FUNGAL ENDOPHYTES THAT CONFER HEAT AND DROUGHT TOLERANCE TO WHEAT

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By

Michelle Hubbard

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Dr. Phyllis Shand
Department of Food and Bioproduct Sciences
University of Saskatchewan
Saskatoon, Saskatchewan
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ABSTRACT

Fungal endophytes can improve plant tolerance to abiotic stresses such as heat and drought. I hypothesized that the six endophytic fungi SMCD 2204, 2206, 2208, 2210, 2214 and 2215 would promote heat and drought tolerance in wheat during both seed germination and at later developmental stages. The Vujanovic and Germida laboratories originally discovered these fungi from the roots of Saskatchewan grown wheat (*Triticum turgidum* L.).

I assessed mycomediated enhancement of seed germination (mycovitality) including seedling performance, *in vitro* in terms of percent germination, seedling fresh weight, energy of germination (EG) and hydrothermal time (HTT) of germination. Endophytes SMCD 2206, 2210 and 2215 improved seedling heat or drought resistance, while SMCD 2204, 2208 and 2214 did not.

In the greenhouse and phytotron, I evaluated the ability of the same six endophytes to enhance wheat tolerance for heat or drought stress by measuring photosynthetic stress (PS), carbon isotopic discrimination (Δ), average seed weight (ASW), total seed weight (TSW) and the EG and percent germination of the F_1 seeds produced. SMCD 2206, 2201 and 2215 increased performance of pot-grown wheat under heat and drought.

Epigenetic modifications frequently involve changes in DNA methylation. Methyl-sensitive amplified polymorphism (MSAP) revealed that drought stressed wheat seedlings colonized with SMCD 2206 had DNA methylation patterns more similar to those of unstressed plants (with or without the endophyte) than to uncolonized drought stressed plants. Plant DNA sequences – similar to a cytochrome p450 EST and three transposable elements (TEs) – were differentially methylated between endophyte-free and endophyte colonized drought stressed plants.

I tested the hypothesis that the endophyte-free progeny of SMCD 2206 colonized wheat grown in the phytotron or greenhouse under heat or drought stress would have heightened resistance for the same abiotic stressors to which their parents were exposed, compared to uninoculated first generation plants. Data on PS, ASW, TSW and Δ showed that F_2 plants incompletely inherited stress tolerance.

This research demonstrated that fungal endophytes SMCD 2206, 2210 and 2215 improve wheat tolerance for heat and drought both *in vitro* and in pot studies. If field trials produce similar results, these isolates could be agriculturally important.

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
ABA	Abscisic acid
AM	Arbuscular mycorrhizae
ANOVA	Analysis of variance
ASW	Average seed weight
CFU	Colony forming units
DDM1	Decreased DNA methylation 1
DNA	Deoxyribonucleic acid
DSE	Dark septate endophyte
ECM	Ectomycorrhizae
EF	Elongation factor
EG	Energy of germination
EST	Expressed sequence tag
ETR	Electron transport rate
FABS	Food and Bioproduct Sciences
FWC	Field Water Capacity
HTT	Hydrothermal time
LSD	Least significant difference
LTR	Long terminal repeat
MET1	Methiltransferase1
MSAP	Methyl-sensitive amplified polymorphism
NPQ	Non-photochemical quenching
NSERC	Natural Sciences and Engineering Research Council
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEG	Polyethylene glycol
PS	Photosynthetic stress
RII	Relative interaction intensity

RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROS1	Repressor of silencing 1
SD	Standard deviation
SE	Standard error
SMCD	Saskatchewan Microbial Collection Database
TE	Transposable elements
TSW	Total seed weight
WHC	Water holding capacity
WUE	Water use efficiency

1.0 INTRODUCTION

Symbiotic associations between endophytic fungi and plants are ecologically important and globally prevalent. Fungal endophytes are able to improve plant resistance to biotic and abiotic stress. This ability is likely to become increasingly important in both natural and agricultural systems as global climate change forces plants to cope with increasingly adverse growing conditions. However, fungal endophytes are incompletely understood and merit further study.

1.1 Significance of work

In this project, I focused on both scientific and practical aspects of mycobiont-plant interactions under abiotic stress conditions. In essence, I aimed to increase scientific understanding of the mechanisms involved in wheat-endophyte symbiotic association and then to utilize this relationship to enhance desired agronomic traits in wheat under heat or drought stress. On the applicative side, the use of fungal endosymbionts to increase plant tolerance for heat and drought stress has great agricultural and ecological benefits. This is especially true given that anthropogenic climate change increases the likelihood of climate instability and hence temperature and water stress experienced by plants (IPCC 2007). According to Lobell et al. (2011) the climate variability over the last few decades resulted in a 5.5% drop in worldwide wheat yield. Growing human populations require increasing food supplies. Thus, it is critically important to investigate ways in which the impacts of climate change on staple food crops, such as wheat, can be alleviated. The use of mycobionts to confer abiotic stress tolerance would help mitigate crop lose and facilitate expansion of agriculture into marginal land. Also, it would reduce the need for irrigation and help plants withstand specific stresses without genetic modification such as those used by Morran et al. (2011) to improve wheat drought tolerance. While there is compelling evidence that fungal endophytes can allow plants to survive abiotic stress which non-colonized plants cannot (Márquez et al. 2007), the mechanisms by which this stress tolerance is conferred are not well understood. Ameliorating this gap in understanding would have tremendous scientific significance. In particular, a more complete grasp of the impact of endophyte colonization on plant epigenetic inheritance, molecular and proteomic modifications, would not only advance scientific insight, but could facilitate more appropriate use of fungal endosymbionts.

1.2 Hypothesises and objectives

I hypothesized that fungal endophytes would improve wheat tolerance for heat and drought. This heat and drought resistance would be reflected in improved seed germination *in vitro*; reduced stress in mature plants; increased grain yield; epigenetic modification of host plants; and elevated performance of a second, endophyte-free, generation of wheat.

My objective was to test the hypothesis by evaluating the germination of wheat inoculated with each of the six endophytic fungi *in vitro*. I also aimed to assess the performance and grain production of mature wheat. Furthermore, I explored whether wheat was epigenetically modified by endophyte colonization. Finally, I sought to determine if mycobiont-conferred heat and drought tolerance were heritable in the absence of the fungus.

2.0 LITERATURE REVIEW

2.1 Fungal endophytes confer abiotic stress tolerance to plants

Fungal endophytes can be defined as fungi which live inside plant tissues for all or part of their life cycle without causing disease symptoms (Wilson 1993; Wennström 1994; Saikkonen et al. 2004). There is evidence that fungal endophytes can protect plants exposed to various abiotic (Rodriguez et al. 2008), biotic (Prestidge and Gallagher 1988; Latch 1993) and/or both abiotic and biotic (Hahn et al. 2008) stresses. Endophyte colonization has been shown to enhance plant tolerance for many abiotic stresses including heat (Márquez et al. 2007; Khan et al. 2012b), drought (Hahn et al. 2008; Gibert et al. 2012), freezing, UV-B radiation (Draggen 2007), nutrient deprivation, salinity (Maggio et al. 2003; Waller et al. 2005; Khan et al. 2012a), alkaline conditions (Bu et al. 2012) and heavy metal contamination (Zhang et al. 2006; reviewed in Rodriguez and Redman 2008; Li et al. 2012). Mycomediated enhancement of performance at the germination stage is known as mycovitalism (Vujanovic and Vujanovic 2007). This process is seen as a modern tool for plant biotechnology (Vujanovic 2007). In addition, fungal endophytes have been reported to increase tolerance for abiotic stress in mature monocots and dicots (Márquez et al. 2007; Rodriguez et al. 2008).

Although the ability of fungal endophytes to improve heat and drought tolerance in host plants has been well established (Márquez et al. 2007; Hahn et al. 2008; Sun et al. 2010; Khan et al. 2012b), the mechanism(s) by which this takes place are incompletely characterized. Putative mechanisms by which endophytic fungi interact with their hosts include plant expression of stress-related genes, or production of stress-hormones (Sherameti et al. 2008). Symbiotic fungi may present differential intracellular or intercellular symbiotic colonization structures in response to plant health or stress status (Abdellatif et al. 2009). Fungal endophytes may also promote the accumulation of osmotically active or non-structural carbohydrates (Richardson et al. 1992). These mycobionts may produce or stimulate plant production of antioxidant enzymes involved in the scavenging of stress-associated reactive oxygen species (ROS), thereby alleviating the detrimental impacts of stress on plant tissues (Rodriguez and Redman 2005; White and Torres 2010; Torres et al. 2012).

2.1.1 Mycorrhizae confer heat and drought tolerance

Mycorrhizae are fungal endophytes that internally and externally colonize their host plants asymptomatically. Scientific inquiry into mycorrhizae began as early as the 1840s (Rayner

1926). The capacity of these fungi to improve drought tolerance in wheat has been recognized sense the 1980s (Allen and Boosalis 1983; Ellis et al. 1985). Mycorrhizae also improve resistance to heat in asparagus (Matsubara et al. 2000). However, information on the impact of mycorrhizae on heat tolerance in other plants is lacking.

Mechanisms by which mycorrhizae may confer plant drought resistance include changes in plant gene expression (Fan et al. 2011) and/or physiology (Ignacio Querejeta et al. 2012). Various authors suggest that mycorrhizae elevate the ability of plants to withstand drought by improving plant phosphorous, potassium and/or nitrogen nutritional status (Ruiz-Lozano et al. 1995; Subramanian and Charest 1998; reviewed by Augé 2001 and Smith et al. 2010). Colonization by mycorrhizae is also associated with the down-regulation of plant aquaporins, potentially diminishing the loss of plant water to surrounding soil (Porcel et al. 2006). In addition, mycorrhizal extra-radical hyphae may bring plant roots into closer contact with dry soils, facilitating water uptake and/or reducing water lose due to air spaces in the vicinity of the roots (Davies et al. 1992; reviewed by Augé 2001 and Smith 2010). The phytohormone abscisic acid (ABA) also plays a role in mycorrhizae-mediated drought tolerance (Aroca et al. 2008) by altering plant aquaporin protein expression (Ruiz-Lozano et al. 2009). The water use efficiency (WUE) of mycorrhizal plants subjected to drought increases in some studies, and decreases in others, relative to non-mycorrhizal drought stressed plants (reviewed by Augé 2001). As with other fungal endophytes, mycorrhizae elevate plant drought tolerance by increasing the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and peroxidase (Dave and Tarafdar 2012). Mycorrhizae contribute to drought tolerance in their hosts by directly taking up water from the soil; however, this possibility remains unproven (reviewed by Smith et al. 2010). Hence, while much is known about the mechanisms by which mycorrhizae confer drought tolerance to plants, much more remains to be learned. The documented mechanisms of plant-mycorrhizal interactions under drought stress can inform research on the relationships fungal endophytes from Ascomycota and Basidiomycota form with host plants.

2.2 Classifications of fungal endophytes

Examples of fungal endophytes are found in phyla Basidiomycota, Ascomycota and Glomeromycota and can be classified in a variety of ways. Some of the most commonly used classification systems are discussed below.

2.2.1 Mycorrhizae

Although often considered separately from endophytic fungi, mycorrhizae clearly fit the definition of fungal endophytes in that they grow within the roots of up to 80% of land plants (Smith and Read 1997), are non-pathogenic and can improve plant growth and nutrition (Smith and Read 2008). Mycorrhizae are categorized into three main groups: arbuscular mycorrhizae (AM), ectomycorrhizae (ECM; Rinaldi et al. 2008) and mycorrhizae of orchids, or Ericoid mycorrhizae (Allen 1991). Based on both morphological and molecular techniques, Ericoid mycorrhizae belong to phyla Ascomycota (Reed 1989; Hutton et al. 1994; Sharples et al. 2000). These organisms typically colonize the epidermis of hair roots, where they form intracellular hyphal coils (Allen et al. 1989; Briggs and Ashford 2001). Ericoid mycorrhizae are most prevalent in plants growing in nutritionally depleted soils (reviewed in Read 1996; Cairney and Ashford 2002). In contrast, ECM can belong to Ascomycota or Basidiomycota (although members of Basidiomycota appear to dominate the group) and are found in association with the roots of woody plants (Rinaldi et al. 2008). Symbiotic ECM-root associations involve the fungal partner forming a hyphal mantle or sheath around the root tip. In addition, ECM hyphae grow between cortical root cells, forming a structure known as a hartig net (Blasius et al. 1986). Within forest ecosystems ECM facilitate nutrient mobilization, transfer and uptake by plants (Simard et al. 1997). The most well studied group of mycorrhizae are AM, which belong to Glomeromycota. These organisms are compared and contrasted to non-AM fungal endophytes below.

There are a number of key differences between AM and Ascomycota and Basidiomycota endophytes (Hubbard et al. 2011). While AM have unicellular, multinucleate hyphae without cross-walls, other fungal endophytes possess multicellular hyphae (Jun et al. 2002; Peterson et al. 2004). Furthermore, the colonization structures formed by endophytic Ascomycota and Basidiomycota are more complex and varied than those of AM fungi (Ureclay and Battistella 2007; Abdellatiff et al. 2009; Kobae and Hata 2010). For example, non-AM endophytes can form hyphal coils and knots (Abdellatiff et al. 2009), while AM produce vesicles and arbuscules (Ureclay and Battistella 2007; Kobae and Hata 2010). While AM fungi colonize only roots (Kirk et al. 2001), Ascomycota and Basidiomycota endophytes colonize roots and/or aerial tissues and/or generative organs (reviewed by Rodriguez et al. 2009b). Because AM fungi are not present in reproductive organs of their hosts, their host-to-host transmission occurs only horizontally (Peterson et al. 2004). In contrast, non-AM mycobionts can be transmitted vertically

in instances where they colonize seeds (Saikkonen et al. 2002). Fungi classified as AM cannot be cultured in the absence of a compatible plant host (Declerk et al. 2005). In contrast, Ascomycota and Basidiomycota endophytes can be grown as free-living organisms (Márquez et al. 2007). In the presence of stress or in extreme environments the relative prevalence of non-AM endophytes tends to increase, while that of AM tends to diminish (Read and Haselwandter 1981; Medina-Roldán et al. 2008; Schmidt et al. 2008; Perez-Naranjo 2009). Some endophytes belonging to Ascomycota can switch from mutualistic to parasitic interactions with their hosts (Jumpponen 2001; Redman et al. 2001; Declerk et al. 2005). On the other hand AM may have mutualistic and commensal, but not parasitic, relationships with plants (Augé 2001; Ronsheim 2012). While AM can facilitate host nutrient absorption (Brown and Bethlenfalvay 1987; Smith and Read 2008), other endophytes do not appear to provide this service. Both AM and non-AM endophytes can elevate host tolerance for abiotic stress (Duan et al. 1996; Márquez et al. 2007) and/or biotic stress (Cordier et al. 1998; Graham 2001; Shiba and Sugawara 2008).

2.2.2 Clavicipitaceous endophytes

Often referred to as C-endophytes, these endophytic fungi are among the most well characterized endophytes belonging to Ascomycota. Clavicipitaceous endophytes belong to the genera *Epichloë* (telemorph) and *Neotyphodium* (anamorph), order Hypocreales, family Clavicipitaceae (Latch et al. 1984; An et al. 1993). These mycobionts form endophytic associations with the above-ground tissues of cool season grasses such as tall fescue (*Lolium arundinaceum*) and perennial ryegrass (*Lolium perenne*; Bacon and Siegel 1988; Leuchtmann et al. 1994). Fungal symbiotic organs are intercellular (Hinton and Bacon 1985; Siegel et al. 1987; White et al. 1997) and are more abundant and highly branched in basal regions of individual plant organs (Kuldau and Bacon 2008). The degree of hyphal branching is modulated by reactive oxygen species (ROS) levels (Tanaka et al. 2006). Because Clavicipitaceous endophytes colonize the seeds of their hosts, they are transmitted vertically between parental and daughter plants (Siegel et al. 1984). While C-endophytes can improve plant resistance to various biotic and abiotic stressors, including insect herbivory (Rowan and Gaynor 1986) and drought (Kane 2011; Gibert 2012), they can also act as plant pathogens (reviewed in Saikkonen et al. 2004). Despite their relatively narrow host range and intra-group diversity (reviewed in Rodriguez et al. 2009b), C-endophytes can co-exist with other microbial symbionts, such as mycorrhizae (Vicari et al. 2002).

2.2.3 Dark septate endophytes

The term dark septate endophyte (DSE) was coined due to the presence of melanized septa, which appear dark under many staining techniques. Although first recognized as early as the 1920s (Merlin 1922; reviewed by Rodriguez et al. 2009b), DSE remain poorly understood. As reviewed in Mandyam and Jumpponen (2005), DSE are broadly defined as asexual or conidial fungi with melanized hyphae, divided into cellular compartments by septa, which colonize the subsurface (epidermis and cortex) of plant roots both inter and intracellularly. Colonizing DSE hyphae can form diverse and complex structures. For example Abdellatif et al. (2009) proposed a system for describing fungal colonization structures based on direction of hyphal growth relative to the long axis of the root and regularity of fungal cells. These indices illustrate the complexity of DSE morphology, facilitated by the multicellular, septate nature of these endosymbionts.

These fungal organisms are horizontally transmitted from one host plant to another (Jumpponen and Trappe 1998; Vujanovic and Brisson 2002) and colonize plant roots (reviewed in Rodriguez et al. 2009b). Fungi that could be classified as DSE are distributed globally, colonize a broad spectrum of host plants, and tend to be increasingly prevalent in extreme, or stressful, environments (Read and Haselwandter 1981; Perez-Naranjo 2009; Zhang et al. 2011b). These mycobionts often share a host with mycorrhizae (Hatch 1934; reviewed in Jumpponen and Trappe 1998; Girlanda et al. 2002; Vohník and Albrechtová 2011).

The classification of endophytic fungi as DSEs fails to account for the full range of functional, ecological or morphological diversity within fungal endophytes. Some endophytic fungi, such as *Curvularia pubertata*, which colonizes the above-ground tissues of its hosts (Márquez et al. 2007), defy classification as mycorrhizae, C-endophytes or DSE. Hence, there is a need for a more comprehensive system for grouping fungal endophytes.

2.2.4 Other classification systems

Rodriguez et al. (2009b) proposed a novel system for classifying endophytic fungi. This approach is based on breadth or narrowness of host range; plant organs or tissues colonized; level of plant colonization; biodiversity of endophytes within individual hosts; method(s) by which mycobionts are transmitted between hosts; and whether or not any benefits to the host are habitat or stress status specific. Endophytic fungi are divided into four groups by Rodriguez et al. (2009b): Class 1 (Clavicipitaceous), Class 2, 3 and 4 (non-Clavicipitaceous). Class 1 mycobionts possess a narrow host range, being restricted to *Lolium* spp. and *Festuca* spp. grasses (Bacon et

al. 1977). In contrast, endosymbionts belonging to classes 2, 3 or 4 have a broad host range (Arnold and Lutzoni 2007). Furthermore, Class 1 endophytes extensively colonize above-ground host tissues, exhibit low biodiversity *in planta* are both vertically transmitted through seeds and horizontal transmitted from one post-germination host to another and confer benefits to their hosts which are non-specific to the environmental conditions (reviewed in Rodriguez et al. 2009b).

One shortcoming of the Rodriguez et al. (2009b) system is that is does not take mycorrhizal fungi into account. However, this omission is justified by Rodriguez et al. (2009b) based on the fact that mycorrhizal fungi not only colonize plant tissues but also occupy the rhizosphere, whereas some authors define endophytes as being found solely within their plant hosts prior to host senescence or mortality (Sherwood and Carroll 1974; Carroll 1988). However, more recent evidence suggests that other endophytic fungi do sometimes extend into the rhizosphere (Macia-Vicente et al. 2008) and/or soil (Taniguchi et al. 2012), further blurring the lines between mycorrhizae and fungal endophytes.

2.3 Fungal responses to heat and drought

It has long been recognised that fungi are sensitive to abiotic stress both in the field (Toberman et al. 2008) and on artificial culture media (Kim et al. 2005; Ritchie et al. 2006; Esteves et al. 2009). As early as 1874, Heath (1874) reported the apparent ability of heat treatment to kill pathogenic fungi. Nearly a century later, King et al. (1969) isolated heat-resistance fungi from grapes and from vineyard soils and noted that filtration, chemical sterilization or oxygen deprivation could greatly reduce the presence of these microbes. Despite its long history, much remains to be learned about heat tolerant fungi. For example, Suryanarayanan et al. (2011) isolated a fungus capable of withstanding 115 °C heat for up to 2 h.

The most common response to either heat or moisture stress is reduced colony expansion and/or diminished hyphal growth (Kim et al. 2005; Ritchie et al. 2006; Esteves et al. 2009). For example, Kim et al. (2005) found that the colony growth rate of *Sphaeropsis pyriputrescens* increased in a near-linear fashion with increasing temperature between –3 °C and 20 °C, reaching a maximum growth rate at 20 °C to 22 °C, before dropping precipitously as heat stress took effect between 22 °C and 25 °C. Growth was completely absent by 30 °C. These same authors observed a slightly different relationship between radial colony expansion and water potential.

Growth remained nearly unchanged above –1.0 MPa, decreased rapidly between –1.5 and –4.0 MPa, and was close to zero, but still measurable, from –4.0 to –7.3 MPa. Consistently, Ritchie et al. (2006) observed that the potato pathogen *Rhizoctonia solani* grew less rapidly as osmotic and matric potentials declined and that colony growth ceased altogether below –2.0 to –4.0 MPa on laboratory media and –6.3 MPa in soil. Similarly, Esteves et al. (2009) found that drought stress, simulated by changes in matric potential, decreased fungal growth rate. Vujanovic et al. (2001) suggested that the environmental factors – such as temperature and water requirements – in combination with fungal germination, growth, and sporulation, could be important for determination of the fundamental niche of each fungal species.

Another common response of fungi to heat or drought was the accumulation of non-structural carbohydrates. For example, the non-reducing carbohydrate trehalose plays a role in heat and desiccation stress tolerance in yeast (Gadd et al. 1987; Hottiger et al. 1987; Hounsa et al. 1998). Furthermore, *Pochonia chlamydosporia* built up its internal stock of soluble carbohydrates such as glycerol and glucose under increased moisture stress (Esteves et al. 2009).

In the field, the diversity and abundance of fungal soil communities change in response to drought. However, the nature of these changes is variable. While Toberman et al. (2008) reported a decrease in fungal biodiversity and biomass under drought conditions, Hawkes et al. (2011) observed the opposite trend. Research on the ecological responses of fungi to heat or drought stress in the field remains sparse. It is generally thought that fungi fare better than bacteria under such conditions (Abera et al. 2012).

Fungi also respond to abiotic stress by modifying their gene expression and proteomic profiles. For example, a gene in the histidine-to-aspartate phosphorelay system, a major cellular signal transduction pathway in fungi (Gustin et al. 1998), has been implicated in *Neurospora crassa* osmoregulation (Banno et al. 2007). Irmler et al. (2005) employed proteomic tools to identify differentially expressed proteins in an *N. crassa* mutant sensitive to osmotic stress.

2.4 Plant responses to heat and drought

2.4.1 Physiological responses

Responses employed by plants faced with heat or drought stress include escape, avoidance and adaptation (reviewed in Farooq et al. 2009). Because plants are sessile organisms, escape can take the form of shortened lifespan, including earlier flowering time (Araus et al.

2002; review in Farooq et al. 2009). A decrease in the lifespan of crop plants below optimal levels often leads to a decrease in yield (Turner et al. 2001). An earlier flowering time can mean that the plant allocates fewer resources to vegetative biomass and more resources to reproduction and seed production.

Strategies for avoidance of and adaptation to drought, include stomatal closure, reduced leaf surface area (Lorena and Ernesto 2005), presence of a cuticle, or waxy covering on the leaf surface (Jordon et al. 1984; Ristic and Jenks 2002; Smith et al. 2006), increased rooting depth and altered root morphology (Weir and Barraclough 1986; Pinheiro et al. 2005). These phenotypic changes may decrease water loss due to transpiration and increase the plant's ability to take up water from the soil. Increase solute content in the phloem and girdling of the phloem to reduce stem elongation can also permit plant growth under drought conditions (reviewed in Farooq et al. 2009).

On a tissue and cellular level, drought tolerance mechanisms include osmotic adjustment through accumulation of non-structural carbohydrates, or changes in cell wall elasticity (Izanloo et al. 2008; reviewed in Farooq et al. 2009). Osmotic adjustment and the resultant maintenance of turgor may delay irreversible damage to cell membranes and permit the continuation of cellular functions (Elmi and West 1995). Enhanced osmotic adjustment appears to result in the plant experiencing less severe drought stress, leading to reduced abscisic acid (ABA) accumulation (Izanloo et al. 2008).

