>
<td>1.57 a</td>
<td>0.84 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>3.79 a</td>
<td>1.27 a</td>
<td>1.09 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>3.87 a</td>
<td>1.81 a</td>
<td>1.17 a</td>
</tr>
</tbody>
</table>

* Total root biomass was obtained by combining roots in biochar amended layer and the bottom soil layer. Means in each column with the same letter(s) within each DAP and biochar for each variable are not significantly different according to DMRT at 5% confidence level (P>0.05). CMB = Chicken manure biochar, WCB = wheat chaff biochar.
Table 6.4 Effects of two sources of biochar (Experiment 6.2 on mycorrhizal colonisation) after 8 weeks at varying amount with and without fertiliser application in both top and bottom layer in Experiment 6.2.

<table>
<thead>
<tr>
<th>Biochar</th>
<th>DAP</th>
<th>Amount (t/ha)</th>
<th>AM root colonised (%) (top)</th>
<th>AM root length colonised (cm) (top)</th>
<th>AM root colonised (%) (bottom)</th>
<th>AM root length colonised (cm) (bottom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMB</td>
<td>nil</td>
<td>0</td>
<td>41 c</td>
<td>35 d</td>
<td>61.95 a</td>
<td>47.73 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>46 b</td>
<td>45 b</td>
<td>85.75 a</td>
<td>63.99 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>47 b</td>
<td>43 c</td>
<td>83.08 a</td>
<td>71.02 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>51 a</td>
<td>48 a</td>
<td>106.11 a</td>
<td>73.74 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>26 a</td>
<td>16 c</td>
<td>19.54 a</td>
<td>8.65 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>33 a</td>
<td>31 a</td>
<td>30.32 a</td>
<td>26.09 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>28 a</td>
<td>24 b</td>
<td>33.42 a</td>
<td>18.35 a</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>33 a</td>
<td>26 b</td>
<td>28.84 a</td>
<td>16.21 a</td>
</tr>
<tr>
<td>WCB</td>
<td>nil</td>
<td>0</td>
<td>41 b</td>
<td>32 b</td>
<td>58.57 b</td>
<td>53.31 b</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>53 a</td>
<td>47 a</td>
<td>100.61 a</td>
<td>54.77 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>58 a</td>
<td>46 a</td>
<td>98.05 a</td>
<td>59.58 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>58 a</td>
<td>48 a</td>
<td>115.17 a</td>
<td>82.58 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>27 a</td>
<td>19 bc</td>
<td>13.65 c</td>
<td>8.40 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>24 a</td>
<td>21 ab</td>
<td>47.03 ab</td>
<td>34.44 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>32 a</td>
<td>24 a</td>
<td>61.04 a</td>
<td>18.54 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>17 b</td>
<td>17 c</td>
<td>33.61 bc</td>
<td>16.14 b</td>
</tr>
</tbody>
</table>

Means in each column with the same letter(s) within each biochar and DAP for each variable are not significantly different according to DMRT at 5% confidence level (P>0.05). CMB = Chicken manure biochar, WCB = wheat chaff biochar

Microscopy observations of biochars for microbial colonisation

SEM characterisation of the biochar stock demonstrated that chicken manure biochar which comprised of mixture of chicken litter and wood chips had porous properties as found in the wood chips (Plate 6.2a) because chicken manure litter itself did not have a solid porous structure (Plate 6.2 b). The wood chip structure was sharp, with distinct edges. Although the charred chicken manure was observed to contain pores, they were irregular, subangular and sometimes fibrous in structure.
Plate 6.2: Scanning electron micrographs on characterisation of chicken manure biochar with wood chips (a-b) and wheat chaff biochar (c), prepared and observed using Method 4 as in Table 3.2. Micrographs (d-f) showed soil particles (d) and fungal growth (arrow) on the colonised biochar particles of chicken manure biochar with wood chips (e) and wheat chaff biochar (d, f) retrieved from soils after 8 weeks. Scale bar = 200 µm.
Fungal hyphae colonised both chicken manure biochar and wheat chaff biochar particles. Biochar particles or fragments of chicken manure in the chicken manure biochar were difficult to retrieve from soil due to their fragile structure, hence only particles from the wood chip fraction of this biochar source were observed. Soil particles were found attached to most biochar particles observed. Hyphal network observations showed that fungal colonisation of biochar was not extensive (Figure 6.2d-f) either in chicken manure biochar (Figure 6.2e) or wheat chaff biochar (Figure 6.2f).

In comparison to fungal colonisation of Simcoa biochar in Experiment 6.1, fungal colonisation of these two biochars was not extensive.

**Soil microbial biomass C and P**

There were either no effects or very minor effects of CMB or WCB on soil microbial C and soil microbial P in either the top or bottom layers of the pot (data not shown).

**Soil N and P**

In both layers of soil, inorganic N was unaffected by addition of either of the biochars in the absence of DAP addition (P≥0.05; Table 6.5). Similarly, when the soil was amended with DAP, inorganic N was unaffected by application of CMB addition (P≥0.05; Table 6.5). However, WCB application decreased inorganic N in the upper soil layer (P<0.05) but not in the lower soil layer (P≥0.05; Table 6.5). In both layers of soil, available P increased with addition of CMB in the absence of DAP addition (P<0.05; Table 6.5). When the soil was amended with DAP, inorganic N was unaffected by application of CMB (P≥0.05) with the exception of 7.5 t/ha where it increased slightly (P<0.05; Table 6.5). For WCB application in the absence of DAP, there was an increase in available P in the upper soil layer for 5 and 7.5 t/ha, but no effect in the lower layer. When DAP was added with WCB, there was no effect on available P in the upper soil layer (P≥0.05) but there was a decrease for all application levels of WCB in the lower soil layer (P<0.05) with no effect of application level (P≥0.05; Table 6.5).
### Table 6.5 Effects of two sources of biochar on in Experiment 6.2 on soil chemical properties after 8 weeks at varying amount with and without fertiliser application in both top (with biochar) and bottom soil layer in Experiment 6.2.

<table>
<thead>
<tr>
<th>Biochar</th>
<th>DAP</th>
<th>Amount</th>
<th>Nutrients in top and bottom soils</th>
<th>Top soil (water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inorganic N (Top) (Bottom)</td>
<td>Available P (Top) (Bottom)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mg kg(^{-1}))</td>
<td>(mg kg(^{-1}))</td>
</tr>
<tr>
<td>CMB</td>
<td>nil</td>
<td>0</td>
<td>7.12 a 4.60a</td>
<td>1.00 c 0.89 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>8.37 a 4.34a</td>
<td>1.99 c 1.29 bc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6.48 a 4.04a</td>
<td>3.87 b 2.12 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>6.75 a 4.57a</td>
<td>5.63 a 3.10 a</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td></td>
<td>21.20 a 10.17a</td>
<td>15.49 b 28.45 a</td>
</tr>
<tr>
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<td></td>
<td>2.5</td>
<td>20.17 a 8.17a</td>
<td>17.17 ab 19.31 a</td>
</tr>
<tr>
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<td></td>
<td>5</td>
<td>16.01 a 8.67a</td>
<td>17.61 ab 16.30 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5</td>
<td>12.66 a 4.92a</td>
<td>22.06 a 23.75 a</td>
</tr>
<tr>
<td>WCB</td>
<td>nil</td>
<td>0</td>
<td>7.14 a 2.99a</td>
<td>1.02 c 2.81 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>7.22 a 3.67a</td>
<td>1.43 c 1.37 a</td>
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<td></td>
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<td>6.43 a 4.47a</td>
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</tr>
<tr>
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<td>8.17 a 4.62a</td>
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</tr>
<tr>
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<td></td>
<td>26.86 a 6.24 a</td>
<td>19.27 a 28.95 a</td>
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<td></td>
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<td>11.68 b 5.10a</td>
<td>14.65 a 17.70 b</td>
</tr>
<tr>
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<td>7.86 b 4.13a</td>
<td>17.06 a 18.41 b</td>
</tr>
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<td></td>
<td>7.5</td>
<td>10.24 b 3.73a</td>
<td>18.38 a 18.24 b</td>
</tr>
</tbody>
</table>

Means in each column with the same letter(s) within each biochar and DAP for each variable are not significantly different according to DMRT at 5% confidence level (P>0.05). CMB = Chicken manure biochar, WCB = wheat chaff biochar

**Soil pH**

Soil pH (water) as measured only in the upper 5 cm layer of soil was significantly increased at the highest level (7.5 t/ha) of application of both biochar sources without DAP (P<0.05; Table 6.5). On the other hand, when the DAP fertiliser was applied, soil pH did not differ when either of the biochars was added to the soil (P≥0.05; Table 6.5).
6.4 Discussion

Application of biochar as a soil conditioner showed slight stimulation of indigenous AM fungal colonisation on roots of subterranean clover and wheat grown in the two agricultural soils studied here. Application of biochar when either mixed throughout the soil (Simcoa biochar in Experiment 6.1) or banded (for both wheat chaff and chicken manure biochars in Experiment 6.2) did increase mycorrhizal colonisation when measured as either proportion of roots colonised or length of root colonised, however, the effects varied over time, with application of P and in different parts of the root system.

The increase in mycorrhizal colonisation in response to biochar application corresponded with previous observations of AM fungal colonisation being stimulated by biochar application (Ishii and Kadoya, 1994; Solaiman et al., 2010; Elmer and Pignatello, 2011). However, the generally positive findings of Simcoa biochar (woody biochar) on mycorrhizal colonisation (in this chapter) differed from previous studies of biochar and indigenous AM fungi where reduced AM colonisation was reported (Rondon et al., 2007; Warnock et al., 2010) when woody Eucalyptus biochar and lodge pine wood and mango biochar were applied. However, responses can vary with time (Experiment 6.1) and with level of biochar application (Experiment 6.1: the amount of biochar that increased % mycorrhizal root length colonised was not the same at each of the 4 harvest times; Experiment 6.2: WCB in the presence of DAP when applied at 7.5 t/h decreased % root length colonised in comparison to lower levels of application.

Thus, while variability in AM colonisation responses to biochar can be associated with the amount and type of biochar applied, soil type and soil fertility, the time of sampling of roots and method of measurement of mycorrhizas (e.g. as a proportion of the roots colonised vs the length of root colonised) may also influence the conclusions drawn. The response to biochar application may also differ among plant species (in this case subterranean clover and wheat were used in the two experiments, but they were not compared, as they were grown in different soils with different growth conditions and sampling duration. Moreover, mycorrhizal colonisation could also vary according to
host-plant-mycorrhizal-dependency of wheat and subterranean clover plants (Tawaraya, 2003; Li et al., 2005). The higher amounts of Simcoa biochar (0-50 t/ha) resulted in mycorrhizal colonisation of subterranean clover roots of between 56-65% at week 9 while a lower amount (0-7.5) of CMB and WCB (without fertilizer) resulted in between 32-48% mycorrhizal wheat root colonised at 8 weeks. This variability in AM colonisation responses to biochar although in different host plant may be associated with the amount and type of biochar applied and soil characteristics (type and soil fertility).

The optimum amount of biochar in facilitating AM colonisation was hypothesized to be dependant upon the P status of the soil. Within the limits of the design of these experiments, the amount of biochars suitable for soil application appeared to vary according to its source and the P status in soils into which the biochar is applied. This was in line with suggestions by Gaur and Adholeya (2000), Gryndler et al. (2006) and Warnock et al. (2010). Overall, there are several factors to consider in relation to the optimum amount of biochar required to stimulate AM fungi. Soil condition, physical and chemical characteristics of biochar, method of biochar application may influence the degree of response to biochar use. In this case, Simcoa biochar was used at levels 6.5 times higher than that of WCB and CMB, based on Experiment 6.1 and the study of Demspter et al. (2011).

Previously, Yamato et al. (2006) observed that colonisation by AM fungi (% root length colonised) was highest for bark charcoal application in the absence of phosphorus fertiliser application. Furthermore, application of woody *Eucalyptus* biochar at relatively low amounts (1 and 3 t/ha) under field conditions which had received agricultural levels of P as fertiliser, increased mycorrhizal colonisation (Blackwell et al., 2010; Solaiman et al., 2010). The amounts of woody biochar used in Experiment 6.1 were up to 10 times higher than those used in studies by Blackwell et al. (2010) and Solaiman et al. (2010). Despite observations of biochar effects on mycorrhizas formation, transient changes in mycorrhizal colonisation were not associated with changes in plant growth.
Although the effect of banding with other placement methods was not compared in the two experiments presented here, slightly higher microbial biomass and mycorrhizal colonisation was apparent in the top, biochar banded layer of soil. Movement of biochar particles in this short-term study (8 weeks) was not expected and has been previously been shown to take considerable time (Blackwell et al., 2009). Previous studies also showed better stimulation of microbial biomass in the rhizosphere layer than that in bulk soil, however, this needs to be further evaluated.

The underlying mechanisms of interactions between the mycorrhizal fungi and forms of biochar with different physical characteristics are likely to be complex (Warnock et al. 2010), but further evaluation of the hypotheses relevant to different soil conditions, plant species, communities of AM fungi and climatic factors is required. It was not possible to demonstrate whether the physical nature of the biochars used in this study (especially porosity) had any influence on the optimum amount required to stimulate colonisation of roots by AM fungi.

Microscopy evidence supported the proposal in which biochar could be colonised and act as habitat for soil fungi, possibly as one of the mechanisms that could underlying the interactions of biochar and soil fungi which could include naturally occurring AM fungi in soil or at biochar-soil interfaces. However, it was not confirm that the hyphae observed using high resolution microscopy and fluorescence techniques belonged to AM fungi because unsterilised agricultural soils which would have contained a wide range of soil fungi were used here. Biochar observations in previous chapters in this thesis showed that interactions with soil particles, including clogging of biochar pores and surface attachment of soil to biochar. Such effects have the potential to either facilitate or hinder colonisation of biochar by hyphae of AM fungi depending on accessibility to biochar surfaces and connectivity of the internal pore structure of the biochar. It is worth noting that the biochar particles examined here were retrieved from a soil system whereby plants were grown and biochar interaction with roots and soil would have contributed to the formation of micro-aggregates. This was different to that
Chapter 6: Development of Arbuscular Mycorrhizas in Soil Amended with Biochar

in previous chapters (Chapters 3-5) in which all colonised biochar observed had been incubated in soil without plants.

The findings in this study add further visual information on the potential for biochar to contribute to for micro-aggregate formation in association with roots and fungal hyphae. Visual evidence of the biochar-soil-fungal interface suggests that biochar could contribute additional habitat that might benefit mycorrhiza development and trigger improvement in soil conditions such as aggregation in sandy soil, however, the interaction with roots may be important in sandy soils such as those used in these studies because there was relatively little colonisation of biochar particles incubated in the agricultural soils used in Chapters 3 and 4 in the absence of roots.

There was little effect of Simcoa biochar application to soil on soil microbial biomass except for higher microbial biomass C at the higher amount of application (25 and 50 t/ha) which corresponds with the study by Chan et al. (2008). In Experiment 6.2, the application of wheat chaff biochar or chicken manure biochar (2.5 to 7.5 t/ha) in the range of 0 to 7.5 t/ha had no consistent effect on soil microbial biomass. Variation in response may be attributed to differences in biochar properties. Simcoa biochar produced at 600-650°C could be associated with high carbon in terms of chemical properties (Singh et al., 2010a, 2010b, 2012) while both wheat chaff biochar or chicken manure biochar were pyrolysed at 450°C had lower carbon and higher ash contents (Chan et al., 2007, 2008; Downie et al., 2009).
6.5 Conclusion