2.4.2 Molecular signaling responses

Molecular signaling is also involved in plant responses to abiotic stress. For example, phytohormones, such as ethylene (Abeles et al. 1992) and ABA are associated with drought. As soil dries, ABA is produced in the roots and serves as a chemical root-to-shoot stress signal (reviewed in Davies et al. 2005; Shaterian et al. 2005).

Reactive oxygen species (ROS) – ions or molecules which are highly reactive because of unpaired valence shell electrons – are another important component in the plant reaction to abiotic stresses (Wang et al. 2008; Fan et al. 2009; Tyburski et al. 2009). Examples of ROS include such as the superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). These chemical species form as byproducts of normal oxygen metabolism and play important roles in cell signaling. However, ROS accumulation can lead to oxidative damage in cells. Thus, a reduction in ROS levels during stress could enhance stress tolerance as well as

potentially indicating that the plant in question is experiencing a lesser degree of stress or is more tolerant to the stress. Plants may ameliorate ROS induced damage through increased activity of antioxidant enzymes, such as ascorbate peroxidase, catalase, glutathione reductase, dehydroascorbate reductase and monodehydroascorbate reductase (Richardson et al. 1993; Bayat et al. 2009; Yuan et al. 2010).

2.4.3 Photosynthetic responses

Abiotic stress has been observed to limit photosynthetic activity in plants (Björkman and Powles 1984; Valladares and Pearcy 1997; Flexas and Medrano 2002; Zhang et al. 2011a). Drought leads to photosynthetic stress (PS) by inducing stomatal closure in order to limit water loss (Lawlor 1995). However, closure of stomata also reduces CO_2 fixation. In addition, both heat and drought stress can result in damage to, or hinder repair of, photosystem II (Scotnica et al. 2000; Mohanty et al. 2012). Heat stress can also lead to inhibition of the photosynthetic enzyme Rubisco activase, diminishing CO_2 assimilation (Salvucci and Crafts-Brandner 2004). As reviewed by Mohanty et al. (2012), thylakoid organization and membrane structures can also be negatively impacted by abiotic stress, producing further PS.

A variety of techniques are available for measuring PS. A rapid and nondestructive test for photosynthetic stress is maximal photochemical efficiency, $F\text{v}/F\text{m}$, where $F\text{v}$ = [maximum dark adapted fluorescence ($F\text{m}$)] – [minimum dark adapted fluorescent (F_0)]. This test is typically carried out after dark adaptation of the leaves for at least 20 to 25 min. Values for $F\text{v}/F\text{m}$ are inversely proportional to the level of damage to photosystem II (Farquhar et al. 1989). A decrease in $F\text{v}/F\text{m}$ has been detected under heat and drought stress conditions (Karavata and Manetas 1999; Zhang et al. 2011a; Huseynova 2012) and indicates that some of the light energy absorbed by the plant is not being utilized in photosynthesis by photosystem II reaction centres. Hence the $F\text{v}/F\text{m}$ ratio provides information on the maximum quantum yield of primary photosynthetic reactions. Furthermore, the light energy that is not absorbed by PS II can contribute to the formation of ROS, leading to oxidative stress (Chaves et al. 2009; Nishiyama et al. 2011).

Other methods appropriate for assessing PS in drought stressed plants include photosynthetic yield coupled to heat treatment (Burke 2007); Fs/F and F_0 steady state fluorescence test (Flexas 2002); electron transport rate (ETR)/carbon assimilation steady state fluorescence test (Cerovic 1996; Cavender-Bares and Bazzazz 2004); K Step dark-adapted test

(Strasser 2004); stepped actinic test (Flexas 2000); non-photochemical quenching (NPQ) dark-adapted test (Cavender-Bares and Bazzazz 2004); and gas exchange (Marenco et al. 2001; Ko and Piccinni 2010). The Fs/F and F_0 steady state fluorescence test can be used on light adapted C3, C4 and CAM plants. The results of this test show diurnal variation in drought stressed plants (Flexas 2002). The ETR/A steady state fluorescence test is used to estimate the rate of electron transport in PS II and is appropriate for light adapted C4 plants (Cerovic 1996; Cavender-Bares and Bazzazz 2004). Peaks in chlorophyll fluorescence of dark adapted leaves after being illuminated follow a series of steps. The K step is present in leaves exposed to abiotic stressors such as nitrogen deficiency and drought (Strasser 2004). The stepped actinic test is a relatively slow, light adapted test in which Fs values can be used to determine if drought is the cause of PS. Plants use the process of NPQ to convert excited chlorophyll to the ground state without using the energy in photosynthesis. Values for NPQ are larger in plants subjected to heighten drought stress (Cavender-Bares and Bazzazz 2004). Finally, gas exchange is a value measure of drought stress because it is linked to stomatal closure (Siddique et al. 1999). Thus, increased gas exchange corresponds to decreased drought stress.

In order to evaluate PS under heat stress, the following photosynthetic tests are appropriate: quenching and quenching relaxation test (Zhang et al. 2011a); photosynthetic effective quantum yield (Schreiber 2004; Dascaliuc et al. 2007); K step dark-adapted test (Strasser 2004). The quenching and quenching relaxation involve the evaluation of photochemical quenching (Zhang et al. 2011a) or NPQ (Song et al. 2011). The former decreases under increased heat stress, while the latter increases. Photosynthetic yield is a light adapted test. Yield values decrease under heat stress (Dascaliuc et al. 2007).

2.4.4 Gene and protein expression

Plants also respond to abiotic stress with changes in gene expression. Examples of genes whose expression levels are known to be altered by heat or drought stress include a gene for the calcium binding protein calreticulin (Jia et al. 2008), betaine aldehyde dehydrogenase (Zhang et al. 2008), genes encoding copper/zinc and manganese superoxide dismutase (Wu et al. 1999), genes encoding a lipid transfer protein under drought stress in bromegrass (Wu et al. 2004).

Expression of betaine aldehyde dehydrogenase increased in *Jatropha curcas* L. exposed to drought, heat and salt stress (Zhang et al. 2008). Differential expression of genes encoding

copper/zinc, manganese superoxide dismutase in wheat (Wu et al. 1999) and a lipid transfer protein in bromegrass have been observed under drought stress (Wu et al. 2004).

Expression of genes encoding cytochrome P450 proteins can be upregulated in plants experiencing biotic or abiotic stress (Narusaka et al. 2004). In general, cytochrome P450s are enzymes that contain a heme group and catalyze organic oxidation reactions (Schuler 1996; Chapple 1998). In plants, these enzymes are involved in stress responses such as detoxification of herbicides (Sandermann 1992), pathogen attack, drought and salinity (Narusaka et al. 2004).

Proteins involved in plant responses to drought include ethylene-responsive factors (Xiong et al. 2002; Xu et al. 2007), dehydration responsive element-binding factors (Shen et al. 2003; Xu et al. 2008), monoubiquitin (Guo et al. 2008), ABA-responsive element binding proteins and ABA-responsive element binding factor (Kobayashi et al. 2008), glycoside hydrolase enzymes (Konno et al. 2008), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Demirevska et al. 2008), wheat dehydrin DHN-5 (Brini et al. 2007) and protein synthesis elongation factors (EF) EF-Tu and EF-1α in spring wheat (Bukovnik et al. 2009).

2.5 Plants epigenetics

Epigenetics can be defined as changes in gene expression that do not arise from changes in the underlying DNA sequence (Bird 2007). Epigenetic changes can be maintained through cycles of cell division and may be inherited by subsequent generations. The molecular mechanisms of epigenetic changes include histone modification, chromosome rearrangement (Hajkova et al. 2008) and DNA methylation (Boyko and Kovalchuk 2008). Plants have been observed to respond to environmental stress with epigenetic changes (Grant-Downton and Dickinson 2006; Penterman et al. 2007), allowing stress tolerance to pass on to the next generation (Bender 2004; Molinier et al. 2006; Boyko et al. 2007).

2.5.1 DNA methylation and demethylation

In plants, alterations of DNA methylation patterns are the most important and easily studied form of epigenetic regulation. It generally occurs at cytosine residues in CpNpG, CpG or GC sites (Bender 2004; Mirouze and Passzkowski 2011). In symmetrical sites, cytosine methylation signatures can be duplicated as part of DNA replication; in contrast, asymmetrical cytosine methylation requires re-establishment in each nascent daughter strand. Symmetrical CG

cytosine methylation is widely distributed through plant genomes (Mathieu et al. 2007) and is thought to be the most stable form of DNA methylation (Law and Jacobsen 2010).

DNA demethylation of a given DNA sequence generally increases transcription and gene expression (Harrisson et al. 1971; Christman et al. 1977; Hepburn et al. 1983; Finnegan et al. 1998). Furthermore, this process is a common plant response to stress (Zhong et al. 2009; Wang et al. 2011; Zhao et al. 2010; González et al. 2011). Loss of methyl groups can occur passively or actively. In passive demethylation, methyl groups may simply not be maintained during DNA replication or de novo methylation may be impeded (Kress et al. 2001; Kankel et al. 2003; Boyko and Kovalchuk 2008). Active DNA demethylation can involve the DNA glycosylase DEMETER (DME; Zhu et al. 2000; Morales-Ruiz et al. 2006; Penterman et al. 2007) or the DNA glycosylase/lyase repressor of silencing 1 (ROS1; Gong et al. 2002). As discussed by Penterman et al. (2007), active demethylation can counteract the accumulation of stable hyper-methylated regions, or epialleles.

Addition of methyl groups to plant cytosine residues involves methyltransferase activity. The enzyme methyltransferase1 (MET1) is responsible for CpG and CG methylation (Bester et al. 1988; Finnegan et al. 1996; Ronemus et al. 1996; Boyko and Kovalchuk 2008), copying maternal methylation patterns to daughter strands at DNA replication forks (Law and Jacobsen 2010). Hence, MET1 can be said to be involved in DNA methylation pattern maintenance, counteracting passive demethylation that might otherwise take place in successive cycles of DNA replication. Chromomethylase3 is involved in CpNpG methylation (Lindroth et al. 2001; Tompa et al. 2002). This activity is particularity prevalent is genomic regions adjacent to transposable elements (TEs) and has some functional overlap with MET1-mediated CpG methylation (Tompa et al. 2002; Kato et al. 2003). A third group of DNA methylation enzymes are domain rearranged methyltransferases 1 and 2 (Cao et al. 2000; Cao and Jacobsen 2002). These enzymes primarily catalyze de novo addition of methyl groups at asymmetrical sequences (Cao and Jacobsen 2002). De novo DNA methylation can also occur via a process termed RNA-directed DNA methylation (Wassenegger et al. 1994). This mechanism involves small RNA molecules targeting cytosine residues located within homologous sequences using RNA interference systems – including Dicer and Argonaute enzymes – as well as chromatin remodeling factors and plant-specific RNA polymerases (Chan et al. 2004; Xie et al. 2004; Chan et al. 2006). All DNA methylation

pathways act in concert to reduce gene expression and silence transgenes and TEs (Matzke et al. 2009; Furner and Matzke 2011).

2.5.2 Transposable elements

Transposable elements (TEs) are repetitive sequences of DNA that can be mobilized within the genome of plants or animals. Because of their tendency to self-perpetuate, TEs can make up a large proportion of the genomes of both plants and animals (reviewed in Lisch 2009). For example, roughly 90% of the wheat genome is composed of TEs (Devos et al. 2005). TEs can be beneficial to the organisms within which they reside by playing an important role in genetic and epigenetic variability (Kidwell and Lisch 2001; Jordan et al. 2003). On the other hand, TEs can be deleterious, disrupting functional genes, creating mutations or chromosomal damage (McClintock 1950; Kidwell and Lisch 1998, 2000; Kumar and Bennetzen 1999). The expression and mobilization of TEs tends to be triggered by plant stress (Pecinka et al. 2010; Tittel-Elmer et al. 2010; McCue et al. 2012), providing the potential for adaptive gene expression and/or eventual evolution of novel, beneficial phenotypes (Kalendar et al. 2000; Hilbricht et al. 2008). These new traits may arise as a result of TE mobility, trans-generational silencing of nearby genes, or through production of numerous small RNAs, which can impact proximal or distal stress related genes (Hilbricht et al. 2008; Molnar et al. 2010; Dunoyer et al. 2010; reviewed in Mirouze and Paszkowski 2011; McCue et al. 2012).

These mobile genomic features can be broken down into two basic groups: retrotransposons (class I) and transposons (class II). The primary difference between classes I and II is the mode of transposition. Transposons are spliced out of the donor site and subsequently inserted into a recipient location elsewhere in the genome. In contrast, retrotransposons are reverse-transcribed to RNA intermediates before being transcribed to DNA and being inserted back into the host (reviewed in O'Donnell and Burns 2010). Both transposons and retrotransposons can be further sub-divided into superfamilies. Transposon superfamilies include Tc1/*mariner, hAT,* P element, *MuDR/Foldback,* CACTA, *PiggyBac*, PIF/*Harbinger, Merlin, Transib, Banshee, Halitron* and *Maverick* (reviewed in Feschotte and Pritiham 2007). Retrotransposons can be classified as long terminal repeat (LTR) or non-LTR. The former include Ty1/copia, Ty3/copia and retroviruses types (Brosius et al. 2007), while the latter include Short INterspersed Elements (SINEs), Long INterspersed Elements (LINEs) and terminal-repeat retrotransposons in miniature (TRIMs; Brosius et al. 2007).

2.5.3 Histone modification

DNA coils around histones octomers, forming nucleosomes – the structures in which transcription occurs (reviewed in Boyko and Kovalchuk 2008). Tighter coiling leads to decreased transcription and gene expression. The acetylation and methylation status of subunits of histone octomers modulate gene expression by facilitating the formation and maintenance of euchromatin (transcriptionally silent chromatin) or heterochromatin (Bender 2004). Due to the integrated nature of biological systems, histone modifications are interconnected with DNA methylation in that they tend to reinforce methylation pattern imprinting in plants (Boyko and Kovalchuk 2008). For example, histone deacetylase 6 not only functions in histone deactylation but also reinforces cytosine methylation patterns established at CpNpG sites (Aufsatz et al. 2002). Modification of histones is responsive to environmental stressors such as drought (Kim et al. 2008) as are DNA methylation and TE expression and mobility.

2.5.4 Chromatin remodeling

Remodeling of chromatin is another form of stress-responsive epigenetic control in plants (Kim et al. 2010; reviewed by Luo et al. 2011). As reviewed in Boyko and Kovalchuk (2008), numerous factors are involved in plant chromatin remodeling. These factors include SWI2/SNF2 DNA helicases, like heterochromatin protein 1, RNA polymerase Ivb, methyl-CpG-binding domain proteins, and maintenance of methylation 1, which is similar to SWI2/SNF2. This process is frequently complementary to and re-enforcing of DNA methylation patterns and histone modifications (Ben-Porath and Cedar 2001; Geiman and Robertson 2002). For example, enzymes belonging to the SWI2/SNF2 DNA helicase family, such as decreased DNA methylation 1 (DDM1), bring about chromatin remodeling by interfering with interactions between histones and the DNA wrapped around these octomers (Geiman and Robertson 2002). The enzyme DDM1 not only triggers loosening of centromeric heterochromatin (Soppe et al. 2002), but also alters DNA and histone methylation (Singer et al. 2001; Johnson et al. 2002). The fact that DDM1 suppresses TEs (Miura et al. 2001) further demonstrates the interconnected nature of epigenetic mechanisms.

2.6 Inoculation of plants with beneficial microorganisms

Exploration of the most effective way of applying microbial inoculants is merited because of the diversity of inoculation methods available. Inoculation most commonly takes place at the

seed or seedling stage (Zhou et al. 2004; Bailey et al. 2008; Abdellatif et al. 2009). These methods include soil-based inoculation (Zhou et al. 2004; Bailey et al. 2008) seed treatments and *in vitro* methods (Abdellatif et al. 2009). Soil-based methods can employ granular (Kyei-Boahen et al. 2002; Zhou et al. 2004; Hynes et al. 2010) or liquid (Trifonova et al. 2009) delivery of the microorganism(s). Kyei-Boahen et al. (2002) found that soil-based (granular) inoculation of chickpea with rhizobial inoculants was more effective at increasing yield than either peat or liquid seed treatments. Granular formulations can encapsulate the microorganism (Hynes et al. 2010). The composition of formulations is critical, and must ensure: 1) the survival of the microbe; 2) delivery of the inoculum to intended target; and 3) elevated effectiveness of the inoculum (Hynes et al. 2010). Seed treatments require a carrier, which can take the form of peat, wettable powder or liquid, to bind the microbial agent to the seed. Many biotechnology companies produce commercial microbial inoculants in all of the above formats.

2.7 Heat and drought stress application methods

Heat stress can be applied to pot-grown wheat in a variety of ways (Table 2.1). Maximum temperatures tend to range from 30 to 42 °C. Heat is most frequently applied during the grain filling stage (Corbellini et al. 1997; Fokar et al. 1998; Plaut et al. 2004; Tahir and Nakata 2005; Spiertz et al. 2006) and/or as a heat shock of several days duration (Spiertz et al. 2006). Occasionally, heat stress begins shortly after planting (Tripathi et al. 2009), as in the work presented in this thesis. Furthermore, heat stress in pot studies generally mimics natural daily patterns in that higher temperatures are used during the day as compared to overnight (Plaut et al. 2004; Tahir and Nakata 2005; Spiertz et al. 2006).

The application of drought stress in a controlled and reproducible manner is complex. Drought can be applied *in vitro* or *in vivo*. *In vitro* methods of drought application include adjustment of osmotic potential through the addition of sodium chloride (Lang 1967; Ritchie et al. 2006), potassium chloride (Ritchie et al. 2006) or glycerol (Dallyn and Fox 1980) to liquid or solid growth media. Matric potential can also be lowered *in vitro* to simulate drought by supplementing media with polyethylene glycol (PEG; Steuter et al. 1981; Galovic et al. 2005; Ritchie et al. 2006; Sakthivelu et al. 2008).

In vivo drought application methods include greenhouse, phytotron and field studies. In pot studies, drought stress is most commonly measured in terms of percent field water capacity

(FWC) by weight; Table 2.2 summarises a literature survey of methods by which drought has been applied to wheat in pots. Drought is most frequently measured in terms of percent field capacity by weight (Gunes et al. 2007; Zhao et al. 2007; Wang et al. 2008; Ma et al. 2010). However, it can also be measured on the volume of water per volume of soil basis (Saidi et al. 2008) or in terms of water potential (Parent et al. 2010). However, these latter two approaches have the drawback that water may not be evenly distributed throughout the soil or potting mix. Hence water potential measurements may not be representative of the conditions in the pot as a whole. Furthermore, when water is applied to the soil surface in the absence of suction to fully draw down and distribute moisture, plants could experience stress due to reduced soil volume rather than drought per se (F. Walley, *personal communication*).

Both *in vitro* and *in vivo* stress can be applied gradually, in order to mimic most situations found in nature by giving the plants or fungi time to acclimatise. The importance of acclimation has been demonstrated *in vitro* in experiments by Leone et al. (1994) in which potato cell cultures were able to grow in 20% PEG when the PEG concentration was increased gradually, but completely ceased growing when shocked by the same PEG level. Similarly, Saidi et al. (2008) noted that wheat previously exposed to drought stress developed tolerance for drought at a later developmental stage.

Table 2.1 Methods of applying heat to pot-grown wheat.

Study	Measure of Heat	Design	Initiation of Stress	Control Temperatures (°C)	Heat stress Temperature(s) (°C)	Max. Temperature (°C)
Corbellini et al. (1997)	Temperature	Pots periodically rotated to minimize spatial distribution effect. 3 pots (9 plants) 2 reps	Starting 7 d after anthesis	From 10 to 25	13 heat treatments (temperature up to 40) differing in duration	40
Fokar et al. (1998)	Temperature	Completely randomized block design with 4 reps of 8 pots	At anthesis	19 to 28, average of 26	31 to 42 with an average of 38	42
Plaut et al. (2004)	Temperature	8 pots / treatment	Starting 8 d after anthesis	25/18 day/night	30/25 day/night cycle for 3 d	30
Tahir and Nakata (2005)	Temperature	Completely randomized design with 3 reps	Starting 5 d after anthesis	Maximum temperature below 30	38 (3 h) / 18 overnight	38
Spiertz et al. (2006)	Temperature	4 reps	3 d heat shock in grain-filling	18/13 or 25/20 day/night	38/20 day/night, 16 h photoperiod.	38
Tripathi et al. (2009)	Temperature	Unknown	Starting 7 d after planting	25	35	35
					AVE	37
					SD	4.2
					N	6
					SE	1.7

Table 2.2 Methods of applying drought to pot-grown wheat

Study	Measure of Drought	Water Levels	Design	Initiation of Stress	Method of Determining Water Levels	Ave % Field Capacity for Drought
Moinuddin et al. (2005)	% Total water evapo-transpired per 24h, by weight	Drought = 50%	3 reps	Varied; during reproductive stages	Weight	50%
Gunes et al. (2007)	% Field water capacity (FWC)	60% and 40%	4 reps	28 d after planting	Weight	40%
Zhao et al. (2007)	% FWC	100%, 67% and 50%	Randomized blocks, 3 reps	25 d after planting	Weight	50%
Wang et al. (2008)	% FWC	90% for control, water withheld for drought, Soil water content dropped to ~20%	Unknown	90 d after sowing	Soil water content by weight = (weight water in soil)/((weight of dry soil) X FWC) X 100%	20%
Zhang et al. (2009)	Gravimetic water content	33-40% or no water given 1 d after anthesis	Unknown	Varied; 19 d after planting or 1 d after anthesis	Weight	37%
Ma et al. (2010)	% FWC	85% and 60%	5 reps		Weight	60%
					AVE	42.8%
					SD	14%
					N	6
					SE	6%

2.8 Overall rationale for study

The work presented in this thesis aims to explore the capacity of fungal endophytes to confer heat and/or drought tolerance to wheat, as well as the mechanisms involved in plant-endophyte interactions. One of the motivations for undertaking this research is the need for sustainable agricultural food production, especially under a changing climate.

2.8.1 Climate change models and predications

A wide range of climate change models exists and their predications differ markedly. While a general trend for the North American Prairies is towards decreased rainfall and increased temperatures (especially in summer), other areas of the world may experience increased precipitation (IPCC 2007). For example, in the Indian subcontinent, which tends to have a hot climate, an average of 21 climate models presented in the IPCC (2007) report predict an increase in annual precipitation by the years 2080 to 2099. However, precipitation in some parts of India is projected to decrease in the months of December, January and February, compared to 1980 to 1999 levels. Over the same time period, temperatures in India are expected to increase. Arctic regions of North America are another exception to general global trends towards increased heat stress and drought. The IPCC (2007) report calls for elevated temperatures and precipitation levels in large parts of the North American Arctic. Because this area currently experiences temperatures too low for most forms of agriculture, such changes could actually reduce stress or mean that, during any dry periods which do occur, plants could experience drought stress without heat stress. Take together, the above predictions imply that heat and drought are likely to occur both together and separately. However, the uncertainty of future climates, combined with the general trend towards for frequent extreme events, including heat waves and extended dry periods (IPCC 2012), means that research into methods for improving plant tolerance for increased temperatures and moisture deficit are worthwhile.

2.8.2 Potential advantages and pitfalls of fungal endophytes in agricultural applications

Although heat and drought frequently occur together in nature (Craufurd and Peacock 1993; reviewed by Mittler 2006 and Barnabás et al. 2007), there are areas of the world, such as India, where temperatures are high but moisture levels are non-limiting. Hence, studying heat in the absence of drought has both scientific and practical value. The

SMCD endophytes studied here have advantages for agricultural applications. First, they are indigenous to field grown wheat in Saskatchewan and have not been genetically modified, making them more likely to be acceptable to the public. Second, there is no evidence that they are vertically transmitted through seeds from one generation of plants to the next, meaning that these fungi are less likely to spread by means of seeds planted in subsequent years (when they might not be desired). Finally, the SMCD isolates discussed in this thesis can be easily cultured as free-living fungi in the absence of a host plant, facilitating their use in laboratory, pot-based and field studies, as well as agricultural applications.

However, caution is warranted when considering the use of SMCD 2204, 2206, 2208, 2210, 2214 or 2215 (or any other fungal endophytes belonging to Ascomycota or Basidiomycota) in agriculture. Potential pitfalls of the use of endophytic fungi in agriculture include the possibility that the beneficial organisms in question are naturally ubiquitous, meaning that maintaining endophyte-free control plants could prove challenging and inoculation might provide little or no benefit. Furthermore, potential competition for the plant-colonizing niche between the endophyte being applied and other beneficial microorganisms should be considered (Saunders et al. 2010). While root colonizing Ascomycota and Basidiomycota do not seem to exclude mycorrhizal symbiosis (Regvar et al. 2010), little information is available on interactions between different isolates of non-mycorrhizal fungal endophytes or endophytic fungi and beneficial bacteria. Martinuz et al. (2012) showed that individual plants can be simultaneously colonized by a root-inhabiting fungal endophyte (*Fusarium oxysporum*) and a bacterial endophyte (*Rhizobium etli*), both of which control aphid pests. However, co-inoculation with both organisms did not lead to any greater benefit than inoculation with either microorganism on its own. In addition, the host range of SMCD endophytes should be taken into account, both because beneficial endophytes of one plant species can be pathogenic on another host (Redman et al. 2001) and because endophyte-mediated enhancement of weed performance, if it occurs, might be deleterious for the cropping system as a whole. Thus, while fungal endophytes have agricultural potential, careful research is needed before they are widely used.