The objectives of this study were to determine the effect of biochar type and amount in the presence or absence of diammonium phosphate (DAP) fertiliser on mycorrhizal colonisation. The underlying mechanism by which biochar might serve as a potential habitat was investigated using microscopy. AM fungal colonisation and microbial biomass were both measured to determine possible synergies involved at soil-biochar-plant-microbe interfaces. It was hypothesised that (i) mycorrhizal colonisation would increased with increasing biochar increment when phosphorus was not applied, and that (ii) an optimum amount of biochar for facilitating AM colonisation would depend on the P status of the soil. However, biochars derived from wood, wheat chaff or chicken had little effect on plant growth or mycorrhizal colonisation of roots in two agricultural soils. Transient effects were observed with addition of all three forms of biochar to soil. Suitable optimum amounts of biochar application with respect to AM fungi and plant growth vary and can be dependent upon the biochar source and soil. The appropriate amount of biochar for use in agricultural soil needs to consider possible interactions between soil P and AM fungi. Soil nutrient status, biochar placement and host plants can contribute to variable AM responses observed in agricultural soils and thus diminish opportunities for direct comparison. While, the amount of biochar applied to agricultural soil needs to be considered for improved AM symbiosis hence plant growth, there is little evidence that increases in mycorrhizal colonisation in response to biochar application are associated with plant productivity. Variable AM responses to higher levels of application of Simcoa biochar from woody source and to lower levels of application of wheat chaff and chicken manure biochar could be associated with physical and chemical characteristics of biochar. The biochars derived from wood, wheat chaff or chicken manure used in these experiments had negligible effects on soil microbial biomass carbon and phosphorus in the agricultural soils studied.
CHAPTER 7
BIOCHAR AND BIOSOLIDS AS SOIL AMENDMENTS:
INTERACTIONS IN FOUR AGRICULTURAL SOILS

7.0 Abstract

Soils in south-western Australia are generally deficient in nutrients for agricultural production and require management to achieve adequate levels of chemical fertility. Addition of biochar with clay has been suggested as a mean of improving soil carbon storage, texture and nutrient retention of these soils. In previous chapters in this thesis, six biochars were used to amend soils from four agricultural sites in south-western Australia. Here, further evaluation of woody biochar and its potential for ameliorating soil properties including soil microbial biomass and mycorrhizal colonisation in the presence or absence of a lime-clay-biosolids product (LaBC®) was assessed. First, an incubation experiment was conducted for 28 days to compare the effects of contrasting source of woody biochars (Wundowie, Simcoa and activated biochar) on microbial biomass in four WA soils. It was hypothesized that these three woody biochars would result in higher microbial biomass in each soil. Micrographs of Simcoa biochar after 28 days incubation in soil prepared from SEM observations exhibited the presence of soil particles on the biochar. The clayey soil had fewer sand grains and maintained a higher trend of microbial biomass and respiration than did the three loamy sand soils. Activated biochar increased microbial respiration compared to the normal pyrolysed biochars. In a glasshouse experiment using the same four soils amended with one of the biochars (Simcoa), observations were made with or without addition of a lime-clay-biosolid product (LaBC®) to the soils. The combination of Simcoa biochar and LaBC® was expected to improve soil microbial biomass and arbuscular mycorrhizal colonisation and plant growth due to ameliorative values of these amendments. Soil microbial biomass, root length colonised by mycorrhizal fungi and plant grown in Simcoa biochar amended soil was lower than in soil amended with biochar+LaBC® or LaBC® alone after eight weeks in the three loamy sands. However, microbial biomass and mycorrhizal root length colonised or root growth were not responsive to these treatments in the clayey soil.
Chapter 7: Biochar and Biosolids Amendment to Four Agricultural Soils

7.1 Introduction

Biochar amelioration effects on soils can vary depending on the soil carbon content (Kimetu and Lehmann, 2010), soil textural differences (Yeboah et al., 2009) and soil management practices (Beesley et al., 2010). The response of soil microorganisms, including arbuscular mycorrhizal (AM) fungi, to soil amendment with biochar may also vary with land use and agricultural management practices. With respect to AM fungi, biochar application has been assessed for a wide range of soils including sands, sandy loam and sandy clay loams (Ishii and Kadoya, 1994; Elmer and Pignatello, 2011; Table 2.1). The response of biochar in different soils needs further investigation in order to better understand interactions between biochar and soil microorganisms associated with nutrient cycling. For example, reduced mycorrhizal colonisation was found when biochar was applied to a clay loam soil (Rondon et al., 2007) but there was an increase in mycorrhizal colonisation in response to application of biochar in other soil types (Matsubara et al., 2002; Blackwell et al., 2010).

Soils in south-western Australia are commonly coarse textured and this generates problems for agricultural purposes associated with water and nutrient retention. The organic matter content of the soils is also generally low (Jarvis et al., 1996). To overcome these problems, soil management that emphasis organic matter retention such as no till practices (Thomas and Stanley, 2007) or additional clay (McKissock et al., 2002) has been applied to the soil. The use of biochar has been proposed as a means of increasing carbon storage in soil and decreasing leaching of nutrients from sandy soil in this region (Blackwell et al., 2010; Solaiman et al., 2010; Dempster et al., 2012a, 2012b). In addition to biochar, a lime-clay-biosolids product (LaBC®) can be an effective slow release fertiliser capable of ameliorating soil acidity and water repellence (Shanmugam, 2013; Shanmugam et al., 2014) in coarse textured, acidic soil in the Elenbrook Catchment in south-western Australia. Soil amendment with both biochar and clay amended biosolids could alter soil biological processes and via mechanisms that influence water availability and aggregation based on previous work on biochar and biosolids (Knowles et al., 2011; Shanmugam et al., 2014).
The potential for biochar to assist in retention of nutrients in soil and reduce leaching has been demonstrated (Ding et al., 2010; Mukherjee et al., 2011; Mukherjee and Lal, 2013). A biochar and biosolids lysimeter experiment conducted by Knowles et al. (2011) showed that biochar addition to soil could reduce nitrate leaching from biosolids amended soil. Similarly, retention of nutrients by a combination of biochar and clay was associated with reduced cumulative $\text{NH}_4^+$ leaching (Dempster et al., 2012b). Physical properties of biochar, including high surface area and porosity as well as ion exchange capacity, are normally associated with nutrient retention by biochar in soil (Liang et al., 2006).

In this Chapter, the effect of biochar in four soils with different land use histories, organic matter and soil texture was investigated in an incubation experiment, and this was followed by a plant growth experiment with and without soil amendment with the lime-clay-biosolid product, LaBC® (Figure 7.1). It was hypothesized that application of woody biochar(s) to soil would increase microbial responses in the four soils. Physical changes in the biochar after incubation in soil, especially the accumulation of soil particle accumulation on biochar surfaces, would reduce the potential of biochar as a habitat for soil fungi to a lesser extent in the sandy loam textured soils than in the clayey soil. In the glasshouse experiment, it was hypothesized that biochar would interact with LaBC® leading to increased microbial activity.

The objectives of this study were: (i) to use electron microscopy (SEM) to characterise biochar changes after incubation in four soils from south-western Australia with different land use histories, soil carbon and texture, (ii) to determine the effect of woody biochars prepared from a range of pyrolysis conditions and feedstocks on soil microbial biomass when added to four soils with different land use histories, soil carbon and texture, and (iii) to assess the effect of a combination of one of the woody biochars and LaBC® application on soil microbial biomass and mycorrhizal colonisation and growth of subterranean clover in four soils with different land use histories, soil carbon and texture.
Figure 7.1 Conceptual flow of experimental designs of biochar and lime-clay-amended-biosolids product (LaBC®) as soil amendments in Chapter 7 (highlighted in blue box).
7.2 Materials and methods

7.2.1 Experiment 7.1 (Effects of 3 biochars on microbial status of 4 WA soils)

Experimental design
An incubation experiment at 25°C was conducted using three biochars added to four soils collected from the Chittering and Bullsbrook areas of south-western Australia. Biochars were incubated in soil for 4 weeks. The treatments were: no biochar (control), Simcoa biochar, activated biochar and Wundowie biochar, with three replications per treatment. Soil was sampled at day 1, 7, 14 and 28. Each soil (100 g) was thoroughly mixed with 2 t/ha equivalent biochar with particle size 1-4 mm and incubated in 200 ml vials arranged in a completely randomised design, watered to 45% water holding capacity, and incubated in a 25°C constant temperature room. A parallel assay with the same treatments was set up in glass containers to measure carbon dioxide release as described in Chapter 4.

Soil preparation
Soils were collected from three sites in the Chittering Shire, north of Perth. Soil 1 was from an alternative crop trial at Chittering Landcare Land Use Demonstration Project area. Soil 2 was from an agroforestry area at the same Landcare demonstration site and Soil 3 was from a permanent annual pasture, 3 km to the east of this location. Soil 4 was from a pasture near Bullsbrook. The soils were collected from the surface 0-10 cm and sub-samples were sent for analysis to CSBP Soil and Plant Laboratory (Table 7.1; for analyses used see Chapter 4).

Biochar sources
Three biochars were used in the soil incubation experiment (Table 7.2). The two sources of woody biochars (Simcoa and Wundowie biochars) were obtained from Simcoa Ltd, Bunbury, WA and from Best Energy, Australia respectively. Simcoa biochar was made from jarrah wood (*Eucalyptus marginata*) in 2008 by Simcoa Ltd. It was produced at a pyrolysis temperature of 600°C for 24 hours. Wundowie biochar was obtained as described in Chapter 3. Activated biochar was obtained from ANSAC and produced by pyrolysis from Verve samples of *Eucalyptus polybractea* grown in the Narrogin region at a pyrolysis temperature of 700°C. The biochar had been activated by adding steam at
860°C. The biochar particles were thoroughly mixed with soil prior to incubation. Characterisation of Simcoa, Wundowie and activated biochars are presented in Table 7.2.

Table 7.1 Physical and chemical properties of 4 soils used in Experiments 7.1 and 7.2.

<table>
<thead>
<tr>
<th>Soil characteristics</th>
<th>Soils¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Nitrogen (mg kg⁻¹)</td>
<td>Soil 1</td>
</tr>
<tr>
<td>Nitrate Nitrogen (mg kg⁻¹)</td>
<td>1</td>
</tr>
<tr>
<td>Phosphorus Colwell (mg kg⁻¹)</td>
<td>75</td>
</tr>
<tr>
<td>Potassium Colwell (mg kg⁻¹)</td>
<td>46</td>
</tr>
<tr>
<td>Sulphur (mg kg⁻¹)</td>
<td>4.9</td>
</tr>
<tr>
<td>Organic Carbon (%)</td>
<td>1.74</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.033</td>
</tr>
<tr>
<td>pH Level (CaCl₂)</td>
<td>4.3</td>
</tr>
<tr>
<td>pH Level (H₂O)</td>
<td>5.2</td>
</tr>
<tr>
<td>Textural class</td>
<td>loamy sand</td>
</tr>
</tbody>
</table>

¹Soil 1 = alternative crop area (Chittering), Soil 2 = agroforestry area (Chittering), Soil 3 = pasture (Chittering), Soil 4 = pasture (Bullsbrook)

Table 7.2 Characteristics of biochars used in Experiment 7.2.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Biochar Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Simcoa</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>600</td>
</tr>
<tr>
<td>C Content</td>
<td>73.8</td>
</tr>
<tr>
<td>EC (mS/cm)</td>
<td>0.54</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>8.56</td>
</tr>
<tr>
<td>pH (CaCl₂)</td>
<td>7.62</td>
</tr>
</tbody>
</table>

Biochar preparation for microscopy observation

Simcoa biochar characteristics were investigated using scanning electron microscopy before (as reported in Chapters 3 and 4) and after 28 days of incubation in the 4 soils
tested. Prior to incubation, samples of the biochars were treated and prepared as described in Chapter 3 (Table 3.2) except for the following modification. This modification was made based on the objective to observe biochar after removal from soil, especially accumulation of soil particles. After 28 days of incubation in soil, the Simcoa biochar particles were quickly air dried and mounted on stub and described as Method 5 (Table 3.2). Only Simcoa biochar samples were examined from each soil using scanning electron microscopy (SEM) as detailed in Chapter 3.

**Soil analysis**

Soils amended with each of the four different biochar treatments were analysed for microbial biomass carbon, soil microbial biomass phosphorus cumulative carbon dioxide and soil pH, as described in Chapter 4.

7.2.2 Experiment 7.2 (Effects of biochar and LaBC® on soil microbial status and mycorrhizal colonisation of subterranean clover in 4 WA soils)

**Experimental design**

A completely randomised factorial glasshouse experiment was conducted with treatments (i) biochar alone, (ii) LaBC® alone, (iii) biochar + LABC®, and (iv) control (no biochar or LaBC®) applied to the four soils used in Experiment 7.1. There were two plant harvests (4 and 8 weeks). The lime-clay-biosolids product, LaBC®, was produced and provided by the Water Corporation, Western Australia after blending two by-products (lime amended biochar (LAB) from Subiaco Waste Water Treatment Plant and uncontaminated natural clay from Eastern Metropolitan regional Council’s Red Hill solid waste facility). LaBC® was purposely developed for coarse-textured acidic-sandy soils of the Ellen Brook catchment to the south of the Swan Canning Estuary system in south-western Australia (Shanmugam, 2014; Shanmugam et al., 2014). The wet LaBC® formula comprised about 64% dry clay, 3% lime and 6% organic matter from biosolids, 11.6% organic matter, 0.34% total N, 0.07% total P (w/w), alkaline (pH >12) and slightly water soluble according to analysis by the Water Corporation WA (Shanmugam et al., 2014).
Biochar placement, plant maintenance and harvest

Simcoa biochar was selected based on its high porosity and for consistency as previously reported in Chapters 3, 4, 5 and 6. There were two harvests over the eight-week plant growth period. Simcoa biochar and LABC® were applied at 2% (v/v) respectively, and thoroughly mixed in 2 kg soil per pot. The 2% Simcoa biochar and LABC® were applied in the respective treatments following previous findings and experimental treatments as in Chapter 5 (addition of 2% organic v/v) and approximate application range as in Chapter 6. The pots were placed in a completely randomised block design in the glasshouse. Two subterranean clover (Trifolium subterranean L. var. Seaton Park) seedlings were grown in each undrained pot and soil was maintained at field capacity. Subterranean clover was chosen as host plant based on the higher AM root colonisation than for wheat as discussed in Chapter 6.

Soil and plant analyses

Plants and soils were sampled at 4 weeks and 8 weeks after planting. At each harvest, shoot and root biomass were determined (fresh weight) then oven dried at 60°C to constant weight. Sub-samples of fresh roots were used for assessing mycorrhizal colonisation as described in Chapter 6. Soil microbial biomass carbon, soil microbial biomass phosphorus and soil pH were examined from fresh soil (as described in Chapter 4). Fresh roots were retained for quantification of mycorrhizal colonisation as described in Chapter 6.

7.2.3 Statistical Analysis

Analysis of variance (ANOVA) was performed using Statistical Analysis System (SAS) programme version 8.02 for Windows. Comparison of treatment means was checked using the Duncan’s Multiple Range Test (DMRT) at 5% confidence level. All variables taken were analysed by factorial analysis at the time of sampling.
7.3 Results

7.3.1 Experiment 7.1 (Effect of biochars on microbial status of 4 WA soils)

**SEM visualisation of biochar incubated in 4 WA soils**

Scanning electron micrographs of biochar incubated in the four soils showed variation in the extent to which soil was attached to their surfaces (Plates 7.1 and 7.2). Sand grains varying in shape and size (fine to coarse; 20 µm to 100 µm) were observed on biochar particles in each soil (Plates 7.1 and 7.2). Visual differences on soil particles including sand grain coverage were noted among biochars incubated in clayey and loamy sand soils. Approximately 10% of biochar surfaces were clear with few sand grains as observed over 5 fields of views for biochar particles retrieved from Soil 2 (Plate 7.1e) and Soil 3 (Plate 7.2b). This was independent of the textural background of Soils 2 and 3. Some biochar surfaces were blanketed with sand grains as observed for Soil 2 (Plate 7.1d) and aggregates of clay, silt and/or loam particles were present on some biochar particles, particularly for Soil 3 (Plate 7.2a) and loamy sand Soil 4 (Plate 7.2f). Overall, biochar particles incubated in Soil 3 had 10% sand grains (observed over 5 fields of views) attached to their surfaces than for biochar incubated in the other soils.

**Effects of biochars on microbial biomass carbon in 4 WA soils**

There was a significant (P≤0.05) interaction between biochar and soil for soil microbial biomass C during the 28 day incubation. The microbial biomass C varied with type or source of biochar added to each soil at day 1 (Figure 7.2). Application of woody biochars significantly decreased microbial biomass carbon at day 1. All biochars reduced microbial biomass C in Soils 1 and 4. Activated biochar significantly (P<0.05) increased soil microbial C after 24 hours in Soil 2 but not in Soils 3 and 4. All these effects were transient, and where they occurred, they did not persist beyond day 7 (Figure 7.2). In the clayey Soil 3, activated biochar increased soil microbial C at day 28. The highest (200 mg kg⁻¹) level of microbial biomass C was found in activated biochar treatment in clayey Soil 3 (Figure 7.2). Activated biochar resulted in the lowest level of microbial biomass carbon in Soil 1.