3.0 FUNGAL ENDOPHYTES IMPROVE WHEAT SEED GERMINATION UNDER HEAT AND DROUGHT STRESS[1]

3.1 Abstract

Seed germination is a critical life stage for plant survival and timely seedling establishment especially in stressful environments. I hypothesized that fungal endophytes would improve wheat seed germination under heat and drought stress. The hydrothermal time (HTT) model of germination is a conceptual model useful for predicting the timing and energy of germination (EG) under a given set of conditions. The HTT and EG are applied, for the first time, to determine if one or more compatible endophytic fungi enhance heat or drought tolerance in wheat. Fungal endophytes tested dramatically increased the percent of germination, improved EG and HTT values, and diminished wheat susceptibility to heat and drought as measured by fresh weight of seedlings. When colonized by the most effective fungal endophyte, the values of the parameters tested in wheat seeds exposed to heat stress resembled those of unstressed seeds.

[1]This work has been previously published in Hubbard M, Germida J and Vujanovic V (2012) Fungal endophytes improve wheat seed germination under heat and drought stress. Botany. 90: 137–149.

3.2 Introduction

Increasing evidence suggests that endophytic fungus–plant interactions are an important determinant of plant evolution and biodiversity (Davitt et al. 2010; Gundel et al. 2010). Colonization of host plants by endophytic fungi is believed to contribute to plant genotype adaptability to biotic and abiotic stress factors (Waller et al. 2005; Bae et al. 2009). The coevolution process, involving plant hosts and symbiotic fungal organisms, has enabled mycoendosymbionts to develop different levels of specificity for compatible plant genotypes and organs. Endophytic colonization at the seed state is especially critical because of the role of the seed as generative organs in regeneration and dispersion of flowering plants (Baskin and Baskin 2004) and the role of mycobionts as potential drivers of seedling recruitment in natural undisturbed, disturbed, and polluted habitats (Mühlmann and Peintner 2000; Adriaensen et al. 2006; White and Torres 2010). Seed germination is a vital phenophase to plant survival and reproduction in either optimal or stressful environmental conditions. Thus, developing methods by which seedling emergence can be enhanced and predicted under the limitations of heat or drought is valuable. The use of endophytic symbionts is a promising method by which seed germination can be enhanced (Vujanovic et al. 2000) and plants protected from environmental stressors (Waller et al. 2005). Vujanovic and Vujanovic (2007) coined the term 'mycovitality' to refer to this form of plant mycosymbiosis. Fungal endophytes can also increase tolerance for abiotic stress in plants that have progressed beyond the seedling stage (Márquez et al. 2007; Rodriguez et al. 2008).

The hydrothermal time (HTT) model of germination (Gummerson 1986; Bradford 2002) is a tool by which the factors contributing to seed germination can be understood, or by which the germination of seeds of a given plant genotype can be predicted under a specific set of environmental conditions. This model postulates that there are lower and upper limits to the moisture values and temperatures at which seeds of a particular genotype can germinate. Outside of these limits germination will not occur, while between these limits germination will take place and will be more rapid in warm and moist conditions (Bradford 1990, 1995, 2002). The concept of energy of germination (EG), defined in this work as the time, in days, for 50% of seeds to germinate, is closely linked to the HTT model of germination in that both relate to the ability of seeds to break dormancy and initiate germination over time.

The effects of fungus–plant symbiotic relationships on the HTT model of germination and EG are an unexplored area of both scientific interest and practical potential. Understanding how EG and HTT of germination of wheat seeds might be altered by fungal endophytes could be of practical and theoretical importance. The newly acquired fungus-plant interaction data reflecting mycovitality could shed light on the evolution and ecology of the symbiosis at the seed stage and its capacity to enhance seedling emergence, survival, and establishment. From the applicative side, the seed–fungus relationship would also contribute to the plant biotechnology sector by providing insight into a potential tool for increasing food production and reducing crop loss caused by abiotic stress. It would also offer an alternative to the use of genetically modified organisms. This is especially important as anthropogenic climate change increases the likelihood of widespread drought and (or) heat stress in agriculturally important areas (IPCC 2007). In the last few decades, shifts in the climate have been linked to a 5.5% decline in global wheat production (Lobell et al. 2011).

3.3 Hypothesises and objectives

I hypothesized that one or more endophytic fungi would be capable of improving the HTT model parameters under abiotic stressors. This would be detectable in terms of increased rapidity and the percentage of wheat seed germination (EG) under given temperature and moisture conditions *in vitro*. I also hypothesized that there would be a correlation between the endophytes' capacity to enhance drought and heat tolerance in wheat and its ability to tolerate heat and drought as a free-living fungus.

3.4 Materials and methods

3.4.1 Hydrothermal time model of germination and energy of germination

The hydrothermal time model (HTT) (Gummerson 1986) postulates that an individual seed begins to germinate when two conditions are met. First, the sum of daily temperatures, above a minimum cardinal value (T_{min}), accumulated over a period of time, must pass a threshold value (θ_T), measured in degree days. Second, the seed must accumulate sufficient water potential (θ_H). Thus, HTT (θ_{HT}) can be expressed as

$\theta_{HT} = (\theta_H)(\theta_T)$. (Equation 3.1)

According to Köchy and Tielbörger (2007),

$\theta_T = (T_{substrate} - T_{min})\, t$ (Equation 3.2)

with t representing the time elapsed in days, and

$\theta_H = \psi_{substrate} - \psi_{min}$ (Equation 3.3)

in a constant environment assuming that $T_{substrate}$ is equal to or less than the optimal temperature for seed germination. In equation 3.3, $\psi_{substrate}$ and ψ_{min} represent the water potential of the substrate and the minimum water potential at which germination is possible, in MPa, respectively. Consistent with Bradford (2002), equations 3.2 and 3.3 can be substituted into equation 3.1 to yield

$\theta_{HT} = (\psi_{substrate} - \psi_{min})(T_{substrate} - T_{min})\, t$ (Equation 3.4).

However, in the present study, the temperature exceeds the optimal temperature for the germination of wheat (reviewed by McMaster 2009), necessitating the consideration of a maximum temperature (T_{max}) above which germination cannot occur. Thus, equation 3.2 was modified to

$\theta_T = \sqrt{[(T_{substrate} - T_{min})(|T_{substrate} - T_{max}|)]}t$ (Equation 3.5)

where $T_{min} \leq T_{substrate} \leq T_{max}$. If equation 3.5 is substituted for 3.2 in equation 3.4, the following results

$\theta_{HT} = (\psi_{substrate} - \psi_{min}) \sqrt{[(T_{substrate} - T_{min})(|T_{substrate} - T_{max}|)]}t$ (Equation 3.6)

where $T_{min} \leq T_{substrate} \leq T_{max}$.

Energy of germination (EG) can be defined in several ways, including the percentage of seeds germinating after a set time period after planting, relative to the number of seeds tested (Ford-Robertson 1971; Ruan et al. 2002; Dong-dong et al. 2009), or the number of days required to attain 50% of germination (Allen 1958). To integrate EG with the HTT model of germination I chose to use this latter definition, meaning that EG is equal to t in equation 3.2.

3.4.2 Estimation of parameters

The estimation of T_{min} and T_{max} for wheat was based on both information available in the literature and on my own observations. McMaster (2009) summarizes data originating from Friend et al. (1962), Cao and Moss (1989), and Jame et al. (1998), indicating the existence of a curvilinear relationship between wheat development rate and temperature.

Since germination and development of wheat does not take place below 0 or above 40 °C, T_{min} and T_{max} were assigned the values of 0 and 40 °C, respectively.

The parameter ψ_{min} was estimated *in vitro* by germinating wheat seeds grown on potato dextrose agar (PDA; Difco Detroit, Mich., USA) media containing a range of polyethene glycol (PEG) 8000 concentrations (Amresco Inc., Solon, Oh., USA). The water activity (a_w) of PDA alone and PDA containing 8%, 12%, and 16% PEG was measured using the AquaLab 4TE, Series 4 Quick Start, Decagon Devices (Long Sault, Ont., Canada). Water activity is unitless because it is defined as the ratio of the vapour pressure of the water in the sample to the vapour pressure of pure water. Values obtained for water activity were converted to water potential (ψ) using the relationship adapted from Bloom and Richard (2002):

$$\Psi = [(RT)\ln(a_w)]/V \qquad \text{(Equation 3.7)}$$

where *R* is the universal gas constant (8.314 $J\cdot mol^{-1}\cdot K^{-1}$), T is the temperature in degrees Kelvin (ºK), and V is the partial molar volume of water (18 mL/mol). For unit conversions, 1 J/mL = 1 MPa = 10 bar. Water potential is zero for a free water surface or a saturated medium; all other values are negative.

The water activities of PDA and PDA containing 8%, 12%, and 16% PEG were 0.9974, 0.9890, 0.9863, and 0.9825, respectively. These values are equivalent to –0.35, –1.51, –1.88, and –2.41 MPa, respectively, and are consistent with those reported in the literature (Leone et al. 1994).

3.4.3 Plant and fungal material

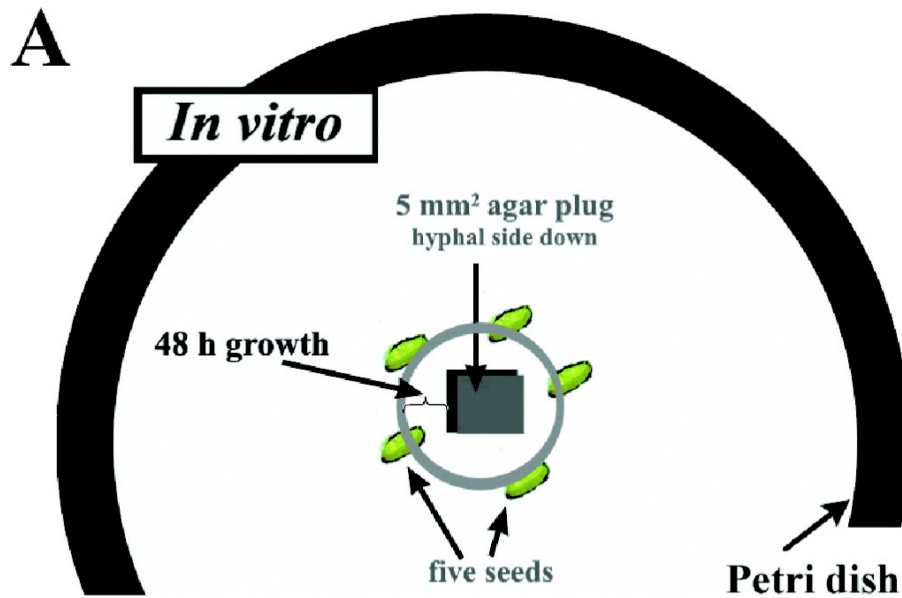
The plant material used was the durum wheat cultivar AC Avonlea, which has low resistance to environmental stressors (Saskatchewan Ministry of Agriculture 2008). The seeds used in the first round of experiments were produced by Paterson Grain (Winnipeg, Man., Canada) in 2008, under field conditions, and not certified to be free of microbes. Seeds used in the second set of experiments were produced by the Agriculture and Agri-Food Canada (AAFC) Seed Increase Unit Research Farm (Indian Head, Sask., Canada) in 2006, under greenhouse conditions, and were certified to be free of microbes (D. Gehl, *personal communication*). Wheat seeds were surface sterilized with 95% ethanol for 10 s, rinsed in sterile distilled water for 10 s, submerged for either 3 min (first round of experiments involving seeds not certified to be free of microbes) or 1 min (second round of

experiments using seeds certified to be microbe-free) in 5% sodium hypochlorite, rinsed three times in sterile distilled water and PDA for germination (Abdellatif et al. 2009). A third seed sterilization method, involving a 3 h exposure to chlorine gas (produced by combining 25 mL 6% sodium hypochlorite with 1.0 mL concentrated hydrochloric acid in a beaker) in a closed plastic box placed in a fumehood (B. Downie, *personal communication*; Rivero et al. 2011) was also tested. The percent germination of seeds subjected to each sterilization protocol and placed on PDA for 3 d is shown in Fig. 3.1B. Only the 3 min submersion in sodium hypochlorite resulted in a significant decrease in germination ($p \leq 0.01$). Seed surface sterilization was intended to eliminate microbes that could compete with the fungal endophytes being investigated. In addition, microbes present on the surface of the seeds could overgrow the Petri dish and emerging seedlings, inhibiting plant growth. All seeds used in the study were determined to be free from microorganisms after sterilization, based on the absence of unintended microbial growth on the Petri plate.

The six endophytic Ascomycota mitosporic fungal isolates (Kiffer and Morelet 2000) deposited in the Saskatchewan Microbial Collection Database (SMCD) SMCD 2204, SMCD 2206, SMCD 2208, SMCD 2210, SMCD 2214, and SMCD 2215 compatible with *Triticum turgidum* L. (Abdellatif et al. 2009; Vujanovic 2007) were used in this study. Fungal organisms were grown on PDA for at least 3 d at room temperature in darkness prior to experimental use.

3.4.4 Fungal endophytes as free-living organisms

Agar plugs (5 mm^2) cut from the margins of the parent colony were placed in the centre of a 90 mm Petri dish containing either PDA alone or amended with 8% PEG (drought). The Petri dish was sealed with parafilm (Pechiney Plastic Packaging, Menasha, Wis., USA) to maintain sterility and placed in a bench-top incubator (Precision Thermo Scientific, model 3522, Nepean, Ont., Canada) at either 23 °C, or under heat stress, 36 °C, in darkness. The diameter of the colony was measured at 24, 48, 72, 96 h, and 5 and 6 d. The changes in diameter were used to calculate colony growth rate. The growth of a minimum of three replicates per fungal isolate was measured.



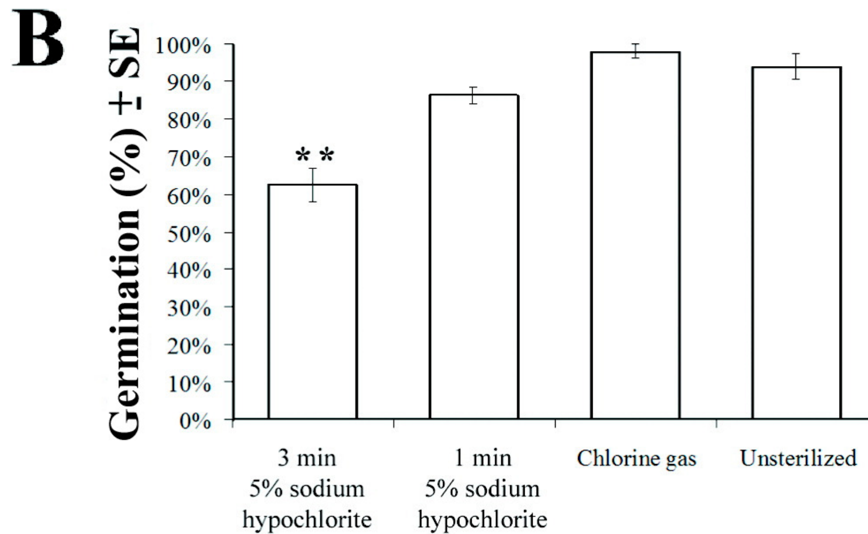


Fig. 3.1 (A) *In vitro* inoculation method. A 5 mm^2 agar plug, cut from the margin of the parent colony, was placed hyphal side down in the centre of a 60 mm Petri dish containing potato dextrose agar (PDA) media. Next, five surface-sterilized seeds were placed a distance equivalent to 48 h hyphal growth from the agar plug and germinated in the dark. (B) The impact of three seed surface sterilization methods on seed germination. Bars labelled with ** are highly significantly different from the other sterilization methods ($p \leq 0.01$, ANOVA, followed by post-hoc LSD test). Error bars represent standard error (SE) of the mean.

3.4.5 Fungal endophytes ability to confer heat and drought tolerance to wheat

Each isolate was applied individually to wheat seeds prior to germination according to the method described in Abdellatif et al. (2010) and shown in Fig. 3.1A. Briefly, five surface-sterilized seeds were placed at a distance equivalent to 48-h hyphal growth from a 5 mm^2 agar plug, placed hyphal side down in the centre of a 60-mm Petri dish. For slow growing isolates, the agar plug of fungal mycelia was placed in the Petri dish 1 to 4 d prior to the introduction of the seeds. The seedlings were germinated for 7 d under abiotic stress and control conditions.

Drought stress was induced using PDA containing 8% PEG. Heat stress was induced in a bench-top incubator in darkness; the temperature was gradually raised by 2 °C every 2 h from 28 °C to 36 °C (i.e., 28 °C for 2 h, followed by 30 °C for 2 h, 32 °C for 2 h and 34 °C for 2 h). In the initial round of experiments, percent germination at 3 d and fresh weight at 7 d was assessed. Each experiment consisted of six Petri plates and was repeated, independently, three times. In subsequent experiments, percent germination was assessed every 24 h for 7 d. Each experiment consisted of 10 Petri plates and was repeated either twice (heat and drought stress combined) or three times (heat stress, drought stress, and control conditions).

The stable internal colonization of wheat roots by the intended fungal endophyte was confirmed by reisolation of the fungal organism from roots that had been surface sterilized to remove an external microbial growth using a procedure modified from Larran et al. (2002). Root fragments (~0.5 cm) were surface sterilized in 95% ethanol for 10 s, rinsed in sterile distilled water for 10 s, submerged for 20 s in 5% sodium hypochlorite, rinsed three times in sterile distilled water, and placed on PDA in a 60 mm diameter Petri dish. The Petri dish was sealed with parafilm and incubated in the dark at room temperature for 4 to 7 d prior to examination.

3.4.6 Statistical analysis

The colony growth rates of free-living fungal organisms grown under heat or drought stress were compared with those of the same organism grown under control conditions using analysis of variance (ANOVA) followed by post-hoc Fischer's least significant difference (LSD) test. Percent germination data was subjected to arcsine transformation prior to statistical analysis (McDonald 2009). Statistical differences between

percent germination after both 3 and 7 d, and fresh weight at 7 d were assessed using a single factor ANOVA to compare all treatments. Subsequently, a post-hoc LSD test was used to evaluate the significance of differences between the no endophyte control and seeds treated with each mycobiont. The level of statistical significance associated with differences between the EG and HTT required to reach 50% germination of endophyte-colonized and control seeds were assessed by evaluating the EG for each of the three independent replicates of the experiment. The resulting data was subjected to an ANOVA and post-hoc LSD analysis. P-values less than 0.05 and 0.01 were considered to be significant and highly significant, respectively. Statistical tests were run with SPSS Inc 2011 (University of Saskatchewan, Saskatoon, Sask., Canada).

3.5 Results

Within each section, the results are organised according to the type of stress: heat, drought, heat and drought, or no stress. Within each type of stress, the results dealing with plant material are presented according to the germinant and (or) seedling traits measured: percent germination at 3 and 7 d, fresh weight at 7 d, EG, and HTT.

3.5.1 Free-living endophytes

The phenotypes of SMCD 2206, 2210, and 2215 were not altered by heat (36 °C), while SMCD 2204, 2208, and 2214 did not grow at 36 °C. The colony growth rates of SMCD 2206 and 2210 were reduced by 36 °C as compared with nonstressed conditions ($p \leq 0.01$), while the growth rate of SMCD 2215 at 36 °C was increased ($p \leq 0.05$) (Fig. 3.2). At 36 °C SMCD 2215 grew the most rapidly, followed in decreasing order by 2206 and 2210 (Fig. 3.2).

The morphology of SMCD 2204, 2206, 2208, and 2215 was not appreciably altered by drought (8% PEG). However, when SMCD 2210 and 2214 were exposed to drought, these two organisms lost their “woolly” appearance and instead acquired a “shiny” or “slimy” appearance. The colony growth rates of SMCD 2204, 2206, 2208, and 2214 were reduced by drought ($p \leq 0.01$, $p \leq 0.01$, $p \leq 0.05$, and $p \leq 0.05$, respectively), while the rate of colony growth of all other endophytes remained unchanged (Fig. 3.2). When drought stress was applied, SMCD 2204 grew at the highest rate followed in decreasing order by 2206, 2210, 2214, 2208, and 2215 (Fig. 3.2).

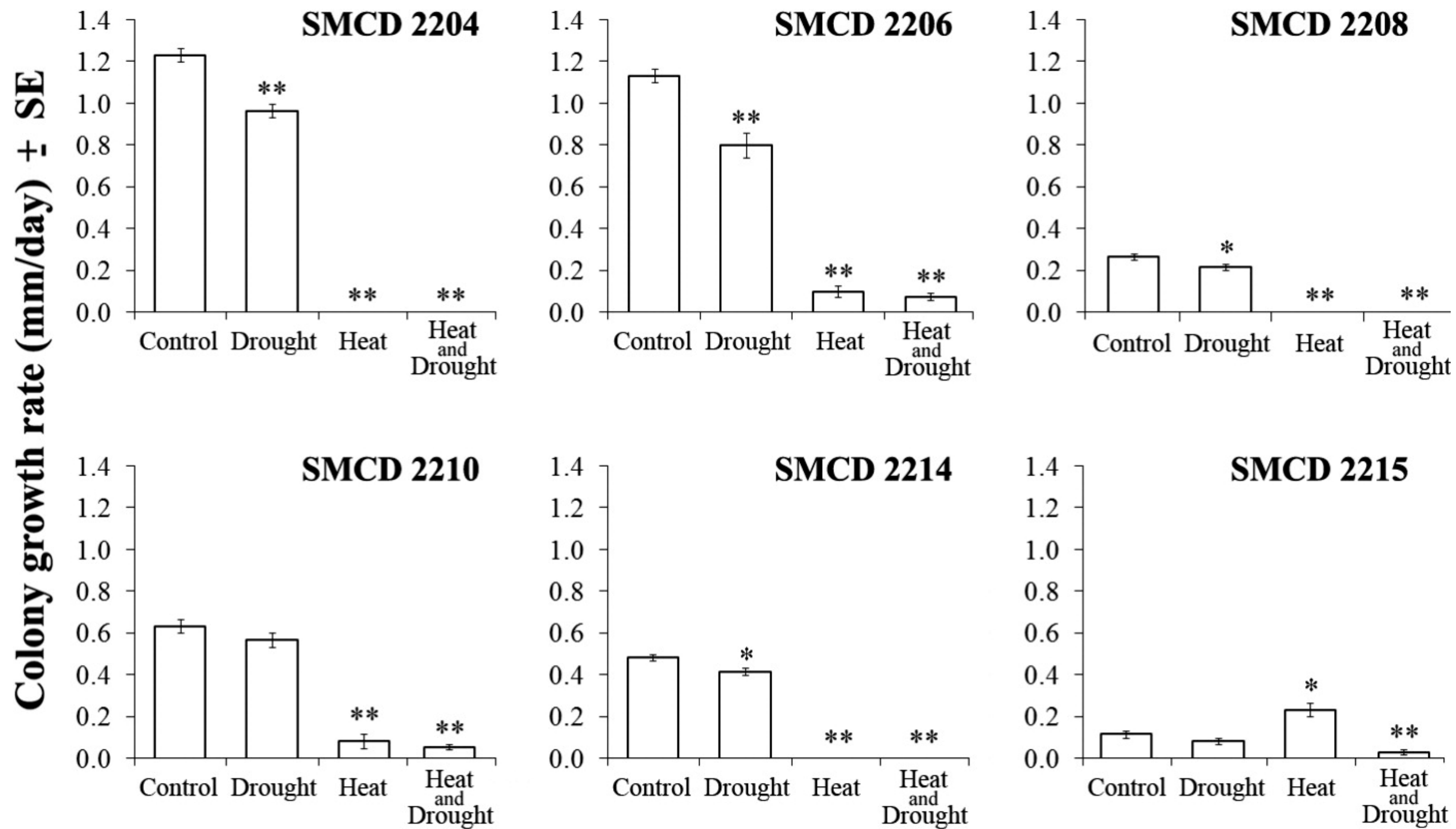


Fig. 3.2 Growth rates of free-living fungal endophytes SMCD 2204, 2206, 2208, 2210, 2214, and 2215 *in vitro* on potato dextrose agar (PDA) under heat stress (36 °C), drought (8% polyethylene glycol (PEG) 8000) stress and control conditions for 5 d and simultaneous heat (36 °C) and drought (8% PEG) for 6 d. Bars labelled with * or ** are significantly, or highly significantly, different from the same endophyte grown under control conditions ($p \leq 0.05$ or $p \leq 0.01$, respectively; ANOVA, followed by post-hoc LSD test). Error bars represent standard error of the mean (SE).