There was no effect of any of the biochars on microbial biomass P in any of the 4 soils (data not shown).
Plate 7.1 Scanning electron micrographs of Simcoa biochar particles incubated in Soil 1 (a, b, c) and Soil 2 (d, e, f) for 4 weeks (28 days) (Experiment 7.1), prepared and observed using Method 5 as in Table 3.2. Scale bar = 200 µm.
Plate 7.2 Scanning electron micrographs of Simcoa biochar particles with soil particles incubated in Soil 3 (a, b, c) and Soil 4 (d, e, f) for 4 weeks (28 days) (Experiment 7.1), prepared and observed using Method 5 as in Table 3.2. Scale bar = 200 µm.
Figure 7.2 Effects of biochars on soil microbial biomass C in 4 soils throughout 28 days incubation period. 1, 2, 3, 4 indicate different soils. Soil 1 = Alternative crop area, Soil 2 = agroforestry area, Soil 3, Soil 4 = natural grassland area. Control = no biochar, Simcoa = Simcoa biochar, Activated C = Activated biochar, Wundowie = Wundowie biochar. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
There was a significant (P<0.05) interaction between biochar source and soil for microbial respiration throughout the 28 days incubation (Figure 7.3). Treatments with the activated biochar in Soils 1, 2, and 3 increased (P<0.05) microbial respiration as early as day 1. Microbial respiration further increased when amended with activated biochar in loamy sand soil (Soils 1, 2 and 4) after 14 days but not in Soil 3 (the clayey soil).

Biochar addition increased soil pH (P<0.05) for all soils throughout the 28 days of incubation. The highest soil pH was recorded at day 1 for Soils 1, 2 and 4 when amended with activated biochar (Figure 7.4). Soil amended with Wundowie biochar had the lowest pH on day 1 but it was highest in Soils 1, 2 and 3 after 28 days.
Figure 7.3 Effects of biochars on soil microbial respiration (cumulative carbon dioxide) in 4 soils throughout 28 days incubation period. 1, 2, 3, 4 indicate different soils. Soil 1 = alternative crop area, Soil 2 = agroforestry area, Soil 3, Soil 4 = natural grassland area. Control = no biochar, Simcoa = Simcoa biochar, Activated C = Activated biochar, Wundowie = Wundowie biochar. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
Figure 7.4 Effects of biochars on soil pH in 4 soils throughout 28 days incubation period. 1, 2, 3, 4 indicate different soils. Soil 1 = alternative crop area, Soil 2 = agroforestry area, Soil 3 = natural grassland area, Soil 4 = natural grassland area. Control = no biochar, Simcoa = Simcoa biochar, Activated C = Activated biochar, Wundowie = Wundowie biochar. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
7.3.2 Experiment 7.2 (Effects of biochar and LaBC® on soil microbial status and mycorrhizal colonisation of subterranean clover in 4 WA soils)

Effects of biochar and LaBC® on plant growth in 4 WA soils

There was an interaction between soil type and soil amendment on shoot growth of subterranean clover at week 8 but not at week 4 (P<0.05; Figure 7.5; data for harvest 1 not shown). There was no shoot growth response to application of biochar alone, LaBC® alone or in combination (biochar+LaBC®) for Soils 1 and 4 (P>0.05; Figure 7.5a). Addition of LaBC® alone and in combination with biochar (biochar+LaBC®) significantly increased (P<0.05) shoot growth in Soils 2 and 3 (Figure 7.5a).

In Soils 1 and 4, root biomass was increased (P<0.05) by addition of biochar and LaBC® and there was an additional effect of combining biochar and LaBC® on root growth in Soil 1 (P<0.05; Figure 7.5b). In Soil 2, there was no effect of biochar on root growth but there was an increase (P<0.05) in response to application of LaBC®. In Soil 3, there was no effect of biochar, LaBC® or biochar+LaBC® on root growth (P>0.05).

In Soil 1, biochar and LaBC® both increased root length, and when they were combined, root length was higher (P<0.05; Figure 7.5c). In Soil 2, only biochar+LaBC® increased (P<0.05) root length, with no effect (P>0.05) of biochar and LaBC® added separately (Figure 7.5c). In Soil 3, LaBC® increased root length, but there was no effect (P>0.05) of biochar and LaBC® in combination on root length (P>0.05). In Soil 4, biochar increased root length (P<0.05) but there was no effect of either LaBC® alone or in combination with biochar on root length (P>0.05).

Effect of biochar and LaBC® on soil microbial carbon in 4 WA soils

There was a significant interaction between soil and amendment (P<0.05) for soil microbial biomass C at harvest 1 (Figure 7.6). Soil 3 had the highest level of soil microbial C and there was no change in soil microbial C in response to amendments applied to this soil (Figure 7.6).

Soils, soil amendments and their interactions significantly (P<0.05) affected soil microbial P at the first harvest but the differences were almost negligible (data not shown). No significant effects of soils or amendments were found for soil microbial P at the second harvest.
Figure 7.5 Effects of biochar and LaBC® on subterranean clover shoot (a) and root biomass (b) and root length (c) in 4 soils after 8 weeks planting period. Soil 1 = alternative crop area, Soil 2 = agroforestry area, Soil 3 = natural grassland area, Soil 4 = natural grassland area. Control = No biochar or LaBC®, Biochar= Biochar only, LaBC® = LaBC® only, Biochar+LaBC®=Biochar and LaBC®. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
Figure 7.6 Effects of biochar and LaBC® on soil microbial biomass carbon in 4 soils after 4 and 8 weeks planting period. Soil 1 = alternative crop area, Soil 2 = agroforestry area, Soil 3 = natural grassland area, Soil 4 = natural grassland area. Control = No biochar or LaBC®, Biochar= Biochar only, LaBC® = LaBC® only, Biochar+LaBC®=Biochar and LaBC®. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
Effects of biochar and LaBC® on mycorrhizal colonisation of subterranean clover grown in 4 WA soils

Soil amendment with either biochar or LaBC® alone did not alter the proportion of mycorrhizal root length colonised in Soils 3 and 4 at either harvest 1 or harvest 2 (Figure 7.7 a, b). Addition of LaBC® generally decreased mycorrhizal colonisation (%) in Soils 1 and 2. Root length colonised (cm) was not different among the amendments for all soils at harvest 1 (data not shown). Mycorrhizal root length colonised was greatest in the biochar+LaBC® treatment in Soil 2 at the second harvest (P<0.05; Figure 7.7c). Biochar increased root length in Soil 1, but not in Soils 2 and 3.

There was a consistent increase in soil pH with application of LaBC® alone and in combination with biochar in each soil (P<0.05; Figure 7.8). Biochar application alone increased soil pH in each soil to a lesser extent than did LaBC® in all four soils (P<0.05; Figure 7.8) after 4 weeks but not after 8 weeks.
Figure 7.7 Effects of biochar and LaBC® on arbuscular mycorrhizal fungi (AMF) root length colonisation (%) (a and b) at week 4 and 8 and root length colonised (cm) of subterranean clover (c) after 8 weeks planting period. 1, 2, 3, 4 indicate different soils. Soil 1 = alternative crop area, Soil 2 = agroforestry area, Soil 3 = natural grassland area, Soil 4 = natural grassland area. Control = No biochar or LaBC®, Biochar= Biochar only, LaBC® = LaBC® only, Biochar+LaBC®=Biochar and LaBC®. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
Figure 7.8 Effects of biochar and LaBC® on soil pH for 4 soils after 4 and 8 weeks planting period. 1, 2, 3, 4 indicate different soils. Soil 1 = alternative crop area, Soil 2 = agroforestry area, Soil 3 = natural grassland area, Soil 4 = natural grassland area. Control = No biochar or LaBC®, Biochar = Biochar only, LaBC® = LaBC® only, Biochar+LaBC® = Biochar and LaBC®. Means followed by the same lower-case (n=12) letters within a soil are not significantly different at P ≤0.05 by Duncan Multiple Range test (DMRT).
7.4 Discussion

Responses to biochar source (Experiment 7.1) and biochar in combination of LaBC® (Experiment 7.2) were studied in four south-western Australian soils.