When challenged by 36 °C heat and drought (8% PEG) simultaneously, SMCD 2204, 2208, and 2214 failed to grow, while SMCD 2206, 2210, and 2215 grew at a significantly slower rate than under control conditions ($p \leq 0.01$) (Fig. 3.2). In control conditions, SMCD 2204 grew the fastest, followed in decreasing order by SMCD 2206, 2210, 2214, 2208, and 2215 (Fig. 3.2).

3.5.2 Response of endophyte-colonized wheat to heat

At 36 °C, colonization by SMCD 2206 and 2215 increased germination after 3 d ($p \leq 0.05$ and $p \leq 0.01$, respectively; Fig. 3.3A), whereas SMCD 2214 decreased ($0.05 < p \leq 0.1$) wheat seed germination at 3 d and SMCD 2204, 2208, and 2210 did not alter this parameter ($p > 0.1$; Fig. 3.3A) relative to the no endophyte heat stressed control. After 3 d, heat stressed seeds that were endophyte-free or colonization SMCD 2204, 2208 or 2214 has lower percent germination than the endophyte-free seed grown at room temperature ($p \leq 0.01$). In contrast, seeds subjected to heat stress and colonized by SMCD 2206, 2210 or 2215 did not differ in terms of percent germination after 3 d, from the endophyte-free, no stress control ($p > 0.1$). After 7 d, 63% and 56% of seeds germinated in co-culture with SMCD 2204 and 2208, respectively. These values were not statistically different ($p > 0.1$) from the 59% germination achieved by the uncolonized control. In contrast, the fungal endosymbionts SMCD 2206, 2210, and 2215 promoted germination after 7 d ($p \leq 0.01$; Fig. 3.4). Compared to the unstressed, endophyte-free control, seeds colonized by no endophyte ($p \leq 0.01$), SMCD 2204 ($p \leq 0.01$), SMCD 2206 ($0.05 < p \leq 0.1$), SMCD 2208 ($p \leq 0.01$) and SMCD 2210 ($0.05 < p \leq 0.1$) had lower germination levels after 7 d, while seeds inoculated with SMCD 2215 did not ($p > 0.1$).

When subjected to 36 °C, the fresh weight of wheat seedlings was stable in co-culture with SMCD 2204, 2206, 2208, 2210, and 2214, while SMCD 2215 significantly increased this parameter in comparison to the endophyte-free, heat stressed control ($p \leq 0.01$ respectively; Fig. 3.3D). The fresh weight of seedlings colonized by no endophyte, SMCD 2204, 2206, 2208, 2210 or 2214 was reduced in relation to the unstressed and uninoculated control ($p \leq 0.01$). The fresh weights of SMCD 2215 colonized and heat stressed seedlings did not differ from those of their uninoculated and unstressed counterparts ($p > 0.1$).

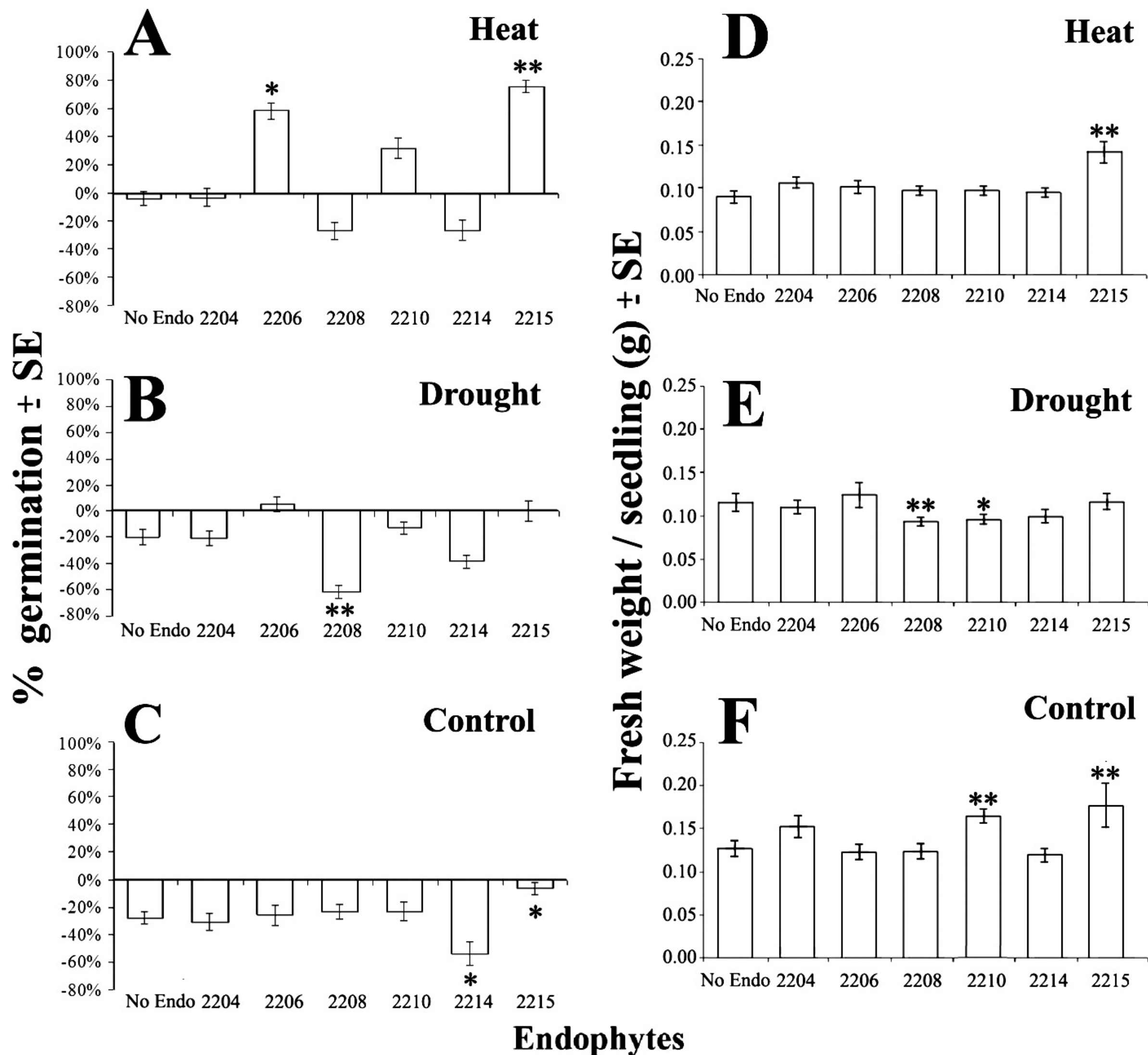


Fig. 3.3 Percent germination and fresh weight of seedlings from initial experiments in which seeds were surface sterilized in 5% sodium hypochlorite for 3 min. Percent germination of wheat seeds *in vitro* after 3 d on potato dextrose agar (PDA) under heat stress (36 °C), drought stress (8% polyethylene glycol (PEG) 8000) and control conditions (A, B, and C) with the *y* axis normalized to percent germination obtained under the same conditions by seeds surface sterilized in 5% sodium hypochlorite for 1 min. Fresh weight of seedlings *in vitro* at 7 d on PDA under heat stress, drought stress, and control conditions (D, E, and F). Bars labelled with * or ** are significantly, or highly significantly, different from the no endophyte control (No Endo; $p \leq 0.05$ or $p \leq 0.01$, respectively; ANOVA, followed by post-hoc LSD test). Error bars represent the standard error of the mean (SE).

The EG for wheat seeds cocultured at 36 °C with fungal endophytes SMCD 2210 ($p \leq 0.05$; Table 3.1; Fig. 3.4) improved compared with endophyte-free seeds. However, SMCD 2204, 2206, 2208, and 2215 did not alter EG ($p > 0.1$; Table 3.1) relative to the control. SMCD 2210 augmented EG to the greatest extent, followed by SMCD 2206 and 2215 (Table 3.1). SMCD 2210 reduced EG to a mere 2 d.

When exposed to heat stress, the HTT required for germination was reduced for wheat seeds colonized by SMCD 2210 ($p \leq 0.05$; Table 3.1), but not any of the other fungi tested ($p > 0.1$; Table 3.1). Endophyte-free wheat seeds needed 50 MPa °C days more than seeds colonized by SMCD 2210 (the most effective endophyte tested) to achieve 50% germination (Table 3.1). There was a negative linear correlation between the HTT necessary for 50% germination and percent germination after 7 d under heat stress (Fig. 3.5).

3.5.3 Response of endophyte-colonized wheat to drought

When subjected to drought stress for 3 d, a diminished percentage of wheat seeds germinated in co-culture with SMCD 2208, compared with endophyte-free seeds ($p \leq 0.01$; Fig. 3.3B), while SMCD 2204, 2206, 2210, 2214, and 2215 did not alter this trait ($p > 0.1$; Fig. 3.3B). After 3 d, all drought stressed seeds, regardless of endophyte colonization status, has lower percent germination than the endophyte-free seed grown on PDA ($p \leq 0.05$ for seeds colonized by SMCD 2206 and 2215; $p \leq 0.01$ for endophyte-free seeds and those colonized by SMCD 2204, 2208, 2210 and 2214). After 7 d, treatment with SMCD 2206, 2210, and 2215 led to an increase in seed germination ($p \leq 0.01$, $p \leq 0.05$, and $p \leq 0.01$, respectively; Fig. 3.4). In contrast, 65% and 67% of seeds cocultured with SMCD 2204 and 2208 had germinated after 7 d (data not shown). Neither of these values differed statistically from the 59% of uncolonized seeds that germinated under the same conditions ($p > 0.1$). Drought stressed seeds inoculated with no endophyte ($p \leq 0.01$), SMCD 2204 ($p \leq 0.01$), SMCD 2208 ($p \leq 0.01$) or SMCD 2210 ($p \leq 0.05$) exhibited reduced percent germination after 7 d in relation to the unstressed no endophyte control, while those colonized by SMCD 2206 or 2215 did not ($0.05 < p \leq 0.1$).

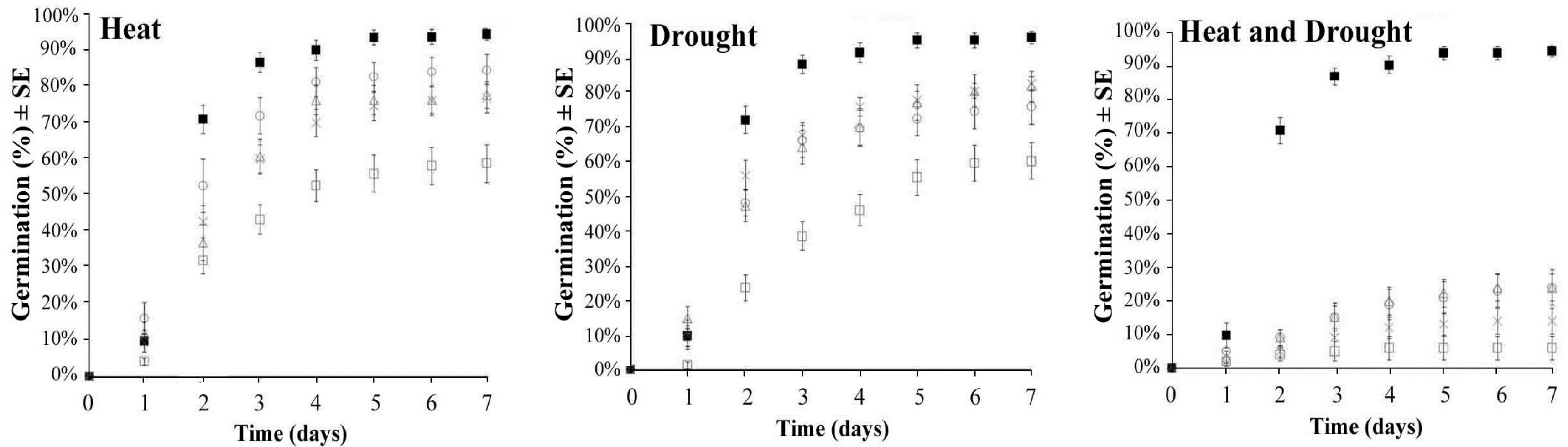


Fig. 3.4 Percent germination over time of wheat seeds co-cultured with the endophytes most effective at conferring abiotic stress tolerance (SMCD 2206, 2210, and 2215) compared with uncolonized, unstressed seeds (positive control; very similar to unstressed seeds colonized by any of the six fungi) and uncolonized, stressed seeds (negative control). Energy of germination (EG) is equal to the time, in days (*x* axis) at which 50% germination (*y* axis) is reached. The symbols "▪", "x", "○", "Δ", and "□" represent the positive control, SMCD 2206 treated seeds, SMCD 2210 treated seeds, SMCD 2215 treated seeds, and the negative control, respectively. Heat and drought treatments correspond to 36 °C and 8% polyethylene glycol (PEG) 8000, respectively. Error bars represent the standard error of the mean (SE). Note: The seeds used in EG determination were from the second round of experiments, and hence sterilized in 5% sodium hypochlorite for 1 min, rather than 3 min.

Table 3.1 Energy of germination (EG) and hydrothermal time (HTT) of seeds grown under heat (36 °C), drought (potato dextrose agar (PDA) media plus 8% polyethylene glycol (PEG) 8000), heat and drought combined, and control *in vitro* conditions. Values are given ± the standard error (SE) of the mean.

	Heat		Drought		Heat and Drought		Control	
Endophyte	**Energy of germination (days)**	**Hydrothermal time to 50% germination (MPa °C days)**	**Energy of germination (days)**	**Hydrothermal time to 50% germination (MPa °C days)**	**Energy of germination (days)**	**Hydrothermal time to 50% germination (MPa °C days)**	**Energy of germination (days)**	**Hydrothermal time to 50% germination (MPa °C days)**
SMCD 2204	3.7 ± 0.3	91 ± 7	2.9 ± 0.3	52 ± 5	2.0 ± 0.8	22 ± 8	1.6 ± 0.2	65 ± 8
SMCD 2206	2.5 ± 0.3	62 ± 7	1.9 ± 0.1 *	34 ± 2 *	2.0 ± 0.8	22 ± 8	1.5 ± 0.2	61 ± 8
SMCD 2208	3.7 ± 0.3	91 ± 7	3.0 ± 0.3	53 ± 5	4.0 ± 1.0	43 ± 10	1.6 ± 0.2	65 ± 8
SMCD 2210	1.8 ± 0.2 *	44 ± 5 *	2.2 ± 0.2 *	39 ± 3 *	1.0 ± 0.5	11 ± 5	1.6 ± 0.2	65 ± 8
SMCD 2215	2.5 ± 0.3	62 ± 7	2.3 ± 0.2 *	41 ± 3 *	1.3 ± 0.2	14 ± 2	1.5 ± 0.2	61 ± 8
No Endo	3.8 ± 0.5	94 ± 11	4.5 ± 0.5	80 ± 8	3.0 ± 1.5	32 ± 15	1.6 ± 0.2	65 ± 8

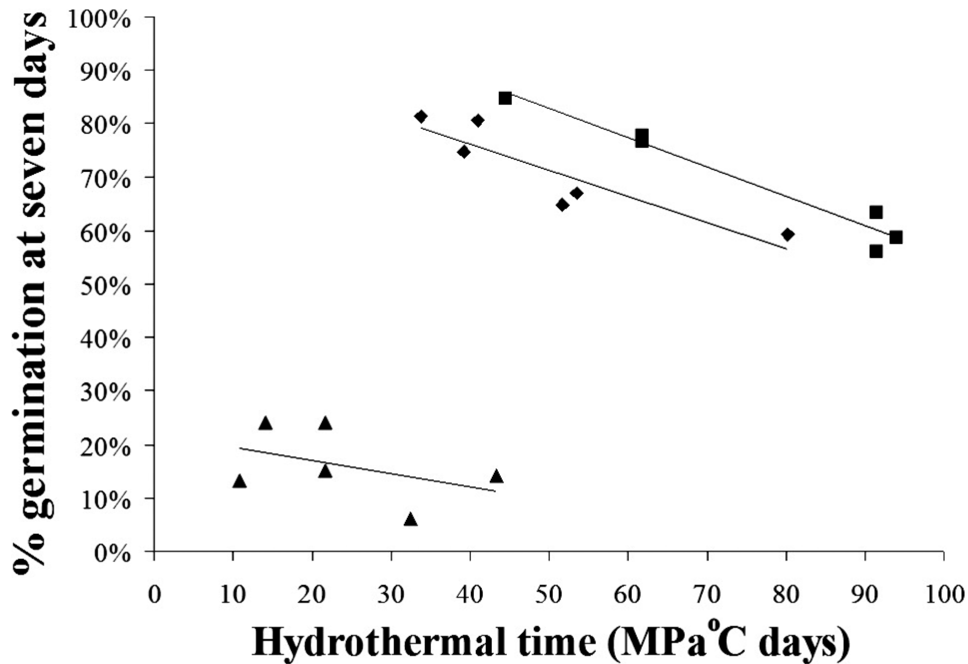


Fig. 3.5 The relationship between hydrothermal time (HTT) required to achieve 50% germination for heat and drought alone and 5% germination for heat and drought combined (*x* axis) and percent germination attained after 7 d (*y* axis). Germination after 7 d and HTT were based on the results of the second round of experiments. The symbols "▪", "◆" and "▲" represent seeds exposed to heat (36 °C), drought (8% polyethylene glycol (PEG) 8000) or both heat and drought stress, respectively. The R^2 values associated with the trendlines are 0.96, 0.80, and 0.18 for seeds exposed to heat, drought, or both heat and drought stress, respectively. Note: the seeds used to determine percent germination at 7 d and HTT were from the second round of experiments, and hence treated with 5% sodium hypochlorite for 1 min, rather than 3 min.

Under drought conditions, SMCD 2208 and 2210 decreased fresh weight after 7 d ($p \leq 0.05$ and $p \leq 0.01$. respectively; Fig. 3.3E). None of the other mycobionts altered this parameter relative to the drought stress uninoculated control ($p > 0.1$; Fig. 3.3E). When compared to the unstressed and endophyte free control, SMCD 2208, 2210 and 2214 depressed fresh weight ($p \leq 0.01$ for all), while inoculation with no endophyte, SMCD 2204, 2206 or 2215 did not ($p > 0.05$ for all).

The EG decreased for wheat seeds co-cultured in drought conditions with all fungal endophytes tested, as compared with endophyte-free seeds ($0.05 < p \leq 0.1$ for SMCD 2204 and 2208 and $p \leq 0.05$ for 2206, 2210, and 2215; Table 3.1). SMCD 2206 improved the EG to the greatest extent, decreasing the time elapsed before 50% germination was achieved after 2.6 d (Table 3.1; Fig. 3.4).

The HTT required for germination was reduced for wheat seeds treated with all fungal endophytes tested under drought stress (Table 3.1). Although uncolonized seeds needed 80 MPa °C days to achieve 50% germination, seeds colonized by endophyte SMCD 2206 (the most effective endophyte tested) required only 34 MPa °C days, representing an important drop of 46 MPa °C days (Table 3.1). There was a visible, negative, linear correlation between the HTT required for 50% germination and the percent germination at 7 d under drought stress (Fig. 3.5). However, the R^2 value associated with this linear relationship was smaller than for the correlation found under heat stress. The ranges of HTTs needed to achieve 50% germination differ between heat and drought stress, with values between 34 and 44 MPa °C days and 80 and 94 MPa °C days being unique to seeds exposed to drought and heat stress, respectively (Fig. 3.5; Table 3.1). The ranges of percent germination after 7 d are similar between seeds exposed to drought and those subjected to heat, though the germination levels of heat-stressed seeds cover a slightly larger range (Fig. 3.5).

3.5.4 Response of endophyte-colonized wheat to drought and heat in combination

Very few wheat seeds germinated when exposed to drought (8% PEG) and heat stress (36 °C) simultaneously (Fig. 3.4). Colonization by endophytic fungi SMCD 2210 and 2215 increased the percent germination after 7 d ($p \leq 0.01$; Fig. 3.4). On the other hand, SMCD 2204, 2206, and 2208 failed to improve this trait ($p > 0.1$). Seeds cocultured with

SMCD 2215 (the most beneficial fungal organism tested) reached 24% germination, four times the level attained by their endophyte-free counterparts (Fig. 3.4).

Because neither uncolonized seeds nor those colonized by any of the endophytes reached 50% germination within 7 d, EG could not be determined and HTT was calculated for 5%, rather than 50%, germination. The time required to reach 5% germination ranged from 24 h to 4 d. None of the endophytes tested decreased the time required to attain 5% germination or HTT values ($p > 0.1$). Overall, the HTT needed to reach 5% germination varied from 11 to 43 MPa °C days ($HTT_{mean} = 23.9$) (Fig. 3.5; Table 3.1).

The range of HTT values for seeds subjected to both heat and drought stress were unique, as compared with the HTT values when either heat or drought was applied alone. There was a negative, linear relationship between HTT required and the percent germination under combined heat and drought stress. However, the R^2 value associated with this linear relationship was smaller than for the correlation found when either heat or drought stress was applied individually (Fig. 3.5).

3.5.5 Response of endophyte-colonized wheat to control conditions

Under nonstressed conditions, SMCD 2215 significantly increased seed germination compared with uncolonized seeds after 3 d ($p \leq 0.01$) (Fig. 3.3C). SMCD 2206, 2208, and 2210 positively impacted, whereas SMCD 2204 did not alter percent of germination. However, SMCD 2214 showed negative impact on seed germination ($p \leq 0.05$) (Fig. 3.3C). In unstressed conditions, SMCD 2204, 2210, and 2215 increased the fresh weight of wheat seedlings after 7 d ($p \leq 0.05$ and $p \leq 0.01$, respectively). Furthermore, SMCD 2206, 2208, and 2214 showed no impact on the fresh weight as compared with uncolonized seedlings (Fig. 3.3F).

In control conditions, EG and HTT parameters were slightly improved by SMCD 2206 and 2215 endosymbionts (Table 3.1). Relatively little alteration in EG and HTT parameters was measured associated with nonstressed wheat seeds in co-culture with different fungal isolates.

3.6 Discussion

The ability of seeds to germinate in favourable conditions is critical for plant adaptability, survival, growth, and reproduction (Baskin and Baskin 2004; Finch-Savage

and Leubner-Metzger 2006). If seeds fail to break dormancy under advantageous conditions, they lose a valuable opportunity for establishment and risk being damaged before another chance for germination occurs. Although the changes in seed vigour (Maleki Farahani et al. 2010) and environmental conditions (Meyer et al. 2000) can influence the alterations in HTT and EG (Köchy and Tielbörger 2007), these parameters can be dramatically improved through a fungus–seed partnership. Indeed, seeds inoculated with compatible mycosymbionts achieved enhanced plant stress tolerance during an early stage of seed germination. In the literature, endophytic fungus-root colonization has frequently been reported to improve stress tolerance in mature plants (Waller et al. 2005; Márquez et al. 2007; Rodriguez et al. 2008). It is likely that both forms of symbiotic relationships (i.e., endophyte-mediated stress tolerance enhancement at the seed germination stage (Vujanovic and Vujanovic 2007) and at the mature plant level) are unique to a give combination of endophytic fungus – plant organ/developmental stage – environmental stress. Such symbiotic relationships could be of interest in plant biotechnology applications (Vujanovic and Vujanovic 2006; Abdellatif et al. 2007) by improving crop traits in heat and (or) drought-stressed environments.

The HTT model can be expanded from a simple assessment of plant–environment interaction to a tripartite fungus–plant–environment model. In essence, mycomediated changes in plant tolerance to environmental stressors were documented. This expanded HTT model is likely to prove instrumental in describing the frequent, quite complex responses observed in field crops. Specifically, this approach could be of considerable practical interest in improving the plant-genotype performances and in better predicting the crop yield in various natural environments (Finch-Savage and Phelps 1993), with or without microbial augmentation. The HTT model also appears suitable for better understanding of the ways in which seeds integrate the signals from their environment to determine when to initiate germination or induce stress tolerance mechanisms (Gummerson 1986; Bradford 2002). The physical environment, in particular minimum and maximum values for temperature and humidity delimit the mycomediated seed germination efficiency. The equations previously employed by the HTT model, which accounted for a minimum cardinal temperature (T_{min}), but not a maximum (Köchy and Tielbörger 2007), were modified in this paper to account for a maximum cut-off temperature (T_{max}) for seed germination.