Microscopy observations on Simcoa biochar particles retrieved from the incubation study (Experiment 7.1) showed that biochar particles were covered by soil particles when incubated in each of the four soils. Microscopy observation from incubation studies (Experiment 7.1) indicated that clayey soil had less sand grain attachment than did the more loamy sand soils. The potential ameliorating effects of biochar source (Wundowie, Simcoa and activated biochar) were then compared within each of the four soils in Experiment 7.2. The clayey soil which had fewer sand grains maintained a higher trend of microbial biomass and respiration than did the three loamy sand soils. The soil attachment to biochar particles may contribute to micro-aggregation, however, the impact of fewer attached sand grains on biochar particles incubated in Soil 3 (and less blockage to biochar pores) could be a contributing factor for a higher trend of microbial biomass and respiration than in the three loamy sand soils which is independent of soil amendment.

Activated biochar pyrolysed at 860°C and further activated by steam stimulated microbial respiration more than the other biochars which had been produced by normal pyrolysis (Simcoa and Wundowie biochars) although this was not reflected in a change in soil microbial C. Activated biochar has been shown to have greater porosity and surface area than normal pyrolysed biochar (Keech et al., 2005; Bird et al., 2008; Downie et al., 2009; Rillig et al., 2010). Activated biochar known for having high surface area could have more advantages than the normal pyrolysed biochar. For example, in a study by Rutto and Mizutani (2006), application of activated charcoal slightly alleviated a detrimental effect of root bark extract which was claimed to be responsible for reducing the benefits derived from mycorrhizal fungi for plant growth. The large surface area of materials such as activated biochar may enhance its ability to absorb organic compounds for soil detoxification purposes as well as improved nutrients and growth resources which promote microbial activities and respiration (Cornelissen and Gustafsson, 2003, 2004; Cornelissen et al., 2005; Foo and Hameed,
2010). Activated biochar owing to a higher pH compared to that of Simcoa or Wundowie biochars could also indirectly improve soil pH conditions, as evident in Soils 1, 2 and 4 after 24 hours incubation. Normal pyrolysed biochar can influence soil microbial biomass and respiration, but may be more limited than that of activated biochar (Chan et al., 2007, 2008; Kolb et al., 2009; Liang et al., 2010; Anderson et al., 2011). Biochars can induce different responses as Wundowie biochar had no effect on microbial biomass C or respiration when incubated in the soil used in Chapter 4, whereas Saligna biochar increased soil microbial C but not respiration and in that study.

There could be several contributing factors which determine the effect of pyrolysed biochar including soil background, the use of an appropriate amount of biochar for different soil types, and biochar characteristics. Dempster et al. (2012a) demonstrated lower microbial biomass carbon associated with similar Eucalyptus biochar to that used in this study when applied to a soil from south-western Australia. A strong influence of soil type on soil respiration could determine level of respiration following biochar application (Kolb et al., 2009). Furthermore, differences in nutrient availability among soils would alter the magnitude of a microbial response (Kolb et al. 2009).

Glasshouse studies (Experiment 7.2) were further conducted to determine the behavior and impact of adding a clay-lime-biosolids product (LaBC®) focusing on improving soil conditions with addition of a normal pyrolysed biochar. Simcoa biochar was selected, rather than activated biochar, for consistency of using ‘model’ and normal pyrolysed biochar as studied throughout this thesis. In response to LaBC® as soil amendment, the findings supported the hypothesis that a combination of Simcoa biochar and LaBC® (Simcoa biochar+LaBC®) would increase mycorrhizal colonisation of roots and microbial biomass C in the glasshouse study, but this was not consistent across soils or time. Furthermore, where a mycorrhizal response was observed, it only occurred for mycorrhizal root length colonised (rather than percentage of mycorrhizal root length colonised). Soil 3, which was clayey, exhibited no response in microbial biomass, percentage of mycorrhizal root length colonisation or mycorrhizal root length to these treatments.
The differences in responses in soils used in this study were in line with the observation that biochar amelioration effects can vary depending on the soil carbon content (Kimetu and Lehmann, 2010), soil textural differences (Yeboah et al., 2009) and soil management practices (Beesley et al., 2010). In the study presented here, the use of 2% biochar (equivalent to 20 t/ha) was in the moderate range compared with that used in Experiment 6.1 (Chapter 6) and slightly lower than that used by Dempster et al. (2012a). Most studies examining the effect of biochar amounts have concluded that an acceptable amount applied for one type of soil and plant species but may not be suitable in another situation (Glaser et al., 2002; Kolb et al., 2009; Blackwell et al., 2010).

As expected, the biochar combined with LaBC® effects may be associated with the added clay and/or lime in the LaBC® and therefore, the benefits of biochar+LaBC® would have been attributed primarily to LaBC® alone, with possible additional benefit of biochar contributing potential habitat for soil microorganisms. The clay and lime components of LaBC® may improve plant growth via nutrient mineralisation and reduced leaching (Dempster et al., 2012b). The combination of clay and biochar had been suggested to have an additional benefit in reducing nutrient leaching (Dempster et al., 2012b). If there had been a benefit from provision of habitat, biochar may protect AM fungal hyphae or stimulate hyphal development while the clay in LaBC® may also support mycorrhizal colonisation through provision of a substrate (Gaur and Adholeya, 2000). Saito and Marumoto (2002) had earlier stated that although the high porosity of charcoal is an effective substrate for saprophytes, it was also favorable for AM fungi. In addition to these effects, some AM fungi can sporulate inside the cavities of expanded clay and on the surface of clay material particles (Norris et al., 1992).

Soil microbial community of each soil is likely to be different, thus affecting the mycorrhizal colonisation for each soils. Furthermore, microbial biomass, the ratio of bacteria to fungi and microbial abundance, including that of AM fungi, could respond differently to biochar and clay or biosolids addition (Dempster et al., 2011; 2012).
7.5 Conclusion

In summary, incubation and glasshouse studies showed that varying soil backgrounds including initial carbon, nutrient status and textural properties of the soils can result in contrasting magnitude of microbial responses with addition of biochar or/and LaBC® to soil. In the incubation study comparing the potential effects of three contrasting biochars prepared from different pyrolysis conditions (Wundowie, Simcoa and activated biochar), sand and soil particles were observed attached to surfaces of the Simcoa biochar which were incubated in the four soils. The clayey soil had fewer sand grains on the surfaces of the Simcoa biochar particles and maintained a higher trend of microbial biomass and respiration than did the three loamy sand soils. Activated biochar had greater potential to increase microbial biomass carbon and respiration compared to the other biochar produced by normal pyrolysis. Further evaluation of the impact of Simcoa biochar and lime-clay-biosolids (LaBC®) on subterranean clover grown under glasshouse conditions for 8 weeks showed little effect on microbial biomass and root length colonised by AM fungi with application of Simcoa biochar alone. The more clayey soil which had the highest soil C and nutrient content was unresponsive to amendment with biochar and/or LaBC® in terms of soil microbial biomass, and mycorrhizal colonisation. In contrast, some response in soil microbial biomass, mycorrhizal root length colonised and plant growth to application of LaBC® alone or with Simcoa biochar were observed in the other three soils, although some of these effects were transient and/or inconsistent among the soils. There was no clear indication of a synergistic effect of adding Simcoa biochar in combination with LaBC® to improve plant productivity in the agricultural soils used.
CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

Three incubation experiments and three glasshouse experiments were conducted to investigate interactions between biochar and soil microorganisms in this thesis. The topics addressed were: (i) the role of biochar added to soil as an amendment and potential habitat for soil microorganisms, and (ii) biochar interactions with soil management and the impact on soil microorganisms, including arbuscular mycorrhizal (AM) fungi (Table 8.1). Associated changes in soil microbial biomass and colonisation of roots by AM fungi were measured in several agricultural soils from south-western Australia following various biochar treatments. While the effects of biochar and other inputs to soil were assessed, the possible mechanism(s) associated with interactions between biochar and soil microorganism, especially where biochar might alter growth, survival and activities of soil microorganisms, was also investigated. Changes in biochar properties following soil amendment were studied from the perspective of habitat provision for fungal colonisation using microscopy techniques for soil incubations in the absence of plants and glasshouse experiments in the presence of plants. Several factors associated with biochar and its management as a soil amendment affecting soil conditions and plant growth are summarised in Figure 8.1.