Fungi are sensitive to the alterations in moisture and heat at both inter- and intraspecific levels (Rodriguez et al. 2008; Abdellatif et al. 2010). According to published data, it appears that the ability of endophytic fungi to confer stress tolerance to a plant host does not necessarily correlate with their ability to tolerate stress as free-living organisms (e.g. Márquez et al. 2007). Fungal endophytes SMCD 2206, 2210, and 2215 were more effective than SMCD 2204, 2208, or 2214 at increasing heat tolerance in wheat as compared with uncolonized controls (summary of effects of all endophytes given in Table 3.2). Physical contact appeared to be a prerequisite for the effects of co-culture to become apparent. Differences in percent germination became pronounced after two or more days in co-culture (Fig. 3.4), by which time fungal growth had led to physical contact being made. SMCD 2206, 2210, and 2215 not only promoted heat tolerance in wheat (Table 3.2) but were able to survive heat stress as free-living organisms, while SMCD 2204, 2208, and 2214 were not (Fig. 3.2). This is consistent with the hypothesis that there would be a positive correlation between an endophytes' ability to confer heat tolerance to wheat and to tolerate heat stress as a free-living fungi. Interestingly, SMCD 2215 was the only endophyte to grow more rapidly under heat stress than at room temperature (Fig. 3.2); this trait may be related to the fungus' ability to confer benefit to wheat seeds subjected to heat stress. Specifically, SMCD 2215 was the sole mycobiont to have a positive impact on fresh weight after 7 d. In the same vein, mycobionts unable to grow as free-living organisms at 36 °C (SMCD 2204, 2208, and 2214; Fig. 3.2) were also unable to enhance wheat tolerance of heat stress. Presumably, SMCD 2204, 2208, and 2214 were able to survive in planta at 36 °C.

As compared with drought, heat stress had a more detrimental impact on not only growth and survival of free-living fungi (Fig. 3.2), but also on EG and HTT (Table 3.1; Fig. 3.5). This was demonstrated by the decreased HTT needed for seeds to reach 50% germination under drought stress as compared with heat for both uncolonized seeds and those colonized by each of the six fungal symbionts (Table 3.1). Notably, the isolates that were unable to survive heat stress as free-living organisms were also incapable of diminishing the EG or HTT of wheat seeds subjected to heat stress. However, all six fungi tested were able both to survive drought as free-living colonies and to lessen the EG and HTT of drought-stressed seeds. However, only SMCD 2206, 2210, and 2215 were able to

accomplish this to a statistically significant degree. This clear-cut relationship between performance without a plant host and ability to improve EG and HTT under heat stress highlights the utility of the HTT model in distinguishing between more effective and less effective beneficial fungal endophytes under stressful environmental conditions.

The benefits conferred by some of the endophytes tested in this paper were quite dramatic. For example, under heat stress, the EG and final percent germination attained by seeds colonized by SMCD 2210 more closely resembled that of the nonstressed seeds (positive control) than that of uncolonized, stressed (negative control) seeds (Fig. 3.4). This highlights the potential scientific and practical value of fungus-mediated plant genotype enhancement.

The combined harmful effects of heat and drought stress are such that none of the endophytes tested are able to restore EG or final germination levels to anywhere near that of their nonstressed counterparts (Fig. 3.4). In addition, the HTT values for seeds subjected to heat and drought simultaneously are distinct from those of seeds exposed to either abiotic stress alone; this can be explained by the fact that two stressors were applied simultaneously and that HTT values were calculated for 5%, rather than 50% germination. The review Mittler (2006) discusses the conflicting adaptation strategies employed by plants to cope with heat versus drought stress. For example, under heat stress, respiration and stomatal conductance tend to increase, while the reverse takes place during drought. These conflicting processes may lead to the observed extreme decline in seed emergence in combined heat and drought. Climate change increases the likelihood of the co-occurrence of moisture and temperature stresses (IPCC 2007) affecting greatly the yield of wheat (Lobell et al. 2011). However, SMCD 2210 and 2215 were both able to increase germination 4-fold (Fig. 3.4) and decrease HTT to less than half its value of uncolonized seeds (Table 3.1). Potentially, a combination of multiple, compatible microorganisms could further promote plant tolerance under simultaneous alteration of heat and drought conditions.

Overall, endophytes SMCD 2206, 2210, and 2215 were the most effective at promoting both heat and drought tolerance, while SMCD 2204, 2208, and 2214 were less efficient (Table 3.2). When exposed to both heat and drought simultaneously, SMCD 2210 and 2215 were most able to confer tolerance (Table 3.2). When all growth conditions (heat,

Table 3.2 Summary of the positive, neutral, and negative impact of each of the fungal endophytes, SMCD 2204, 2206, 2208, 2210, 2214, and 2215, on each of the traits tested under heat (36 °C) (A), drought (8% polyethylene glycol-PEG) (B), heat and drought (C), or control (D) conditions.

A **Heat**	**SMCD 2204**	**SMCD 2206**	**SMCD 2208**	**SMCD 2210**	**SMCD 2214**	**SMCD 2215**
Percent germination at 3 d	- ($p > 0.05$)	↑ ($p \leq 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)
Fresh weight at 7d	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)
Energy of germination	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↑ ($p \leq 0.05$)	n/a	- ($p > 0.05$)
Hydrothermal time	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↑ ($p \leq 0.05$)	n/a	- ($p > 0.05$)
Percent germination at 7 d	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)	n/a	↑↑ ($p \leq 0.01$)
Overall	**Neutral**	**Positive (+3)**	**Neutral**	**Positive (+4)**	**Neutral**	**Positive (+6)**

B **Drought**	**SMCD 2204**	**SMCD 2206**	**SMCD 2208**	**SMCD 2210**	**SMCD 2214**	**SMCD 2215**
Percent germination at 3 d	- ($p > 0.05$)	- ($p > 0.05$)	↓↓ ($p \leq 0.01$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)
Fresh weight at 7 d	- ($p > 0.05$)	- ($p > 0.05$)	↓↓ ($p \leq 0.01$)	↓ ($p \leq 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)
Energy of germination	- ($p > 0.05$)	↑ ($p \leq 0.05$)	- ($p > 0.05$)	↑ ($p \leq 0.05$)	n/a	↑ ($p \leq 0.05$)
Hydrothermal time	- ($p > 0.05$)	↑ ($p \leq 0.05$)	- ($p > 0.05$)	↑ ($p \leq 0.05$)	n/a	↑ ($p \leq 0.05$)
Percent germination at 7 d	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)	- ($p > 0.05$)	↑ ($p \leq 0.05$)	n/a	↑↑ ($p \leq 0.01$)
Overall	**Neutral**	**Positive (+4)**	**Negative (-4)**	**Positive (+2)**	**Neutral**	**Positive (+4)**

C **Heat and Drought**	**SMCD 2204**	**SMCD 2206**	**SMCD 2208**	**SMCD 2210**	**SMCD 2214**	**SMCD 2215**
Energy of germination	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	n/a	- ($p > 0.05$)
Hydrothermal time	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	n/a	- ($p > 0.05$)
Percent germination at 7 d	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)	n/a	↑↑ ($p \leq 0.01$)
Overall	**Neutral**	**Neutral**	**Neutral**	**Positive (+2)**	**n/a**	**Positive (+2)**

D **Control**	**SMCD 2204**	**SMCD 2206**	**SMCD 2208**	**SMCD 2210**	**SMCD 2214**	**SMCD 2215**
Percent germination at 3 d	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↓ ($p \leq 0.05$)	↑ ($p \leq 0.05$)
Fresh weight at 7 d	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	↑ ($p \leq 0.05$)	- ($p > 0.05$)	↑↑ ($p \leq 0.01$)
Energy of germination	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	n/a	- ($p > 0.05$)
Hydrothermal time	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	n/a	- ($p > 0.05$)
Percent germination at 7 d	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	- ($p > 0.05$)	n/a	- ($p > 0.05$)
Overall	**Neutral**	**Neutral**	**Neutral**	**Positive (+2)**	**Negative (-1)**	**Positive (+3)**

Note: The significance of positive or negative impact are classified as either significant, "↑" ($p \leq 0.05$) or highly significant, "↑↑" ($p \leq 0.01$). Significance was assessed using an ANOVA, followed by a post-hoc LSD test.

drought, heat and drought combined, and control) were considered in combination with the level of significance of the benefits (or costs) of the endophytes, SMCD 2215 was the most beneficial, followed in descending order by SMCD 2210 and 2206. SMCD 2204 was neutral, while SMCD 2208 and 2214 were slightly deleterious (Table 3.2). Despite a growing number of published studies on the beneficial effects of mycosymbionts on plant salt-stress tolerance, disease resistance, and higher yield (Waller et al. 2005; Baltruschat et al. 2008; Vujanovic 2008; Fakhro et al. 2010; Khan et al. 2012a), the ability of the endophytic mutualists to confer seed vitality remains poorly understood (Vujanovic and Vujanovic 2007). It is unknown if seed vitality associated with a compatible fungal symbiont is governed by principles similar to root-mycobiont beneficial associations. Potential mechanisms of fungus-mediated vitality include altered intracellular, fungal symbiotic organs (Abdellatif et al. 2009), and expression of stress-related genes, or elicitation of stress-hormones, in colonized plant cells (Sherameti et al. 2008). Symbiotic fungi may also promote the accumulation of osmotically active, or non-structural carbohydrates (Richardson et al. 1992); as well as the plant cell activation of proline biosynthesis, or antioxidant enzymes, to scavenge ROS (Rodriguez and Redman 2005; White and Torres 2010). Increased antioxidant enzyme activity, including catalase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase also seem to play a role in tolerance to abiotic stressors (Yuan et al. 2010) through an altered plant cell physiology (Richardson et al. 1993; Bayat et al. 2009). The ability of fungal endophytes to confer stress tolerance to seed and germinant, transitioning to root-endophyte symbiosis, similar to that described in the literature (Waller et al. 2005; Márquez et al. 2007; Rodriguez et al. 2008) as the plants mature, may provide a novel strategy for mitigating the impacts of global climate change on agricultural and native plant communities.

3.7 Conclusions

Endophytic fungal symbionts are able to confer enhanced resistance to wheat seeds exposed to heat and drought *in vitro*. This increased stress tolerance was measurable in terms of a decreased requirement of HTT for germination and an increased EG of wheat seeds under both heat and drought stress. Fungal isolates SMCD 2206, 2210, and 2215

belonging to Ascomycota are especially promising (Table 3.2). If verified in greenhouse and field trials, the findings of the study could have important implications for the agricultural sector and seed biotechnology. Because relatively little is known about the potential mechanisms by which endophytic fungi confer benefits to their hosts, further *in vitro* and *in planta* investigations into molecular and cellular level interactions are also warranted.

3.8 Connection to the next study

After assessing the ability of the six fungal endophytes SMCD 2204, 2206, 2208, 2210, 2214 and 2215 to improve wheat tolerance for heat and drought at the seed germination stage, it is logical to investigate the possible impacts of these fungi on mature, pot-grown wheat subjected to the same abiotic stressors. I aimed to determine if the same three fungi which most effectively conferred mycovitality to heat or drought stressed wheat – SMCD 2206, 2210 and 2215 – also improve heat or drought resistance in mature and reproductive wheat.

4.0 SYMBIOSIS BETWEEN ENDOPHYTIC FUNGI AND WHEAT INCREASES GRAIN YIELD AND VIABILITY OF SEEDS PRODUCED UNDER HEAT OR DROUGHT STRESS

4.1 Abstract

In order to adapt to stress, plants need to produce high quality, viable seeds. It was hypothesized that the fungal endophytes SMCD 2204, 2206, 2208, 2210, 2214 and 2215 would increase grain yield and potential reproductive success of wheat exposed to heat or drought. The aim of this study was to assess wheat stress tolerance in terms of photosynthetic stress (PS), average seed weight (ASW), total seed weight (TSW), relative interaction intensity (RII), as well as time to 50% germination and percent germination of seeds produced under heat stress, drought stress or well watered conditions. All endophytic fungi either increased or had to no impact on heat or drought tolerance. Endophyte SMCD 2206 was the most beneficial, followed by SMCD 2210 and 2215. It was further hypothesized that carbon isotope discrimination (Δ) values would be lower, indicating higher water use efficiency (WUE), in seeds produced by drought stressed wheat colonized by SMCD 2206, 2210 or 2215, relative to those produced by endophyte-free, drought stressed plants. However, the opposite proved to be the case. Overall, endophytes SMCD 2206, 2210 and 2215 have the potential to improve wheat adaptation to heat and drought. The mechanisms by which this occurs warrant further research.

4.2 Introduction

Fungal endophytes that colonize plant roots, but not aboveground tissues, are an incompletely understood group of organisms in terms of interactions with their hosts, ecological roles or taxonomic positions. Symbiotic associations between plants and root-colonizing Asco and Basidiomycota can increase plant tolerance of heat and drought (Sherameti et al. 2008; Perez-Naranjo 2009; Sun et al. 2010; Chapter 3). These interactions take place in both seedlings and mature plants. In Chapter 3, I found that three of the root-colonizing Ascomycota used in this study improved wheat heat and drought resistance in terms of seed germination. This type of symbiotic relationship between seeds and fungi has been termed mycovitality by Vujanovic (2007). Abdellatif et al. (2009) observed that SMCD 2204 – one of the isolates used in this study – formed both inter- and intra-cellular colonization structures. These structures differed between living and killed roots. This suggests that the health of the host plant can impact the fungal partner, as well as vice versa. Mature plants subjected to drought stress can also benefit from fungal endophyte colonization. Perez-Naranjo (2009) found that endophyte colonization led to increased growth in grass species subjected to drought stress. Consistently, the root-colonizing fungus *Piriformospora indica* (Basidiomycota) confers drought tolerance to seedling and mature *Arabidopsis* (Sherameti et al. 2008) and to Chinese cabbage (Sun et al. 2010). Both Sherameti et al. (2008) and Sun et al. (2010) found that *P. indica* alters the expression of drought-associate genes in the leaves of drought stressed plants. This endophyte also increases the activity of plant antioxidant enzymes under drought stress (Sun et al. 2010). Hence, the ability of root-colonizing endophytes to improve plant resistance for heat or drought stress is an exciting area of research that merits further study.

Plant tolerance of abiotic stress has been successfully evaluated using photosynthetic stress (PS), carbon isotope discrimination (Δ) and grain yield. The photochemical capacity of photosystem II is a measure of PS and is calculated from the ratio of variable chlorophyll fluorescence (Fv) to maximal chlorophyll fluorescence (Fm). This trait has been used as an indicator of heat and drought stress in wheat (Yang et al. 2002; Paknejad et al. 2007). Plant height has also been used as a measure of wheat drought tolerance and linked to grain yield (Khan et al. 2010). Values for Δ are inversely related to water use efficiency (WUE) and

positively related to grain yield in wheat (Mohammady et al. 2009). Together, PS, plant height, Δ and grain yield are useful in assessing wheat tolerance for abiotic stresses.

4.3 Hypothesises and objectives

Here I assessed the ability of six root-colonizing fungal endophytes – SMCD 2204, 2206, 2208, 2210, 2214 and 2215 – to increase heat and drought stress tolerance in established, vegetative wheat by measuring PS. I hypothesized that 1) the PS of wheat grown in heat or drought conditions would be diminished by each of the six fungi relative to the uninoculated control. In contrast, I hypothesized that 2) PS would not differ between endophyte-free and endophyte treated wheat grown in well-watered conditions. In order to test this hypothesis, I measured PS in heat stressed plants colonized by each of the six fungi. I compared the results to those obtained from uninoculated plants grown in the same conditions. The same process was followed for plants grown under drought stress and under well-watered and non heat stressed conditions. In addition, I hypothesized that 3) all six endophytes would increase the average seed weight (ASW) and total seed weight (TSW) produced under heat or drought stress compared to endophyte-free control plants grown in the same conditions. I also postulated that seeds 4) produced by endophyte-colonized heat or drought stressed plants would reach 50% germination faster and achieve a higher percent germination than seeds produced by uninoculated plants exposed to the same conditions. To test the above hypothesises I evaluated the impact of these endophytes on seeds produced in terms of ASW, TSW, time required for 50% of seeds to germinate and percent germination. Contrary to my initial hypothesis, I observed that, SMCD 2204, 2208 and 2214 were less able than SMCD 2206, 2210 and 2215 to confer heat and drought tolerance. This led me to formulate the hypothesis that 5) these latter three isolates would improve WUE (lower Δ) of drought stressed wheat relative to endophyte-free, drought stressed controls.

4.4 Materials and methods

4.4.1 Plant and fungal material

The parental seeds of the durum wheat cultivar AC Avonlea were produced by Paterson Grain (Chapter 3). As in Abdellatif et al. (2009), seeds were surface-sterilized in

95% ethanol for 10 s, rinsed in sterile distilled water for 10 s, submerged for 3 min in 5% sodium hypochlorite (Javex) and then rinsed three times in sterile distilled water.

The six endophytic Ascomycoteous mitosporic fungal isolates used in this study were originally isolated from the roots of durum wheat *Triticum turgidum* L. grown at field sites in Saskatchewan, Canada. They were isolated and characterised by Drs Vujanovic and Germida and preserved in the Saskatchewan Microbial Collection Database (SMCD) and known as SMCD 2204 (Class Dothideomycetes), and SMCD 2206, 2208, 2210, 2214 and 2215 (Class *Incertae sedis*). All six isolates are easily culturable on potato dextrose agar (PDA; Difco Detroit, Michigan, 48201-2532, USA) in the absence of a host plant. Fungal isolates were grown on PDA at room temperature (23ºC) for at least 3 d prior to experimental use.

4.4.2 Inoculation

This paper discusses two experiments. The first experiment utilized *in vitro* inoculation. In the second experiment, inoculations were done in pots. In the first experiment, surface-sterilized seeds were co-cultured *in vitro* for 7 d with one of the fungal endophytes. This was done under non-stressed conditions (PDA, room temperature [23ºC]), drought stress (PDA amended with 8% polyethylene glycol 8000 [PEG, Amersco Inc., 6681 Cochran Road, Solon, OH 44139 USA]) or heat stress (36ºC) as described in Chapter 3. The resulting seedlings were transferred to 2 L plastic pots containing 300 g (dry weight) of autoclaved, Sunshine mix 4 (SunGro Horticulture Canada Ltd., 200 Burrard Street, Suite 1200, Vancouver, BC, V7X 1T2, Canada) at water holding capacity (WHC). Three seedlings were planted per pot. Over a three week period, three batches of up to three pots per treatment were started each week for three weeks, for a total of nine pots per treatment. When more than nine uncontaminated seedlings were available in a batch, a sub-set was selected at random.

In the second experiment, each of the fungal isolates was applied to wheat AC Avonlea cultivar seeds prior to germination as described in Abdellatif et al. (2010). Briefly, five surface-sterilized seeds were positioned at a distance equivalent to 48 h hyphal growth from a 5 mm^2 agar plug, placed hyphal side down in the centre of a 2 L plastic pot filled with 300 g (dry weight) of autoclaved, Sunshine mix 4 potting soil. The seeds and agar plug were then covered with a 3.5-4.0 cm layer of Sunshine mix 4. There were nine pots per

treatment. For both experiments, pots containing plants were placed in either a phytotron *Conviron* PGR15 growth chamber (Controlled Environments Ltd., Winnipeg, MB) for heat stress or in a greenhouse for drought stress and control treatments. The pots were arranged in a randomized block design. The locations of blocks and/or individual pots were changed every 14 d or more frequently. All data presented originated from experiment two, with the exception of photosynthetic stress (PS) data, which came from experiment one.

4.4.3 Application of heat or drought stress

Heat stress was induced in a 1.22 x 2.44 m controlled environment reach-in *Conviron* growth chamber in the phytotron at University of Saskatchewan, College of Agriculture and Bioresources from June to October 2009 (first experiment) and August 2009 to January 2010 (second experiment). In the first experiment, plants were held at non-stress temperatures, averaging 24 ºC both day and night until the most mature seedlings attained stage 12 on the Zadoks scale (Zadoks et al. 1974), equivalent to the two leaf seedling stage, within 7 to 14 d. Subsequently, the temperature was increased to a constant 36 ºC. Day length was 14 h. Heat stress of constant 36 ºC proved to be too severe. None of the plants subjected to it produced any seeds. Hence, heat treatment was amended in the second experiment to mimic daily temperature cycles with lower temperatures during the night. In the second experiment, seedlings that would be subjected to heat stress were maintained under a non-stress temperature regime for the first 10 to 14 d after planting, or until the most mature seedlings reached Zadoks stage 12 (Zadoks et al. 1974). This regime consisted of 8 h at 16 ºC in darkness, 1 h at 20 ºC in darkness, 4 h at 20 ºC exposed to light, 6 h at 24 ºC exposed to light, 4 h at 20 ºC exposed to light and 1 h at 20 ºC in darkness. The temperature and photoperiod were intended to mimic a typical Canadian Prairie summer growing season (Madsood et al. 2005; Grant et al. 2009). Seedlings were acclimated to heat stress over a 7 d period via a daily cycle of 10 h at 18 ºC in darkness and 14 h exposed to light. After being held at 18 ºC for 10 h, the temperature was increased at a rate of 2 ºC h^{-1} from 18 ºC to 32 ºC and held at 32 ºC for 2 h. Next, the temperature was decreased by 2 ºC h^{-1} from 32 ºC to 18 ºC. This regime aimed to resemble a hot, but not overly extreme, summer day in the Canadian Prairies. After acclimation, heat stressed plants were subjected to the following 24 h temperature cycle: 10 h at 20 ºC in darkness. This was followed by 2 h at each of 25 and 30 ºC exposed to light. Next seedlings were subjected to 6 h at 36 ºC in

daylight and 2 h at each of 30 and 25 ºC exposed to light. Day and night time relative humidity was held at 50% and 100%, respectively. In both experiments, light was produced by a mixture of T12VHO 4200K fluorescent and 60W incandescent bulbs. This led to an average light intensity in the growth chamber of 393 µmol m^2 s^{-1} (Baird et al. 2010). I compensated for any impacts of variability in the chamber environment by using randomized blocks and moving plants within the chamber. The primary purpose of this paper was to determine how the fungal endophytes influence wheat traits under a given set of conditions. Hence, the absence of a heat stress-free phytotron chamber does not prevent achievement of the above aim.

Drought stress was induced from June to October 2009 (first experiment) and August 2009 to January 2010 (second experiment). During this period, daytime highs in the greenhouse ranged from 21 to 33 ºC and overnight lows from 17 to 19 ºC. Relative humidity ranged from 50 to 100%, with the lower and higher values generally occurring in late afternoon and overnight, respectively. On sunny days, natural sunlight provided irradiation. On cloudy or winter days with a shorter photoperiod, 1000 watt high pressure sodium light bulbs supplemented sunlight. These bulbs were suspended from the ceiling roughly 2 m above the plants. In the first experiment, drought stressed plants were grown at 25% soil water content by weight. Well-watered plants were grown at 100% water holding capacity (WHC). During the first experiment an acclimation period of 7 d began after seedlings reached Zadoks stage 12 (Zadoks et al. 1974) in 10 to 14 d. During acclimation the volume of water added to each pot daily was decreased by 50 mL per pot per day, from 300 mL per pot per day down to 50 mL. In the second experiment control plants were watered to 100% WHC daily, while drought stressed plants were watered to 100% WHC weekly. This drought regime was adopted to imitate reduced irrigation frequency (Singh et al. 2012). Because pots have a limited volume and Sunshine mix 4 has high drainage, the pots used in this study have less capacity for water storage than field soil. Hence, more frequent watering was needed in the current study than in the field study conducted by Singh et al. (2012).

4.4.4 Photosynthetic stress

Maximal photochemical efficiency is inversely proportional to damage to photosystem II (Farquhar et al. 1989b). This parameter was used to assess photosynthetic stress (PS) experienced by wheat plants grown under heat, drought or well-watered

conditions in the first experiment. A decrease in maximal photochemical efficiency is associated with increased heat or drought stress (Karavata and Manetas 1999). Maximal photochemical efficiency is equal to Fv/Fm, where Fm and Fv represent maximum and variable dark-adapted fluorescence. Fv is calculated from the equation

$$Fv = Fm - F_0, \quad \text{(Equation 4.1)}$$

where F_0 is minimum dark-adapted fluorescence. When plants started in each of the first two batches reached Zadoks stages 31 to 37 (Zadoks et al. 1974), PS was measured in the second youngest leaf on the tallest stem of each plant. Measurements of PS were made after a 20 min dark adaptation period, using a hand-held OPTI-SCIENCES OS-30P Chlorophyll Fluorometer (8 Winn Avenue, Hudson, NH, 03051, USA).

4.4.5 Plant height, total seed weight, average seed weight and relative interaction intensity

Plant heights were measured once plants reach maturity and spikes had formed. Mature spikes were collected and the seeds cleaned by hand. Dry seeds were weighed on a Mettler Toledo PG802-S laboratory balance. The seeds from all plants in all pots subjected to each treatment were weighed to give total seed weight (TSW). For seeds produced under well-watered or drought conditions, a minimum of 30 groups of 10 seeds were selected at random and weighed. Because of the lower number of seeds produced by heat stressed plants, all seeds from this treatment were randomly divided into groups of 10 seeds and weighed. The resulting data was used to calculate the average seed weight (ASW) per 10 seeds produced for each treatment.

The TSW from each treatment were used to assess the relative interaction intensity (RII) between endophyte and host. RII was calculated from the formula

$$RII = (B_{E+} - B_{E-})/(B_{E+} + B_{E-}) \quad \text{(Equation 4.2)}$$

where B is the weight of all seeds with (E+) or without (E-) the endophytic fungus (modified from Armas et al. 2004). Values for RII range from -1.0 to +1.0. Negative RII values indicate an antagonistic plant-symbiont relationship. Values at or close to zero denote a commensal interaction and positive values point to mutualism (Armas et al. 2004).

4.4.6 Germination of seeds

Seeds produced in the greenhouse or phytotron by (E-) and (E+) plants were allowed to germinate on sterile, moist 42.5 mm Whatman No. 5 filter paper (Schleicher and Schuell, BioScience, Inc., 10 Optical Avenue, Keene, NH 03431, USA). The filter paper was placed in the centre of a 60 mm Petri dish and saturated with 1 mL of sterile distilled H_2O (adapted from Ali et al. 1994). Five seeds were arranged on each Whatman filter paper. Subsequently, the Petri dish was sealed with parafilm and incubated at room temperature (23 ºC) in the dark for 14 d. Time to 50% germination was determined by measuring the time in days needed for germination to occur in half of the seeds produced by plants subjected to a given treatment.

4.4.7 Carbon isotope discrimination

Water use efficiency (WUE) is generally defined as the biomass accumulated (Sinclair et al. 1984) or seed yield (Hochman et al. 2009) divided by water consumed. This parameter is negatively correlated with carbon isotope discrimination (Δ) in plants (Farquhar and Richards 1984). In addition, lower plant Δ values are indicative of increased stomatal closure and thus decreased stomatal conductance (Khan et al. 2007). In preparation for Δ analysis, seeds were dried in a 70°C oven for at least 4 d and ground to a fine powder with a Spex SamplePrep 8000D Mixer/Mill®. Three well-mixed subsamples of 3.5 ± 0.4 mg from each treatment were analysed in a Europa 20:20 continuous flow isotope ratio mass spectrometer interfaced with a Robo-Prep elemental analyser. Laboratory standard samples were inserted between groups of eight samples. These standards were composed of finely powdered field green pea (^{13}CN.PEAGRN) and calibrated to samples supplied by the International Atomic Energy Association (IAEA) in Vienna, Austria. The carbon isotope composition ($\delta^{13}C$) of each sample was calculated according to the relationship

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000, \qquad \text{(Equation 4.3)}$$

expressed in units of per mil (‰) (Farquhar et al. 1989a). R_{sample} and $R_{standard}$ represented the ratio of ^{13}C to ^{12}C in the sample and in the Peedee belemite carbonate formation standard, respectively. The equation

$$\Delta = (\delta_a - \delta_p)/(1 + \delta_p), \qquad \text{(Equation 4.4)}$$

was used to determine Δ. The symbols δ_a and δ_p denote the carbon isotope composition of

the atmosphere and the plant sample, respectively. I assumed δ_a to be equivalent to -8‰ (Johnson et al. 1990).

4.4.8 Statistical analysis

For each trait – PS, plant height, ASW, time to 50% germination, percent germination of seeds and Δ – an analysis of variance (ANOVA) was performed to compare endophyte-colonized plants in each treatment (heat, drought and well-watered) to their endophyte-free counterparts. Each ANOVA was followed by a post-hoc Fischer's least significant difference (LSD) test. P-values less than an alpha level of 0.05 and 0.01 were considered significant and highly significant, respectively. Percent germination of seeds after a 14 d incubation period was subjected to arcsin transformation prior to statistical analysis (McDonald 2009). Linear correlation was used to explore the relationship between Δ and TSW. Statistical tests were run using SPSS Inc. 2011.

4.5 Results

4.5.1 Photosynthetic stress

In experiment one, the photosynthetic stress (PS) experienced by heat stressed plants was diminished by fungal endophytes SMCD 2206 and 2210 ($p \leq 0.05$ for both) compared to the uninoculated, heat stressed plants. However, neither these two isolates, nor any of the others, reduced PS of heat stressed plants relative to the no endophyte control grown in the absence of heat stress ($p > 0.05$ for all). Endophytes SMCD 2204, 2208, 2214 and 2215 had no effect on PS compared to the both the heat stressed endophyte-free control ($p > 0.05$; Fig. 4.1A). In addition, there was no difference between the uninoculated plants subjected to heat stress or their heat stress free uncolonized counterparts ($p > 0.05$). When exposed to drought stress SMCD 2208 ($p \leq 0.01$), 2210 ($p \leq 0.05$), 2214 ($p \leq 0.01$) and 2215 ($p \leq 0.01$) decreased PS compared to the endophyte-free drought stressed control. On the other hand, SMCD 2204 and 2206 had no effect on PS compared to the drought stressed no endophyte control ($p > 0.05$; Fig. 4.1B). The PS levels of drought stressed plants treated with any of the six mycobionts were no different from those of uninoculated and well-watered plants ($p > 0.05$). Uncolonized drought stressed plants exhibited PS similar to that of endophyte-free well-watered plants ($p > 0.05$). In well-watered conditions, PS did not differ between uncolonized plants and those colonized by each fungal endosymbiont ($p > 0.05$; Fig. 4.1C).

4.5.2 Plant appearance and height

Under heat stress, plants colonized by SMCD 2206 and 2215 differed visually from their uncolonized counterparts (Fig. 4.2A). When subjected to drought stress, plants inoculated with SMCD 2206, 2210 and 2215 appeared larger than uncolonized and drought stressed plants (Fig. 4.2B). Heat stressed plants inoculated with SMCD 2210 or 2215 were taller than heat stressed and endophyte-free plants ($p \leq 0.05$; Fig. 4.2C). Plants subjected to heat stress and colonized by SMCD 2206 were also taller than their uninoculated counterparts ($p = 0.052$; Fig. 4.2C). When subjected to drought stress, plants inoculated with SMCD 2206, 2210 and 2215 appeared larger than uncolonized and drought stressed plants (Fig. 4.2B). However drought stressed plants colonized by any of the six endophytes did not differ significantly in height from the no endophyte drought stressed controls ($p > 0.05$; Fig. 4.2D). Endophyte colonized and well-watered plants do not differ from their uninoculated counters in terms of appearance or height ($p > 0.05$; Fig. 4.2E). Plants exposed to heat or drought stress were shorter than the uninoculated and well-water plants, regardless of their inoculation status ($p > 0.05$ for all).

4.5.3 Average seed weight

When exposed to heat stress, plants colonized by fungal endophytes SMCD 2204, 2206, 2210 and 2215 exhibited an increase in the average weight per 10 seeds produced, or average seed weight (ASW) compared to the endophyte-free control ($p \leq 0.01$). However, colonization by SMCD 2208 or 2214 had no impact on ASW relative to the same heat stress control ($p > 0.05$; Fig. 4.3A). Plants subjected to drought stress and colonized by SMCD 2206, 2208, 2210 and 2215 also had a greater ASW than uninoculated plants ($p \leq 0.01$). The ASW arising from drought stressed wheat colonized by SMCD 2204 or 2214 did not differ from that of their uncolonized counterparts ($p > 0.05$; Fig. 4.3B). Under well-watered conditions, none of the fungal endosymbionts tested altered the ASW compared to that of the endophyte-free well-watered control ($p > 0.05$; Fig. 4.3C).

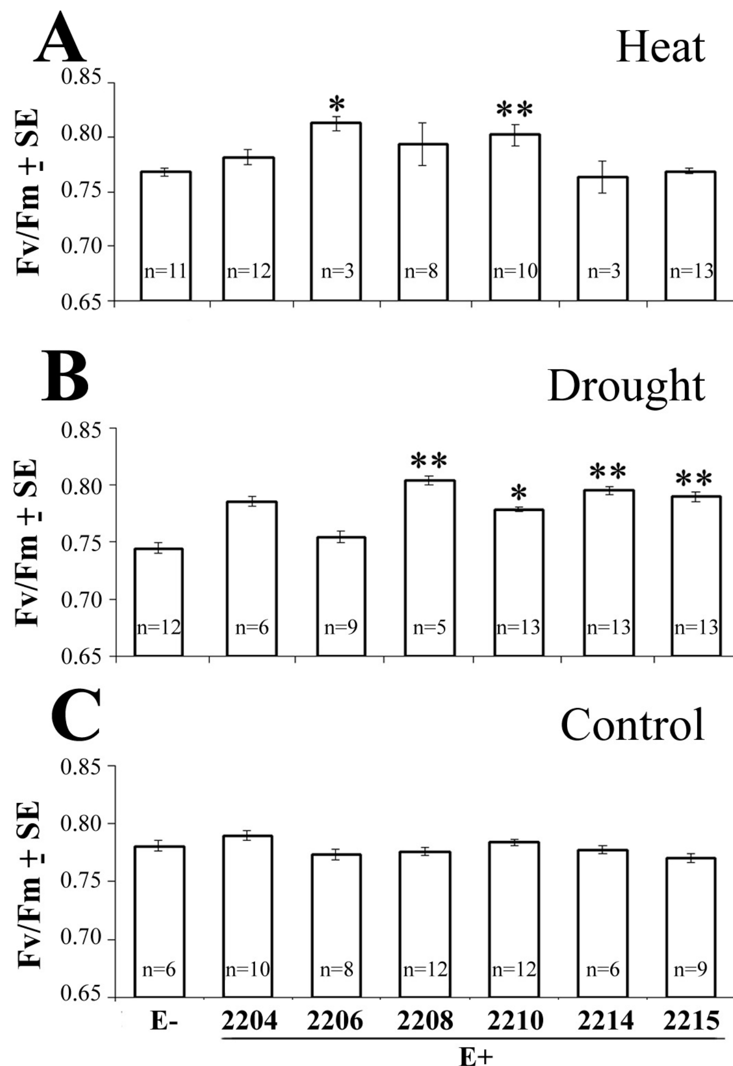


Fig. 4.1 Maximal photochemical efficiency (*F*v/*F*m ratio) measured in 1 month old plants grown under (A) heat stress, (B) drought stress and (C) well-watered conditions. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. A decrease in the *F*v/*F*m ratio indicates increased stress. Asterix (*, **) indicate statistical differences from the endophyte-free control ($p \leq 0.05$ or $p \leq 0.01$) according to an ANOVA, followed by post-hoc LSD test. Error bars represent the standard error (SE) of the mean. The symbol "n" refers to sample size.

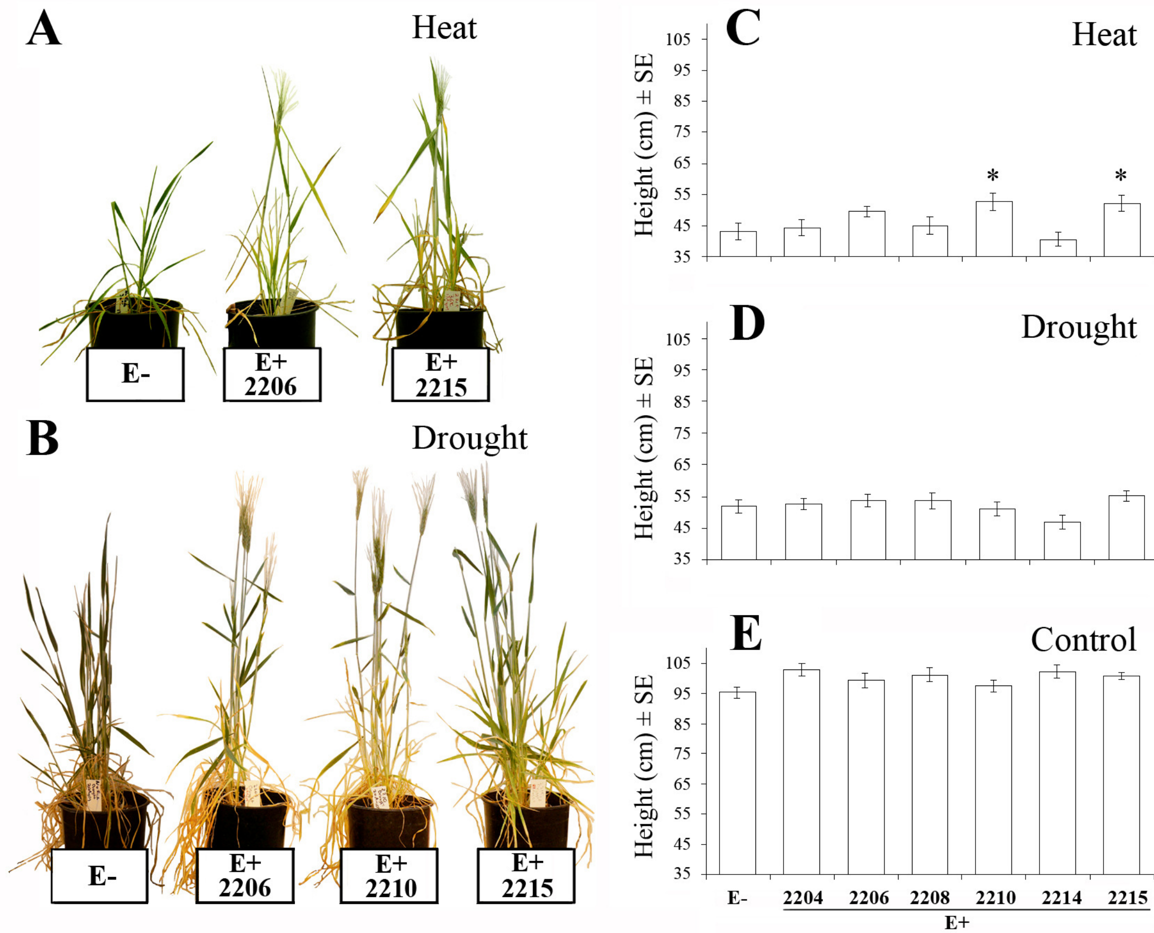
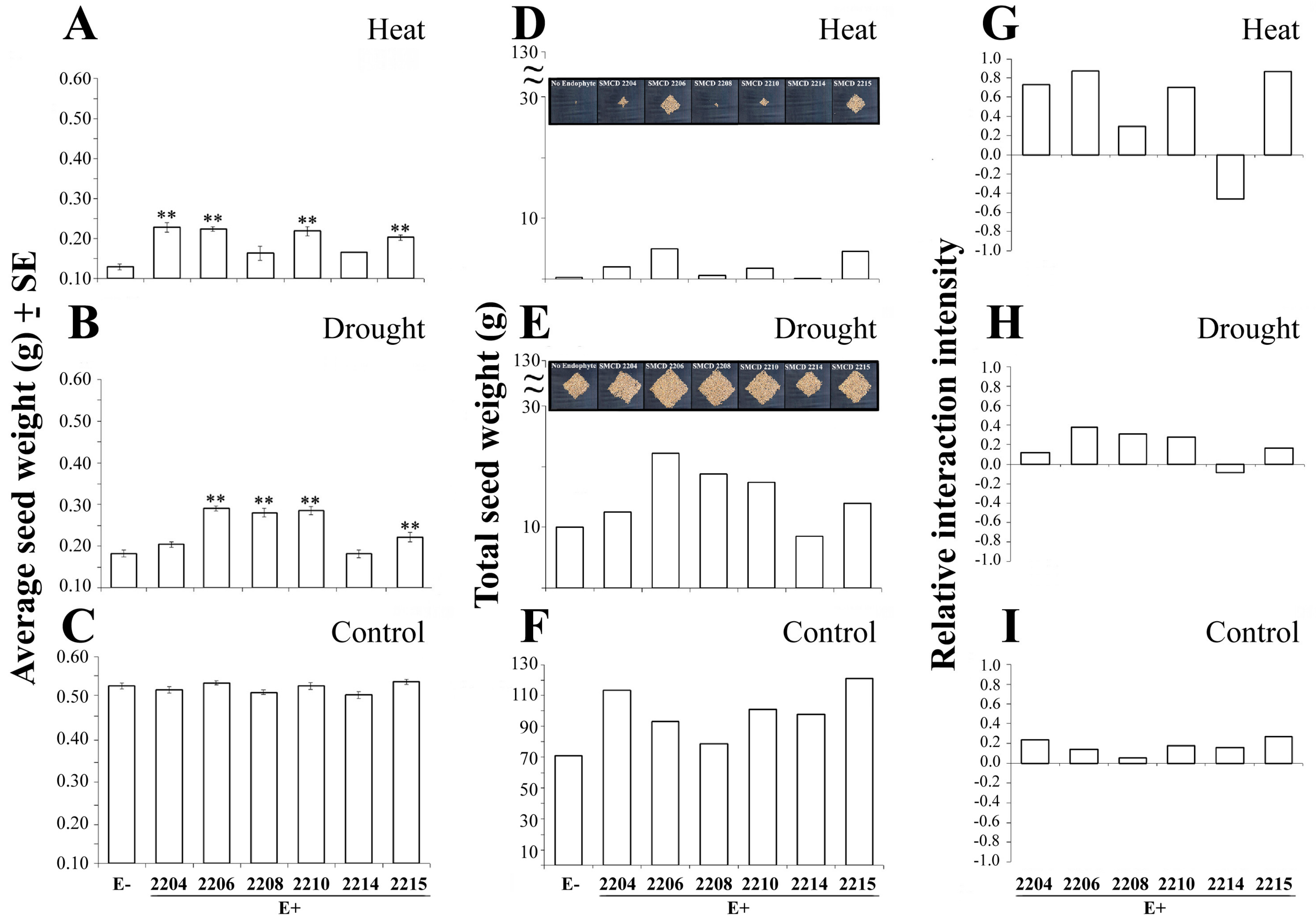


Fig. 4.2 Effect of fungal endophytes on plant appearance (A and B) and height (C, D and E) after exposure to (A and C) heat, (B and D) drought stress or well-watered conditions (E). The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Asterix (*) indicate statistical differences from the endophyte-free control ($p \leq 0.05$) according to an ANOVA, followed by post-hoc LSD test. Error bars represent the standard error (SE) of the mean.



A
Heat
B
Drought
C
Control
Average seed weight (g) ± SE
D
Heat
E
Drought
F
Control
Total seed weight (g)
No Endophyte
SMCD 2204
SMCD 2206
SMCD 2208
SMCD 2210
SMCD 2214
SMCD 2215
G
Heat
H
Drought
I
Control
Relative interaction intensity
E-
2204
2206
2208
2210
2214
2215
E+

Fig. 4.3 (A to C) Average seed weight (ASW) per 10 seeds produced, (D to F) total seed weight (TSW) of all seeds from all pots and (G to I) relative interaction intensity (RII) between wheat and each of the fungal endophytes are shown for plants grown under (A, D and G) heat, (B, E and H) drought and (C, F and I) control conditions. Values for RII range from -1.0 to +1.0. Positive, neutral or negative values indicate mutualism, commensalism or antagonism, respectively. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Asterix (**) indicate statistical differences from the endophyte-free control ($p \leq 0.01$) according to an ANOVA, followed by post-hoc LSD test. Error bars represent the standard error (SE) of the mean. Appearance of seeds produced by drought-stressed wheat plants (insets on D and E) are also shown.

4.5.4 Total seed weight

Colonization of wheat by fungal endophytes SMCD 2204, 2206, 2208, 2210 and 2215 increased the total seed weight (TSW) produced by heat stressed plants by 6.4, 15.7, 1.8, 5.6 and 14.4 fold over that from the endophyte-free control, respectively. In contrast, SMCD 2214 colonization resulted in a drop in TSW to only 37% of the endophyte-free control. Endophytes SMCD 2206 and 2215 increased TSW to the greatest extent (Fig. 4.3D). Under drought stress, SMCD isolates 2204, 2206, 2210 and 2215 resulted in TSWs being enhanced by 1.3, 2.2, 1.9, 1.8 and 1.4 fold, respectively. However, inoculation with SMCD 2214 reduced the TSW to 84% of the control. Isolate SMCD 2206 increased TSW most dramatically, followed, in decreasing order, by SMCD 2208, 2210, 2215 and 2204 (Fig. 4.3E). Under well-watered conditions, endophytes SMCD 2204, 2206, 2208, 2210, 2214 and 2215 increased the TSW, compared to the endophyte-free control, by 1.6, 1.3, 1.1, 1.4, 1.4 and 1.7 fold, respectively (Fig. 4.3F).

4.5.5 Relative interaction intensity

When subjected to heat, the relative interaction intensity (RII) was positive between wheat and SMCD 2204, 2206, 2208, 2210 or 2215 and negative between wheat and SMCD 2214 (Fig. 4.3G). Wheat and SMCD 2206 (0.09) had the highest RII, followed in decreasing order by SMCD 2215 (0.87), 2204 (0.73), 2210 (0.70), 2208 (0.30) and 2214 (-0.46). The mean RII between heat stressed plants and endophytic fungi was 0.50 ± 0.21. Plants grown in drought conditions had positive RIIs with all endophytes tested except SMCD 2214 (Fig. 4.3H). The highest RII was between wheat and SMCD 2206 (0.38). In drought conditions, the mycobionts SMCD 2204, 2208, 2210, 2214 and 2215 had RII with their host of 0.12, 0.31, 0.27, 0.09 and 0.17, respectively. Under drought stress, the average wheat-endophyte RII was 0.19, with standard error of 0.07. In well-watered conditions, the plant-symbiont RII values were positive for all fungi tested (Fig. 4.3I), with an average of 0.17 ± 0.03. Isolate SMCD 2215 had the highest RII (0.26), followed by SMCD 2204 at 0.23, 2210 at 0.18, 2214 at 0.16, 2206 at 0.14 and 2208 at 0.05. The standard errors given above are not shown in Fig. 4.3G, H and I because they the standard error of the average RII values associated with all six endophytes under a given set of conditions.

4.5.6 Time to 50% germination

Time to 50% germination can be used as a measure of stress tolerance and seed viability (Chapter 3). A shorter time to 50% germination indicates greater seed viability or environmental adaptation. Wheat plants cultivated at elevated temperatures and colonized by fungal endophytes SMCD 2206, 2210, 2214 or 2215 produced seeds with a shorter time to 50% germination than uncolonized wheat ($p \leq 0.01$). In contrast, colonization by SMCD 2204 had a neutral impact on time to 50% germination ($p > 0.05$) and 2208 negatively altered this trait ($p \leq 0.05$). Seeds arising from endosymbiont-free parents attained 50% germination in an average of 3.5 d. Those produced by plants inoculated with SMCD 2204 reached 50% germination in 3.4 d. Colonization of parent plants by SMCD 2206, 2210, 2214 or 2215 led to production of seeds which reached 50% germination after 2.3, 2.3, 1.9 and 3.0 d, respectively (results for SMCD 2206, 2210 and 2215 shown in Fig. 4.4A). SMCD 2208-treated plants gave rise to seeds with a time to 50% germination of 4.7 d.
All endophytic fungi tested shortened the time to 50% germination of seeds produced by drought stressed parents ($p \leq 0.01$). Uncolonized plants exposed to drought stress gave rise to seeds that took an average of 10 d to reach 50% germination. Seeds from plants colonized by SMCD 2204, 2206, 2208, 2210, 2214 and 2215 took 4.3, 2.8, 4.0, 3.1, 6.1 and 4.6 (results for SMCD 2206, 2210 and 2215 shown in Fig. 4.4B) d, respectively, to reach the same level of germination.

When wheat was grown under well-watered greenhouse conditions, none of the fungal endosymbionts tested had a significant impact on the time to 50% germination of seeds produced ($p > 0.05$). The variability between seeds produced by well-watered endophyte-free and well-watered colonized plants was much less than under heat or drought stress. If parental plants were not inoculated, seeds attained 50% germination in an average of 2.7 d (dark boxes in Fig. 4.4A and B). Under the well-watered conditions, wheat treated with SMCD 2204, 2206, 2208, 2210, 2214 and 2215 gave rise to seeds which reached 50% germination in 2.4, 2.1, 2.3, 2.3, 2.4 and 2.5 d, respectively.