TOPIC 1: Biochar as a potential habitat for soil microorganisms and as a soil amendment - characterisation and associated issues

Selection of the form of biochar was an important factor in determining potential effects on soil microorganisms, especially when one of the underlying mechanisms involving biochar (i.e to serve as a microbial habitat) was the main aspect being studied. The woody biochars used throughout this thesis (Chapters 3, 4, 5, 6, 7) (Table 8.1) were used based on the assumption that their pores would provide habitat for fungal hyphae. They were highly porous, and potentially suitable as microbial habitat.
Table 8.1 Summary of experiments, main findings and probable mechanisms according to investigations in each chapter of this thesis.

<table>
<thead>
<tr>
<th>Chapter 3</th>
<th>Methodology and Microscopy&lt;br&gt;Objective: identifying biochar as potential habitat for soil microorganisms</th>
<th>Biochar is heterogenous&lt;br&gt;Soil incubated biochars had soil attached to the pores and surfaces</th>
<th>Biochars are porous and potential as microbial habitat in soil but biochars differ widely in their porosity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 4 - Incubation</td>
<td>Effects of woody biochars and particle sizes on microbial biomass&lt;br&gt;Objective</td>
<td>Moora (loamy sand)</td>
<td>woody</td>
</tr>
<tr>
<td>Chapter 5 - Incubation</td>
<td>Effects of woody biochar with crop residues on soil microbial status and soil P</td>
<td>Cunderdin (loamy sand)</td>
<td>woody</td>
</tr>
<tr>
<td>Chapter 6 - Pot experiments</td>
<td>Effects of application amount of Simcoa biochar on mycorrhizas and plant growth&lt;br&gt;Effects of plant vs animal derived biochar with fertiliser on mycorrhizas and plant growth</td>
<td>Cunderdin, Minginew (loamy sand)</td>
<td>woody, chicken manure biochar, wheat chaff biochar</td>
</tr>
<tr>
<td>Chapter 7 - Incubation and pot experiments</td>
<td>Effects of biochars on 4 different soils&lt;br&gt;Effects of biochar and commercial biosolids lime-clay amendment on mycorrhizas and plant growth</td>
<td>4 WA soils (3 loamy sand soils vs 1 clayey soil)</td>
<td>woody</td>
</tr>
</tbody>
</table>
Figure 8.1 Examples of interactions associated with biochar as a potential habitat for soil organisms and as a soil amendment investigated in this research.
High porosity of biochar is normally associated with retention of the original cellular structure of the plant materials in the biochar (Keech et al., 2005; Downie et al., 2009; Verheijen et al., 2009; Uchimiya et al., 2010; Anderson et al., 2011). With respect to habitat potential of other types or sources of biochar used in this research (such as chicken manure biochar and wheat chaff biochar), their potential as habitat could not be compared and ruled out although they may be able to deliver similar effects to those of porous woody biochars. This relates to structural modifications such as physical changes and interactions with soils which were not within the scope this study. Furthermore, some biochars tested were mixtures of materials, such as the chicken manure biochar used in Chapter 6 which included wood chips. Interestingly, biochar from plant waste such as wheat chaff biochar had potential as habitat although it may not retain its structure after deposition in soil. Thus, further comparisons of feedstocks such as animal vs plant parts vs woody biochars are required to improve understanding of their potential as microbial habitats in soil.

Characterisation of biochars (Chapter 3) revealed heterogeneity which complicates assessment of their potential as a microbial habitat (Chapter 3). The heterogeneity of pores, surfaces and mineral composition occurred not only between biochars, but also within a biochar source and resulted from different pyrolysis conditions and feedstock (Downie et al., 2009; Verheijen et al., 2009). Furthermore, heterogeneity of internal and external surfaces of biochar was associated with variation in mineral and elemental composition among and within biochar(s) as detected using X-ray diffraction (XRD), energy dispersive X-ray (EDS) analysis and SEM imaging. Such variability could influence the potential of biochar as a habitat in soil and underpins the mixed responses in microbial activities to biochar (Ascough et al., 2010a, 2010b; Moskal-del Hoyo et al., 2010).

While physical characterisation is a major requirement for evaluating or predicting the potential of biochar as a microbial habitat, technical issues were encountered while characterising biochar such as misleading BET area surface data due to inadequate technical procedures for which details are often not provided (Chapters 3 and 4).
Differences in total (BET) surface area as a factor of pyrolysis temperature can be correlated with micropore abundance (Downie et al., 2009) and external surface area is more likely to be associated with biochar particle size distribution (Chapter 4) and may comprise a small amount of the total surface area of biochar. The impact of cracks and occurrence of tars in the pores of biochar remains unknown. Furthermore, comparison or measurement of physical and chemical properties of biochar (porosity and surface area, the presence of tar and blockage of biochar pores by soil particles), prior to and after biochar deposition to soil were beyond the scope of this study. Effects and complication of soils entering and covering the biochar particle need further study.

This research was more focused on fungi than bacteria. Examination of Simcoa biochar indicated that it could be colonised by fungal hyphae prior to its use as a soil amendment (see Chapter 3). Biological inspection of biochar prior to soil application could be done as part of preliminary characterisation in studies where fungal colonisation of biochar is recorded after incubation in either soilless media or soil using light, fluorescence and electron microscopy.

Biochar surfaces and pores could offer a habitat to soil organisms as already suggested (Warnock et al., 2007; Lehmann et al., 2011). With biochar pores ranging from less than 1μm to 200μm, many pores would be accessible to fungal hyphae in soil (Swift et al., 1979), including hyphae of AM fungi (Saito, 1990). Fungi, especially saprophytic fungi, were expected to extensively colonise biochar particles due to their association with decomposing organic matter (Ascough et al., 2010b; Moskal-del Hoyo et al., 2010). However, while the degree of colonisation may be extensive on biochar surfaces, colonisation of biochar pores by fungal hyphae may be minimal (Chapter 3). The extent to which fungal colonisation occurs in biochar especially the internal micro-environment may not be as extensive as has been claimed. Overall, studies of biochars after incubation in soil revealed evidence of the larger pores of sizes 100 μm as pore range preferred for fungi colonisation (Chapter 3). Despite the potential of positive influences of biochar on fungal colonisation, the heterogeneity of biochar produced
from various sources of organic matter, charring temperature, particles size and amount applied to soil could all affect fungal growth.

Since measurement of microbial biomass also involved soil bacterial biomass and given that this study did not directly investigate bacteria, the MBP and MBC measurements could also be attributed to bacterial community changes. This aspect warrants further investigation as the soil microbial community of each soil is likely to be different, perhaps affecting the biomass and mycorrhizal responses to biochar in each soil. Further investigation of biochar pores with respect to the bacterial community would have provided more complete understanding of mechanisms of how biochar affects soil microorganisms overall. Previous studies have explored beneficial effects of biochar to bacteria (Kumar et al., 1987; Steiner et al., 2004). Bacterial communities could be more sensitive and responsive to biochar (Rondon et al., 2007; Cheng et al., 2008). The influence of pH has been identified as a regulating factor for bacterial abundance and activity following biochar addition to soil (Steiner et al., 2004; Rillig et al., 2010). Although investigation of fungi was the central focus of the microscopy observation of biochar as a microbial habitat in this study, the occurrence of bacterial clusters was also observed although this was difficult to observe and unclear at the magnification used. Previously, adhesion of bacteria to biochar has been reported (George and Davies, 1988; Jin, 2010). However, the level of bacterial adhesion to biochar pores and surfaces may vary based on large biochar pore curvature, pore size, precipitates and electric current forming on biochar surfaces (George and Davies, 1988; Cheng et al., 2008).

Assumptions about biochar habitable spaces for soil microbes based on studies conducted in artificial environments (Ascough et al., 2010b) could have overlooked soil interactions with biochar, especially on biochar pores. In this study, soil particles adhered to biochar surfaces and clogging of smaller biochar pores occurred. The consequence of the occurrence of soil in pores and the attachment of soil to biochar surfaces on fungal colonisation is not known and could be overcome by in situ and 3D studies.
TOPIC 2: Biochar interactions with soil management and the impact on soil microorganisms, including arbuscular mycorrhizal (AM) fungi

When applied to soil, biochar type or source, application amount and factors associated with soil management were expected to influence the degree of microbial responses. Biochar could experience changes including abiotic and biotic (microbial) oxidation (Zimmerman, 2010; Jones et al., 2011). However, these changes in biochar especially for woody feedstocks, had small or negligible impacts on soil microbial biomass for soils taken from a range of agricultural areas in south-western Australia as used in this study. Biochar particle size is not expected to affect microbial biomass in these soils although it may be important for microbial attachment. The application amount of biochar acceptable for one soil type and plant would vary with biochar source, soil background and management.