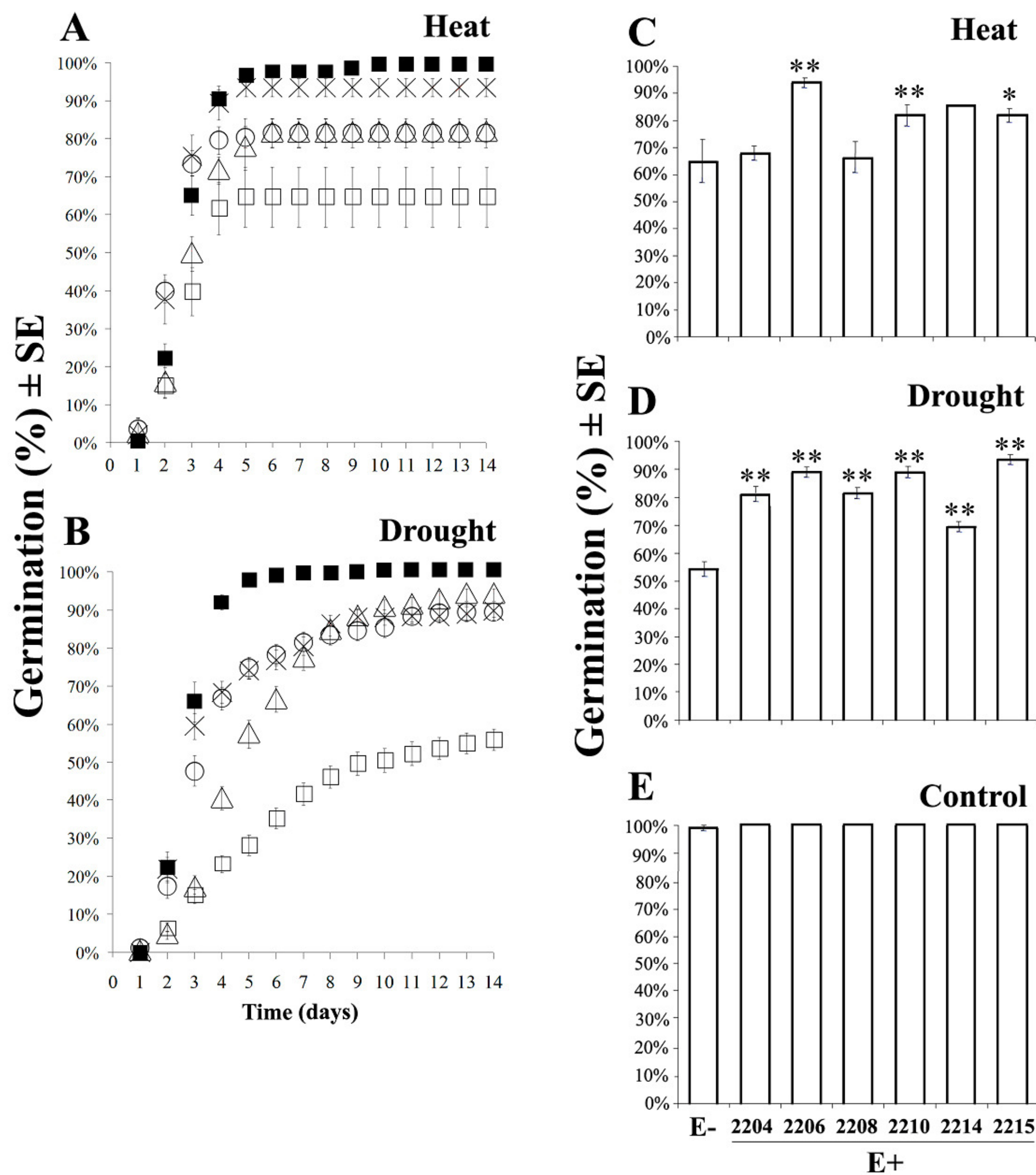


Fig. 4.4 Percent germination over time of seeds produced by plants exposed to (A) heat or (B) drought stress. Seeds produced by unstressed plants colonized by any of the endophytes pattern of germination over 14 d closely resembled that of seed produced by unstressed, uninoculated plants (indicated by the symbol "■"). Seeds produced by heat or drought stressed plants treated with each the three most beneficial fungal endophytes, SMCD 2206, 2210 or 2215, are represented by the symbols "X", "○" and "Δ", while seeds arising from stressed and endophyte-free plants are denoted by "□". The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization in the parental plants, respectively. All seeds are (E-). Percent germination after 14 d at room temperature, produced by plants grown under (C) heat, (D) drought or (E) control conditions are shown. In (C), (D) and (E) the symbol (E-) corresponds to the symbol "□" from (A) and (B), not the symbol "■". Asterix (*, **) indicate statistical differences from the endophyte-free control ($p \leq 0.05$ or $p \leq 0.01$, respectively) according to an ANOVA, followed by post-hoc LSD test. Error bars represent the standard error (SE) of the mean.

Compared to the endophyte-free control, heat stressed plants produced seeds with a higher percent germination if inoculated with SMCD 2206 ($p \leq 0.01$), 2210 ($p \leq 0.01$), or 2215 ($p \leq 0.05$; Fig. 4.4C). Drought stressed plants produced seeds with a higher percent germination after 14 d if the parents were treated with any of the six endophytes tested ($p \leq 0.01$; Fig. 4.4D). Treatment with SMCD 2215 had the most positive impact, followed by SMCD 2206 and 2210. SMCD 2214 was the least effective fungal isolate tested (Fig. 4.4D). All seeds produced by heat or drought stressed plants, regardless of their inoculations status, attained a lower percent germination than those produced by the endophyte-free unstressed control ($p \leq 0.01$ for all). None of the mycobionts altered the percent germination of seeds produced by well-watered plants (Fig. 4.4E).

4.5.7 Carbon isotope discrimination

Seeds produced by drought stressed plants colonized by SMCD 2206, 2210 or 2215 had higher carbon isotope discrimination (Δ) values (decreased stomatal closure and lower water use efficiency (WUE)) than endophyte-free drought stressed plants ($p \leq 0.01$; Fig. 4.5A). Isolate SMCD 2215 elevated Δ most, followed in decreasing order by SMCD 2206 and 2210. In contrast, Δ values of seeds arising from well-watered plants were lower if the maternal plants were treated with SMCD 2206, 2210 or 2215 ($p \leq 0.01$; Fig. 4.5A). There was a positive correlation between Δ values from seeds produced by well-watered and drought stressed plants and TSW from the same plants ($p \leq 0.05$; $R^2 = 0.56$; Fig. 4.5B).

4.6 Discussion

I had hypothesised that endophytes SMCD 2204, 2206, 2208, 2210, 2214 and 2215 would all reduce photosynthetic stress (PS) in heat or drought stressed wheat, but not well-watered wheat, relative to endophyte-free plants grown in the same conditions. Endophytic fungi SMCD 2206 and 2210 promote heat stress tolerance by lowering photosynthetic stress (PS), increasing F_v/F_m values (Fig. 4.1A). Drought stressed wheat colonized by SMCD 2208, 2210, 2214 or 2215 reduced PS compared to the drought stressed endophyte-free control (Fig. 4.1B). When heat, drought and well-watered conditions are all considered, SMCD 2210 had the most beneficial impact on PS, followed by SMCD 2208, 2214, 2215 and 2206. Consistently, the root-colonizing Basidiomycete, *Pirifomospora indica* increases F_v/F_m values of *Arabidopsis* (Sherameti et al. 2008) and Chinese cabbage (Sun et al. 2010) plants subjected to drought. Surprisingly, the F_v/F_m values of well-watered plants without

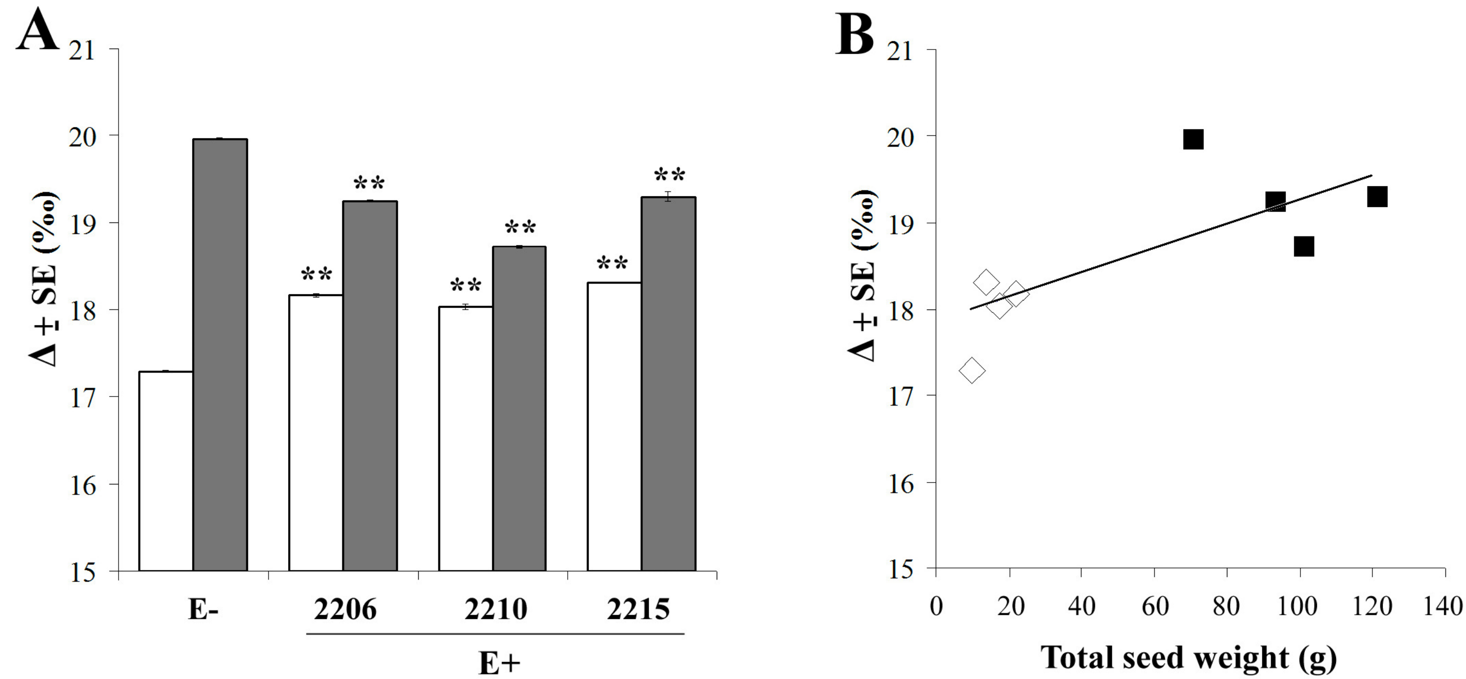


Fig. 4.5 (A) Carbon isotope discrimination (Δ) of seeds produced by uncolonized plants or plants inoculated with each of the three most beneficial endophytic fungi, SMCD 2206, 2210 or 2215 are shown. Unfilled bars represent seeds produced under drought stress, while grey bars denote seeds arising from plants grown under non-stressed conditions. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Asterix (**) indicate statistical differences from the endophyte-free control ($p \leq 0.01$) according to an ANOVA, followed by post-hoc LSD test. Error bars represent the standard error (SE) of the mean. (B) Linear regression analysis for Δ versus total seed weight. The symbols "◊" and "■" represent drought and well-watered treatments respectively. The R^2 value was 0.56 for the drought and well-watered plants combined ($p \leq 0.05$).

endophyte colonization were similar to those of heat stressed endophyte-free plants (Fig. 4.1A and C). The low *F*v/*F*m values of well-watered plants are inconsistent with well-watered plants being truly stress free. Based on the observation of Ma et al. (2005) that wheat *F*v/*F*m values were highest when wheat was grown in soil at 60% field water capacity, perhaps well-watered plants were actually over watered. Possibly, the endophytes do not alleviate PS due to overwatering. The plants in phytotron were watered every 2 d whereas the well-watered plants in the greenhouse were watered daily. This fact, combined with the higher evaporation associated with higher temperatures, means heat stressed plants did not experience overwatering. Endophytes not only reduced PS (Fig. 4.1) and improved appearance and increase plant height (Fig.4. 2), but also enhanced wheat generative capacity compared to the stressed, no endophyte control, but not the well-watered uninoculated control.

Under both heat and drought, SMCD 2206, 2210 and 2215 increased average seed weight (ASW; Fig. 4.3A and B) and grain yield, or total seed weight (TSW; Fig. 4.3D and E). Consistently, SMCD 2206, 2210 and 2215 increase percent germination and decreased time to 50% germination of wheat seeds *in vitro* under heat or drought stress (Chapter 3). In addition, SMCD 2206, 2210 and 2215 had positive relative interaction intensities (RII) with wheat under both heat and drought (Fig. 4.3G and H). This indicates a mutualistic relationship (Armas et al. 2004). The ability of mycosymbionts to increase TSW was especially noteworthy. For example, colonization by isolate SMCD 2206 increased TSW produced by plants subjected to heat or drought stress by 15.7 and 2.2 fold as compared to their uncolonized counterparts grown under the same conditions. This shows that heat inhibits TSW to a greater degree than drought or that the heat stress applied in this experiment was more extreme than the drought treatment.

Isolates SMCD 2206, 2210 and 2215 provided more dramatic benefits to plants subjected to heat as compared to drought. The heat stress applied appeared to be more detrimental than drought. This conclusion is supported by four key observations. First, inoculation with SMCD 2206, 2210 or 2215 significantly increased plant height under heat stress (Fig. 4.2C), but not under drought stress (Fig. 4.2D). Second, treatment with endophytic fungi results in greater multiplication of TSW in plants exposed to heat stress than in those subjected to drought. Third, when plants were grown under well-watered

conditions, none of the fungal isolates tested had as great an impact on wheat performance (measured in terms of PS, plant height, ASW and TSW) as they did under heat or drought. Fourth, the average RII values associated with the interactions between these three mycobionts and their host were more positive under heat as compared to drought (Fig. 4.3G and H). In stress-free conditions RIIs were lower still (Fig. 4.3I). As proposed by Burdon (1987) and Cheplick (2009), increased abiotic stress tends to result in RII values that are more positive for mutualistic interactions. Conversely, more negative RII values are observed under higher levels of stress for antagonistic relationships. Hence, the RII values (Fig. 4.3G, H and I) clearly demonstrate that the heat stress applied in this study was more severe than the drought stress. Exploitation of the heightened benefits of treatment with mycobionts in more extreme environments could prove advantageous.

More of the fungal isolates tested were able to confer drought tolerance than were capable of promoting heat tolerance. All six organisms improved the performance of drought stressed plants to at least some extent. In contrast, SMCD 2208 and 2214 had a net neutral impact on wheat tolerance for heat and SMCD 2204 improved only TSW and ASW. In addition, all six mycobionts increased percent germination of seeds produced under drought stress (Fig. 4.4E). In contrast, only SMCD 2206 and 2210 had a comparable impact on this parameter when plants were exposed to heat (Fig. 4.4D). This difference in response to the two abiotic stressors may be explained by the inability of some of the endophytes (SMCD 2204, 2208 and 2214) to survive *ex planta* at 36ºC (Chapter 3). Interestingly, SMCD 2204 and 2214 lead to some benefit to wheat exposed to heat stress, despite being unable to survive on PDA at 36 ºC. Possibly the fungal hyphae were able to encounter and colonize the seeds or seedlings before a temperature lethal to the free-living fungus was reached. Endophytic fungi can have a beneficial impact on their hosts at high temperatures which neither the endosymbiont nor host could tolerate alone (Márquez et al. 2007). This fascinating aspect of endophyte-plant symbiosis suggests that further investigations into mycomediated heat tolerance are merited both in germinating seeds and at later phenophases.

Plants grown in drought conditions and colonized by SMCD 2206, 2210 or 2215 produced seeds with elevated carbon isotope discrimination (Δ) compared to those produced by uncolonized plants in the same conditions (Fig. 4.5A). In contrast, these three fungi all

lowered that Δ of seeds produced by well-watered wheat, relative to the endophyte-free control (Fig. 4.5A). Plant Δ values are negatively correlated with water use efficiency (WUE) and positively linked to grain yield in wheat (Mohammady et al. 2009). Zacharisen et al. (1999) found a weak relationship between Δ and seed yield, suggesting that Δ alone is an inadequate measure of productivity. Consistent with the findings of Mohammady et al. (2009), I observed a positive correlation between TSW and Δ (Fig. 4.5B), indicating a possible link between the legacy of parental plants and the properties of seeds. The Δ data suggest that inoculation with SMCD 2206, 2210 or 2215 reduced WUE of drought stressed wheat, while increasing grain yield. The aforementioned data is contrary to the hypothesis that these three mycobionts would increase WUE. A potential interpretation of this finding is that drought stressed plants colonized by SMCD 2206, 2210 or 2215 were able to extract more water from the environment by yet to be elucidated mechanism(s). This increased water could then have been used less efficiently. An alternative explanation is that Δ is tissue specific. Xu et al. (2007) observed differences in Δ between wheat leaves and grain. In addition, because higher Δ values have been linked to increased stomatal conductance, elevated Δ values could indicate that the endophyte colonized plants were experiencing less drought stress and were able to maintain greater stomatal openness. Consistent with my findings, these authors also noted a positive association between grain Δ values and grain yield across environments with differing water availability. The Δ values of seeds produced by SMCD 2206, 2210 or 2215 inoculated plants were nearer to those produced by well-watered plants. This implies that endophyte colonized plants experienced less severe drought stress than their uncolonized counterparts grown in the same conditions.

The time to 50% germination and percent germination of seeds produced by plants subjected to abiotic stress and colonized by SMCD 2206, 2210 or 2215 resembled seeds arising from well-watered maternal plants more closely than seeds arising from heat or drought stressed, endophyte-free plants (Fig. 4.4). These results are comparable to those obtained *in vitro* in Chapter 3. For example, *in vitro* inoculation of heat stressed seeds with SMCD 2206 led to a 22% increase in germination (Chapter 3). In the current study, treatment of drought stressed parental plants with SMCD 2206 resulted in a 33% increase in seed germination over those produced by uninoculated plants grown under the same conditions. This increase in seed germination could be meaningful in the field. A 33%

elevation in germination would mean that one third fewer seeds would need to be planted to produce the same number of plants. However, additional lab and greenhouse studies are merited prior to undertaking field trials. One factor that should be address in *in vitro* and pot-based studies prior to field experiments is the fact that endophyte-free plants are very rare in nature (Bacon and White 2000). Endophyte-free controls are a useful tool in preliminary studies aimed at teasing out the impact of each fungus. However, future greenhouse trials using unsterilized field soil (containing naturally occurring microorganisms) could provide valuable data in later studies.

The increase in percent germination of seeds produced by endophyte-treated over those produced by uncolonized plants grown under the same abiotic stress are remarkable given that all seeds are endophyte-free (Fig. 4.4D and E). In the literature, transgenerational impacts of stress have been observed in other plants. Verhoeven et al. (2010) reported the inheritance of epigenetic changes brought about by exposure to stress in asexual dandelions. Furthermore, Whittle and Krochko (2009) found that parental exposure to heat stress resulted in elevated heat tolerance in subsequent generations of *Brassica napus*. The intergenerational impacts of mycobiont colonization documented in this study imply a link not previously demonstrated in relation to plant-endophyte interactions.

Follow-up studies on the ways in which fungal endosymbionts interact with their hosts to promote tolerance for environmental stress is merited. Given the multigenerational impacts of heat and drought stress and endophyte colonization, the hypothesis that heritable epigenetic changes are involved in plant-mycobiont interactions is worth investigating. A common mechanism of epigenetic change is DNA methylation (Razin and Cedar 1992; Henderson and Jacobsen 2007; Boyko and Kovalchuk 2008). Hence, the fact that DNA methylations patterns are altered in drought stressed rice (Wang et al. 2011) as well as in salt stressed wheat (Zhong et al. 2009) suggests that fungal endosymbiosis may induce epigenetic changes in heat or drought stressed host plants. Elucidating how SMCD 2206, 2210 and 2215 interact with germinating seeds (mycovitality; Vujanovic 2007) and adult plants would be a valuable next step in better understanding the roles of fungal endophytes in plant evolution and stress tolerance.

4.7 Conclusions

Fungal endophytes SMCD 2206, 2210 and 2215 have the capacity to enhance wheat tolerance for heat and drought stress, increasing TSW more than ten- and two-fold, respectively. These three endophytic fungi were also able to promote mycovitality *in vitro*, validating the usefulness of laboratory-based screening techniques. Because the mechanisms by which endophytic fungi interact with host plants are incompletely understood, but are hypothesized to involve epigenetic reprogramming of the plant host, future *in vitro* and *in planta* research into the cellular and molecular mechanism(s) by which these endophytes interact with their hosts is merited. Pending the results of further laboratory and pot-based experiments, field trials involving SMCD 2206, 2210 and/or 2215 could be justified. If the greenhouse and phytotron results presented in this work can be duplicated in field trials, these organisms would be useful to the agricultural sector.

4.8 Connection to the next study

Based on the results obtained in Chapters 3 and 4, I judged SMCD 2206 to be the most promising endophytic fungus. Because the mechanisms by which the SMCD endophytes interact with their hosts are unknown, I chose to explore whether SMCD 2206 confers osmotic tolerance to wheat by epigenetically modifying its host.

5.0 FUNGAL ENDOPHYTE COLONIZATION OF WHEAT UNDER OSMOTIC STRESS COINCIDES WITH ALTERED DNA METHYLATION

5.1 Abstract

Drought stress is one of the greatest limiting factors to agricultural production worldwide. The endophytic fungus SMCD 2206 improves wheat tolerance for drought. However, the mechanism(s) by which this mycobiont interacts with its host are not known. Methyl-sensitive amplified polymorphism (MSAP) was used to test the hypothesis that endophyte colonization of drought stressed seedlings coincides with modification of the methylation status of CCGG sequences in plant genomic DNA. In endophyte-free wheat seedlings, drought stress resulted in both DNA methylation and demethylation events, with the overall trend being towards decreased genomic methylation. The DNA methylation patterns observed in drought stressed wheat seedlings co-cultured with SMCD 2206 resembled those of unstressed controls (with or without the endophyte) much more closely than they resembled those of endophyte-free, drought stressed plants. Consistent with the documented involvement of mobile genomic elements in plant epigenetic modification, DNA sequences isolated from some of the most prominent of MSAP bands that were polymorphic between endophyte free and endophyte colonized drought stressed plants, were similar to a CACTA type transposon and two retrotransposons of Gypsy and Copia types. Another polymorphic band was similar to a wheat cytochrome p450 EST. These findings shed new light on plant-endophyte associations, showing that SMCD 2206 colonization of drought stressed wheat coincides with epigenetic modifications of the host plant. Further studies on DNA methylation combined with plant gene expression levels could provide additional information on these epigenetic changes. This is the first time sequence-specific epigenetic changes in plants have been linked to fungal endophyte colonization.

5.2 Introduction

Drought poses a severe threat to global agricultural productivity, and is likely to become more intense and frequent as climatic change intensifies (IPCC, 2007). In the last few decades, shifts in the climate are linked to a dramatic decrease in global wheat production (Lobell et al. 2011). Simultaneously, the worldwide demand for cereal is increasing by roughly 1.5% annually, while genetic alteration in wheat elevate yield potential by approximately 1% leaving a shortfall of around 3.3 million metric tons of wheat per year (Sayre et al. 1997; FAOSTAT 2010 http://faostat.fao.org/). Hence, exploring alternate, sustainable methods of increasing wheat grain production from sub-optimal environments is attractive. A promising approach is the use of fungal endophytes that improve plant performance under abiotic stress (Singh et al. 2011). Symbiotic interactions between wheat and compatible endophytic fungi from Saskatchewan Microbial Collection and Database (SMCD) have been reported by Abdellatif et al. (2009). The results presented in Chapter 3 demonstrate that some SMCD mycobionts confer mycovitality (Vujanovic and Vujanovic 2007) to wheat seeds subjected to heat or drought stress. Although the molecular mechanism by which mycovitality takes place is not well understood I hypothesized that SMCD strains can induce epigenetic changes in plant stress tolerance.

Epigenetic changes involve alterations in gene expression while the underlying DNA sequence remains constant. This process plays a role in plant responses to stress, including drought (Labra et al. 2002; Wang et al. 2011) and salinity (Lu et al. 2007; Zhong et al. 2009). Because DNA methylation is an important mechanism of epigenetic change (Holliday and Pugh 1975; Bender and Fink 1995), it is logical to use this phenomenon as a marker for epigenetic modifications.

Another advantage of exploiting differences in DNA methylation states to learn about plant epigenetics is that molecular approaches for unlocking this information are readily available. Methyl-sensitive amplified polymorphism (MSAP) – which is a modification of the amplified fragment length polymorphism (AFLP) technique – is a well validated method for assessing the methylation state of cytosine residues in CCGG sequences (Li et al. 2008; Wang et al. 2011). This is done by exploiting the differential methylation sensitivities of the CCGG-cleaving isoschizomers *Hpa*II and *Msp*I (Mcclelland 1981). Isolating and sequencing DNA fragments that are differentially methylated between

treatments are able to uncover additional information on the types of genes or genomic sequences involved in plant responses to stress (Lu et al. 2007; Mason et al. 2008). Hence, a similar approach investigating any differences between inoculated and uninoculated osmotically stressed seedlings could provide further insight into mycomediated stress tolerance.