Microscopy observations on biochar varying in particle size (Chapter 4), or with organic residues (wheat/canola) (Chapter 5), or with increasing amount of biochar application (Chapter 6), provided no evidence of differences in fungal colonisation on biochar. While these observations showed that biochar particles could be suitable as fungal habitat, the extent to which biochar contributed to microbial habitat in the soils as a whole is not clear and in the circumstances examined it is likely to be limited.

The underlying effects of biochar placement method on soil microbial biomass (whether biochar is deposited in bands or distributed throughout the soil) needs to be investigated over longer time frames than those which were included in this short term research. Experimental comparison of biochar placement in various soils should also be investigated in longer term studies. Although there was little evidence of soil microbial biomass being affected by biochar management in the coarse-textured sandy soils tested throughout this thesis, compared to the more clayey soil, biochar placement method may need to differ with soil type.
Addition of wheat and canola residues (Chapter 5) with two sources of woody biochar showed evidence that the presence of these crop residues similarly influenced fungal colonisation of the biochar using fluorescence and SEM microscopy, in soil where the addition of the residues altered microbial activity. Similarly, colonisation of roots by AM fungi was only marginally affected by biochar application. Thus, although there was little apparent influence of biochar on growth of mycorrhizal hyphae in roots, soil aggregation was observed in sandy soils when amended with woody biochar where plants were present (Chapter 6).

Overall, the interactions of biochar-soil and soil microorganisms can be summarised into several processes and factors associated with biochar:

i) **Accumulation of soil particles in biochar pores, and/or on soil on biochar surfaces**

Following biochar incorporation into soil, chemical and physical changes in biochar properties (Downie et al., 2009) and interactions between soil particles (e.g. clay and sand particles) and biochar (Chia et al, 2010; Joseph et al., 2011) may influence microbial colonisation. However, there was little evidence that biochar management (type or amount) with or without other soil amendments (organic residues) influenced fungal colonisation of biochar (when observed using fluorescence and SEM) in the experiments presented here. Laboratory studies showed the possibility that estimates of fungal colonisation on biochar may be overestimated compared to that which occurs in soil (Chapter 3).

In the case of soil attached to biochar surfaces and pores, the porosity, pore size and the connectivity among available pore spaces could have some influence on the ability of soil solution to diffuse through the biochar and for microbial colonisation. In addition, soil particles found cementing and covering biochar pores and surfaces could affect the surface area measurement and overshadow potential effects of varying biochar particle size or porosity. The relatively low level of fungal colonisation observed in biochar
pores in this study of several soils typical of agricultural areas in south-western Australia raises questions to whether or not pore connectivity and soil particles either limit or enable fungal hyphal extension into or exploration of biochar pores. It is possible that the cell structure of the biochar arising from parent (plant) anatomical features might limit pore connectivity throughout the biochar and consequently restrict fungal hyphal exploration.

ii) Changes of physical characteristics of biochar

In addition to comments made above (Topic 1), previous studies (Ascough et al., 2010b; Moskal-del Hoyo et al., 2010) did not justify the importance of structural weaknesses in biochar and how cracks in the biochar may affect exploration and penetration by fungal hyphae. It is not known whether or not fungal colonisation on biochar surfaces relies on structural weakness to penetrate into biochar micro-environment and pores.

Factors associated with biochar application to soil (banded layer vs bulk soil)

While the scope of this microscopy investigation focused on the biochar surfaces and microenvironment, there is also a need to study the behavior of microorganisms in bulk soil where biochar is deposited. There was little evidence that application of biochar alone to soil stimulated microbial biomass within the time frame used here (up to 12 weeks), though interactions of biochar did occur with the presence of added crop residues (Chapter 5) or fertiliser (Chapter 6). One of the contributing factors could be associated with the biochar placement method and location of biochar and soil being sampled and examined throughout the designated study period.

Assessment of microbial biomass and respiration in most experiments were analysed using bulk soil, except in Experiment 6.2 (Chapter 6) where microbial biomass and AM root colonisation were sampled and analysed in both top soil (which included the band of biochar) and bottom soil (soil without biochar band). This sampling method in Experiment 6.2 which divided soil into 2 layers (top and bottom soil) differed from the other experiments where biochar were homogenously mixed throughout the soil. Only a
small effect of biochar on microbial biomass was observed in Chapters 4, 5, and 7 or Chapter 6 (Experiment 6.1). Similarly, in Experiment 6.2, no effect of biochar amount observation was noted. However, mycorrhizal colonisation in roots either retrieved from the bulk soil or from lower soil (bottom) layer (without biochar) was lower than in the top soil containing the band of biochar. This raises the need to further understand biochar roles and impacts on microorganisms in areas where biochar is deposited area compared with the bulk soil. Although in practice, banding biochar near the root zone is highly recommended, the mechanisms and impact on the soil microorganisms in rhizosphere or in banded and bulk soil is poorly understood.

**Insights and overview**

Mechanisms by which biochar can provide potential habitat for soil microorganisms can be categorised from three perspectives: a) the biochar micro-environment, b) the biochar-soil interface, and c) the proximity of biochar and soil.

a) **Biochar micro-environment:**

Once incorporated into soil, biochar particles and their surfaces and pores were readily covered by soil particles. These are the first interactions allowing microbial attachment on surfaces or in pores. Microorganisms may find protection and/or consume the readily available resources from biochar at this point. Over time, the soil particles may either enter the pores and enhance or limit microbial colonisation within the biochar micro-environment by reducing access of air and moisture. Cracks and other physical or chemical changes of biochar could also allow or restrict more microbial colonisation of the biochar. Larger pores are preferable for fungal colonisation as demonstrated via SEM observations. The importance of larger pores and pore connectivity could also play a role in the survival of microorganisms within the internatl structure of biochar.
b) Biochar-soil interface:
At biochar-soil interfaces, fungal hyphae acted as a bridge between soil and biochar by creating microaggregates.

c) Proximity of biochar and soil:
Localised effect of biochar in soil (in close proximity of biochar deposition in soil) or dilution of biochar (in bulk soil) can result from the placement method of biochar its application in soil leading to differences in local density of biochar particles. Theoretically, separate assessment of AM colonisation and microbial biomass within the band of biochar (top soil) from the bulk soil (bottom soil) could provide further insight into biochar-microbe interactions which may otherwise be overridden by soil dilution effects. Furthermore, the role of fungal hyphae colonising biochar particles should be further examined in relation to decomposition of biochar, the creation of microaggregates around biochar particles and the extent of fungal colonisation in the biochar microenvironment compared to the bulk soil. Further investigation may reveal localised microbial-biochar interactions (i.e. close to where biochar is deposited) in comparison to what could be a dilution effect of biochar in soil more distant from where the biochar was deposited.
Conclusion

Biochar application to the soils studied in south-western Australia involves complex interactions with soil and soil management practices. Quantification of biochar pore distribution and size was inconclusive, although microscopy observation supported the claim that biochar could be a potential habitat for soil microorganisms in these short term incubation and glasshouse studies. Soil particles deposited on biochar surfaces and in pores could mask and influence the role of biochar in these locations. Overall, the biochars used in these short-term incubation and glasshouse studies could lead to beneficial effects on AM fungi and soil microorganisms but responses may vary according to biochar source, soil background, host plant and whether other soil amendments were present. When biochar is applied with organic amendments, the mineralisation of biochar could be enhanced, but a concurrent effect of biochar on organic matter could also be important. On the contrary, although significant interactions between biochar and fertiliser have been shown, the optimal amount of biochar applied to soil when interacting with fertilisers may vary with biochar source and soil tested as well as with the host plant. Characterisation of biochar as a soil amendment and habitat could be further extended through (i) methodological determination of biochar as potential habitat for soil microorganisms using 3D techniques, prior to and after biochar deposition in soil (ii) clarification of the role of pore connectivity in the biochar micro-environment, (iii) understanding the role of soil particulate matter and its impact in biochar micro-environment, (iv) the possible role of biochar labile compounds and tars on microbial survival within biochar, and (v) potential differences in microbial colonisation when biochar is applied to the bulk soil compared with banding.
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