Variations in overall cytosine methylation levels have been spectroscopically measured in diseased plants colonized by mycorrhizae (Dugassa et al. 1996) and MSAP used to detect epigenetic changes associated with salt-stress in wheat (Zhong et al. 2009) and drought stress in rice (Wang et al. 2011). However, the exploration of endophyte-plant interactions via assessment of site-specific changes in plant DNA methylation is novel.

5.3 Hypothesises and objectives

I hypothesized that SMCD 2206 improves wheat seedling performance under osmotic stress by altering wheat DNA methylation, leading to differential gene expression. My objective was to test the above hypothesis by using MSAP analysis to search for these modifications in DNA methylation. Following MSAP, I aimed to sequence DNA extracted from polymorphic bands.

5.4 Materials and methods

5.4.1 Plant and fungal material

Seeds of the durum wheat cultivar AC Avonlea were obtained from Agriculture and Agri-Food Canada (AAFC) and treated as in Chapter 3. In short, seeds were affirmed to be free of subsurface microbes and surface-sterilized. Seeds were germinated *in vitro* under control (-0.35 MPa) and drought (-1.51 MPa) conditions, consisting of potato dextrose agar (PDA; Difco Detroit, Michigan, 48201-2532, USA), PDA amended with 8% (w/v) of polyethene glycol (PEG) 8000 (Amresco Inc., Solon, OH), respectively.

The mitosporic Ascomycete deposited in the Saskatchewan Microbial Collection Database (SMCD) and referred to as SMCD 2206 was originally isolated by Dr. Vujanovic in 2005 from wheat *Triticum turgidum* L. growing in Saskatchewan, Canada. The fungus was grown on PDA at room temperature in darkness for at least 3 d before experimental use.

Wheat seeds were co-cultured with SMCD 2206 prior to germination according to the method described in Abdellatif et al. (2010). Briefly, five surface-sterilized seeds were placed at a distance equivalent to 48 h hyphal growth from a 5 mm^2-agar plug, placed hyphal side down in the centre of a 60 mm Petri dish. The seedlings were germinated for 8 d under drought or control (stress-free) conditions prior to DNA extraction.

5.4.2 Methyl-sensitive amplified polymorphism

A flowchart of the overall process of methyl-sensitive amplified polymorphism (MSAP) is shown in Fig. 5.1. Genomic DNA was extracted from freshly excised leaves of 8 d old seedlings using DNeasy Plant Mini Kit (Qiagen, 2900 Argentia Road, Unit 23, Mississauga, ON, L5N 7X9) according to the manufacturer’s instructions. The intensity of bands corresponding to the resultant DNA was compared to that of the Low DNA Mass Ladder (Invitrogen, 5250 Mainway, Burlington, ON, L7L 5Z1) to estimate concentration.

In order to assess differences in CCGG site methylation, two restriction enzyme digestions were carried out concurrently on each of the two replicates from each treatment, using a protocol modified from Zhong et al. (2009). Hence, there were a total of four restriction enzyme digests per treatment. Briefly, 500 ng of genomic DNA was combined with 10 units of *Eco*RI (Promega, 2800 Woods Hollow Road, Madison, WI 53711 USA) and 20 units of one of the isoschizomers *Hpa*II or *Msp*I (Promega) and incubated in a total volume of 20 µl for 6 h at 37 °C. The isoschizomers *Hpa* II and *Msp*I cleave CCGG sequences and are sensitive to the methylation state of their cleavage sites (Table 5.1; Fig. 5.2A). Unmethylated CCGG sites are cut by both *Hpa*II and *Msp*I. Semi-methylated CCGG sites, which are methylated on one strand of DNA, but not the other, are cut by *Hpa*II, but not *Msp*I. *Msp*I cleaves methylated CCGG sequences while *Hpa*II does not. A site is considered methylated if it has methyl group on each strand of DNA on the inner C and G. Neither *Hpa*II nor *Msp*I cut hyper-methylated CCGG sites. Hyper-methylated sites have either a methyl group on the outer C and outer G, or two methyl groups on each strand.

Table 5.1 Methylation states of CCGG sequences cut by *Hpa*II and/or *Msp*I.

Enzyme	Sites cut		Sites not cut		
*Hpa*II	C C G G C C G G	^{Me}C C G G G G C C	C^{Me}C G G G G C$_{Me}$C	^{Me}C C G G G G C C$_{Me}$	^{Me}C^{Me}C G G G G C$_{Me}$C$_{Me}$
*Msp*I	C C G G C C G G	C^{Me}C G G G G C$_{Me}$C	^{Me}C C G G G G C C	^{Me}C C G G G G C C$_{Me}$	^{Me}C^{Me}C G G G G C$_{Me}$C$_{Me}$

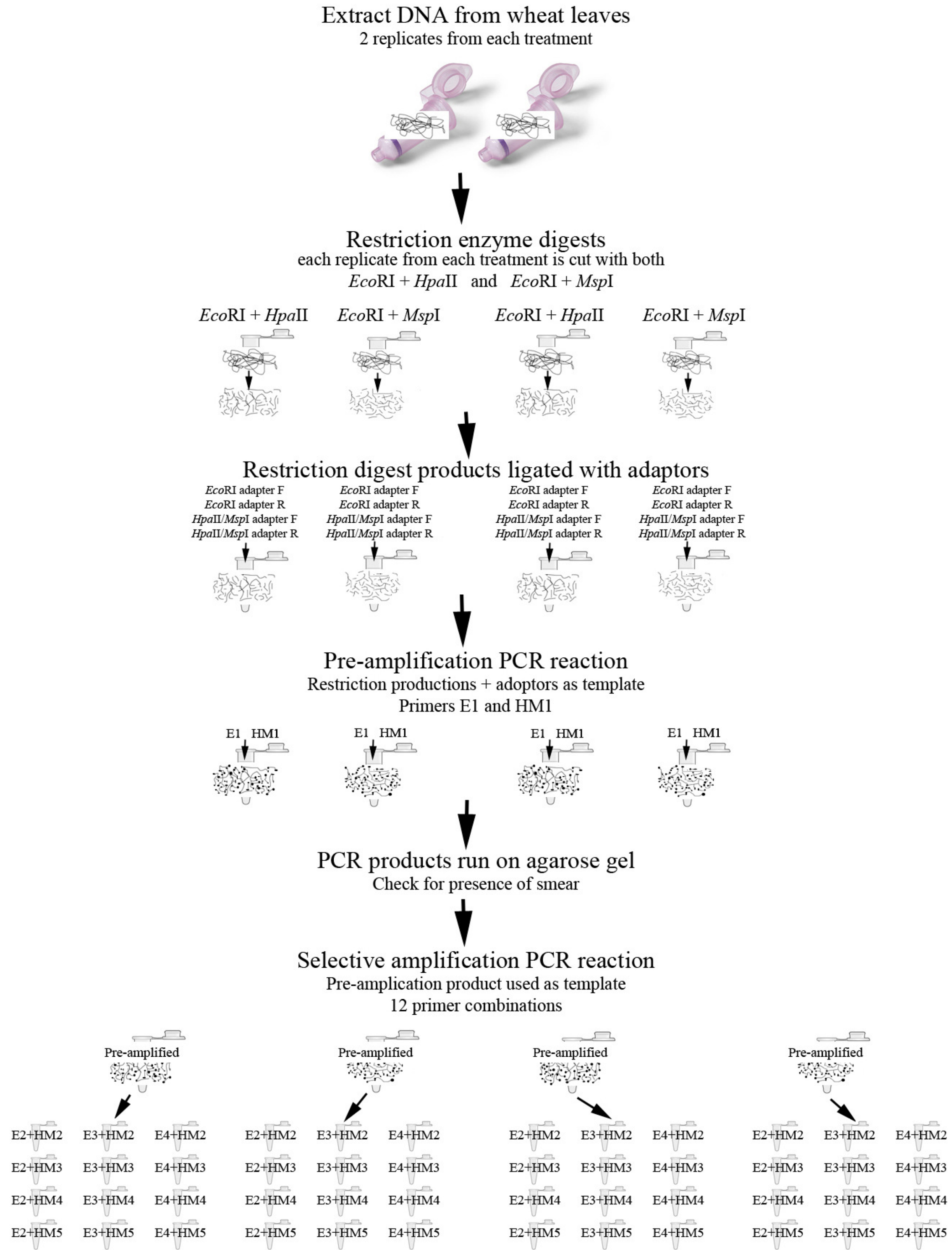


Fig. 5.1 The process of methyl-sensitive amplified polymorphism (MSAP). Adaptors are represented by black dots on the ends of restriction digestion fragments.

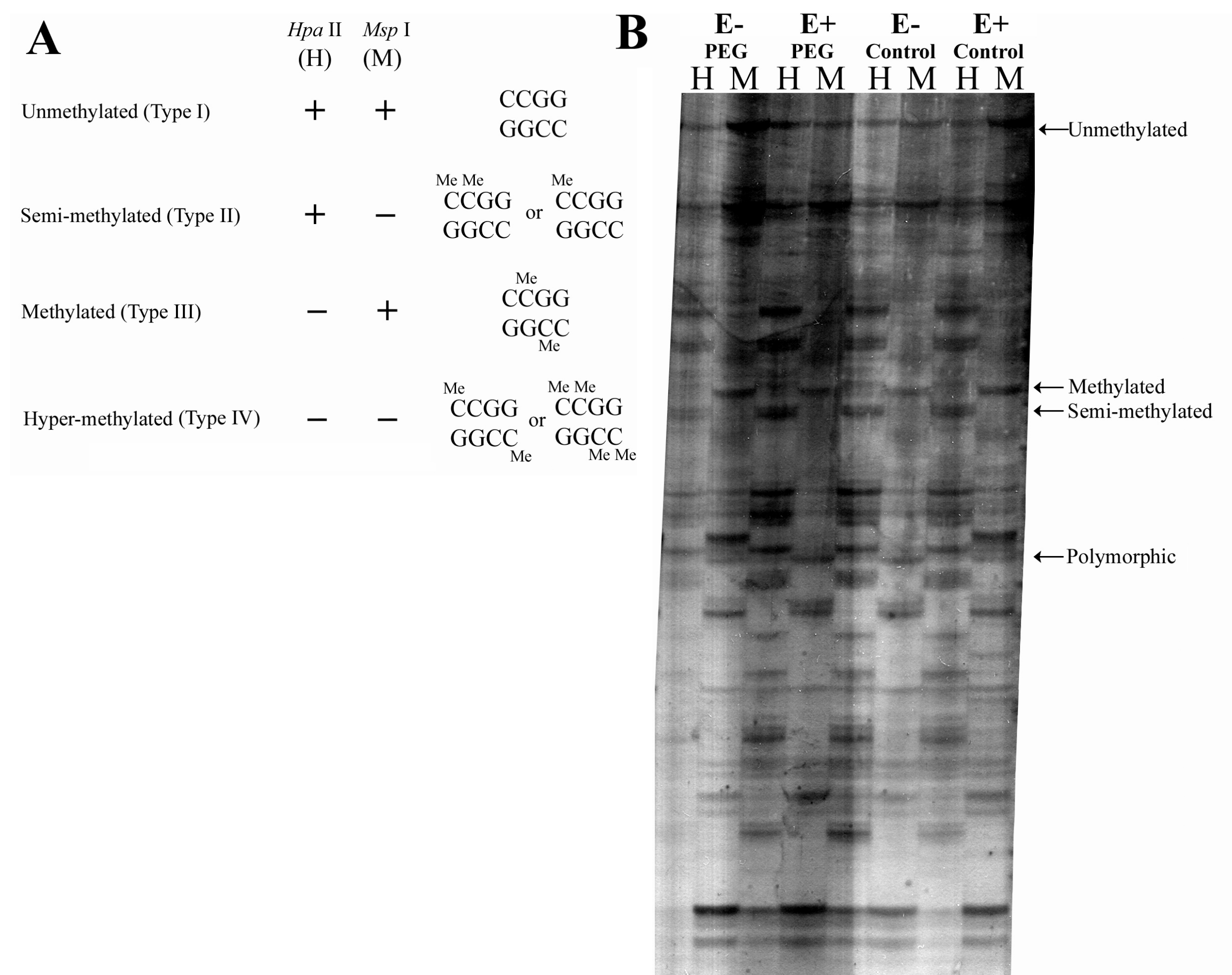


Fig. 5.2 Methyl-sensitive amplified polymorphism (MSAP) banding patterns. (A) Methylation sensitivities of the restriction endonucleases *Hpa*II (H) and *Msp*I (M) (from REBASE) and banding patterns resulting from methyl-sensitive amplified polymorphism (MSAP) analysis. The symbol "+" indicates the presence of a band, which in turn means that digestion took place. Conversely, "-" represents the absence of a band. "Me" indicates a 5' methylation of the associated cytosine residue. (B) An example of banding patterns produced by primer pair E4 and HM4 run on a 5% polyacrylamide gel after silver staining with arrows indicating examples of the phenomenon named. The symbols "E-" and "E+" denote DNA extracted from uninoculated and inoculated plants, respectively.

Adapters were then ligated to the digestion fragments produced in the *Eco*RI and *Hpa*II and *Eco*RI and *Msp*I reactions described above using a protocol modeled off of Zhong et al. (2009). As in the digestion, four ligation reactions were carried out for each treatment. A ligation mixture, consisting of 5 pmol *Eco*RI adapter F and *Eco*RI adapter R (sequences given in Table 5.2; Invitrogen), 50 pmol *Hpa*II / *Msp*I adapter F and *Hpa*II / *Msp*I adapter R (sequences given in Table 5.2; Invitrogen), 0.2 mM ATP and 2 units T4 DNA ligase (Promega), was added in 10 µl aliquots to the results of digestion. The ligation was incubated for 18 h at 4 °C and terminated at 65 °C for 20 min.

Table 5.2 Names and sequences of primers and adapters employed in methyl-sensitive amplified polymorphism (MSAP) analysis.

	Primer or adapter	Sequence (5' - 3')
	*EcoR*I adapter F	CTCGTAGACTGCGTACC
	*EcoR*I adapter R	AATTGGTACGCAGTCTAC
Primers	E1	GACTGCGTACCAATTC+A
	E2	GACTGCGTACCAATTC+AAC
	E3	GACTGCGTACCAATTC+ACG
	E4	GACTGCGTACCAATTC+ACT
	E5	GACTGCGTACCAATTC+AGT
	*Hpa*II / *Msp*I adapter F	GATCATGAGTCCTGCT
	*Hpa*II / *Msp*I adapter R	CGAGCAGGACTCATGA
Primers	HM1	ATCATGAGTCCTGCTCGG+T
	HM2	ATCATGAGTCCTGCTCGG+TAA
	HM3	ATCATGAGTCCTGCTCGG+TCC
	HM4	ATCATGAGTCCTGCTCGG+TTC

The restriction enzyme fragments ligated to adaptors were amplified through two rounds of PCR. A non-selective pre-amplification PCR was followed by a selective PCR reaction. The pre-amplified PCR reaction was carried out as described by Zhong et al. (2009). For each ligation reaction (four per treatment), one pre-amplification PCR occurred. In short, 2 µl of the ligation product (template) was mixed with 40 ng each of primers E1 and HM1 (sequences given in Table 5.2; Invitrogen), 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 1 unit of Taq polymerase (Invitrogen) to a total volume of 15 µl. The PCR was run at 94 °C for 60 s, 25 cycles of 30 s denaturing at 94 °C, 30 s annealing at 56 °C and 60 s extension at 72 °C, followed by 10 min at 72 °C. The presence of a smear of

fragments between 100 and 1000 bp in length was confirmed prior to the PCR product being diluted 1/10 in 0.1 X TE.

Next, the products of the pre-amplification were amplified using a selective PCR (Zhong et al. 2009). The selective PCR used 12 primer pairs (Invitrogen; Table 5.2; Fig. 5.1). Thus, for every treatment, the four pre-amplification reactions led to 4x12=48 selective PCR reactions. Each reaction contained the same components and volume as the pre-amplification PCR, except for the template (5 µl of the pre-amplification product) and the primers (30 ng of the *Eco*RI primer (E2, E3, E4 or E5) and 40 ng of *Hpa*II / *Msp*I primer [HM2, HM3 or HM4]). The following PCR was run at 94 °C for 60 s, followed by 36 cycles of 30 s at 94 °C, 30 s at annealing temperatures which diminished from 65 °C in the first cycle to 56 °C for the final 23 cycles by 0.7 °C per cycle, and 60 s at 72 °C. The PCR ended with a 10 min final extension at 72 °C. The selective PCR products were mixed 1:1 with formamide dye (98% formamide, 10 mM EDTA, 0.01% w/v bromophenol blue and 0.01% w/v xylene cyanol) and denatured for 4 min at 95 °C. Subsequently, 7.5 µl of selective amplification products were separated by electrophoresis on denaturing polyacrylamide gels (5% acrylamide 19:1, 7 M Urea) in 1×TBE buffer at 80W. The polyacrylamide gels were silver stained, dried overnight and reproduced on X-ray film (FujiFilm, MI-DUP, 7-3, Akasaka 9-chome, Minato-ku, Tokyo 107-0052, Japan). The X-ray film was scanned and imported into Adobe PhotoShop 6.0 (345 Park Avenue, San Jose, California, 95110, USA) for band analysis. Images of gels were analyzed by adjusting the contrast and brightness for optimal band visibility in segments of several centimeters at a time. Bands were entered into a spreadsheet containing “0”s and “1”s, where “0” represented the absence and “1” the presence of a band.

The entire MSAP procedure was performed in duplicate from two different DNA extractions. Only bands that could be detected in gels from both replicates were scored and evaluated. The matrix of “0”s and “1”s were interpreted as shown in Figure 5.2A. As in Lu et al. (2007), methylation status was described as type I, II, III or IV. These types are defined as unmethylated, semi-methylated, methylated or hyper-methylated. The presence of a band in both the *Hpa*II and *Msp*I lanes (indicating digestion of the site by both enzymes) meant that the CCGG site was unmethylated, or a type I site. The presence of a band in the *Hpa*II lane, but not in the *Msp*I lane indicated semi-methylation (type II).

Conversely, the absence of a band in the *Hpa*II lane, paired with the presence of a band in the *Msp*I lane, signified that the site was methylated (type III). The absence of a band in either lane indicated a hyper-methylated site (type IV). A total of four gels, with 48 wells each, were run and analyzed. A portion of an example gel is shown in Figure 5.2B.

5.4.3 Cloning and sequencing of polymorphic methyl-sensitive amplified polymorphism fragments

Several of the most prominent reproducible polymorphic bands were cut out of the rehydrated gel, suspended in 20 µl of 1X EB and re-amplified (using the same primer pairs and reaction profile as the selective amplification PCR), according to a method similar to that of Lu et al. (2006). PCR products were ligated into a T-vector (Promega) and cloned into TOP10 heat shock competent *E. coli*. The plasmids were purified using a Qiagen miniprep kit and sent for sequencing at the National Research Council – Plant Biotechnology Institute (Saskatoon, SK, Canada). Sequence data was entered into the public databases PlantGDB (http://www.plantgdb.org/) and NCBI (http://www.ncbi.nlm.nih.gov). In addition, translated nucleotide queries of proteins databases were performed on NCBI.

5.5 Results

5.5.1 Genomic DNA methylation

Seedlings grown in drought conditions without the endophytic fungus SMCD 2206 (E-) had the most unmethylated CCGG sites (304 out of 624 sites or 48.7% of sites). Endophyte colonized (E+) seedlings exposed to drought had 287 unmethylated sites, corresponding to 46.0% of sites. Both (E-) and (E+) seedlings germinated on PDA had 283 (45.4% of sites) and 288 (46.2% of sites) unmethylated sites, respectively (Fig. 5.3).

The percentage of semi-methylated sites was lower in (E-), drought-stressed seedlings than in (E+), drought afflicted seedlings or in unstressed (E-) or (E+) seedling (Fig. 5.3). The four treatments described above were semi-methylated at 221, 244, 251 and 247 sites, respectively. These numbers equate to 35.4, 39.1, 40.2 and 39.6% of sites (Fig. 5.3).

Methylated sites were most frequently observed in DNA from (E-) seedlings exposed to drought (89 sites, corresponding to 14.3% of sites; Fig. 5.3). In contrast, only

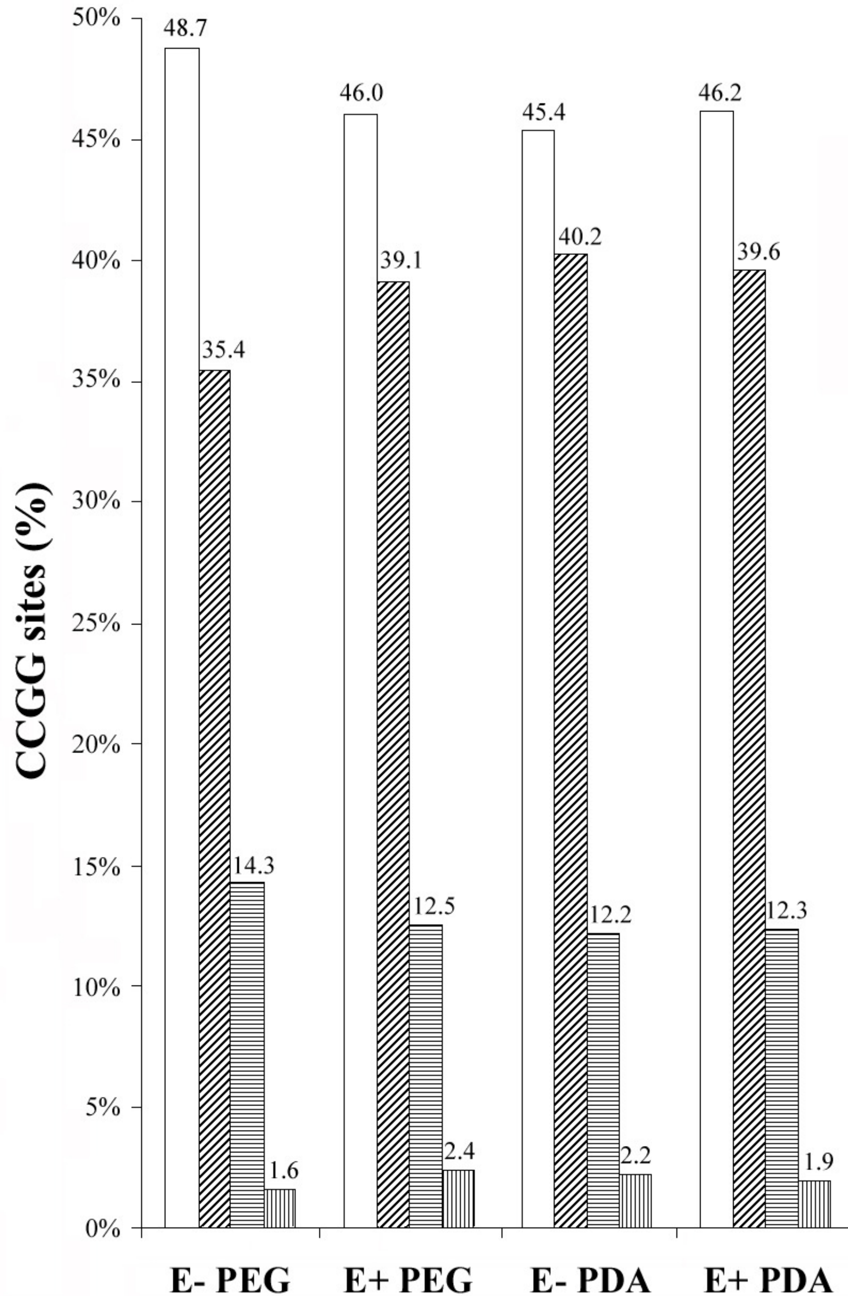


Fig. 5.3 Overall methylation status of CCGG sites. Percentage of sites shown by methylation-sensitive amplified polymorphism (MSAP) to be unmethylated (type I, empty bars ☐), semi-methylated (type II, diagonal cross-hatching ▨), methylated (type III, horizontal cross-hatching ▤) or hyper-methylated (type IV, vertical cross-hatching ▥) in DNA extracted from seedlings germinated under each of four treatments. These treatments were endophyte-free (E-) and subjected to drought stress (potato dextrose agar (PDA) amended with 8% polyethylene glycol [PEG]); SMCD 2206 colonized (E+) and drought stressed (PEG); uncolonized (E-) and stress-free (PDA); SMCD 2206 treated and unstressed (E+ PDA). Percentages are given above the bars.

12.5, 12.2 and 12.3% of sites (78, 76 and 77 sites) were methylated in (E+) seedlings subjected to drought or (E-) or (E+) seedlings grown on PDA (Fig. 5.3).

Hyper-methylation was observed at 15 sites (2.4% of sites) in the DNA extracted from (E+) seedlings germinated under drought-stress (Fig. 5.3). Genomic DNA from drought stressed, (E-) plants displayed hyper-methylation at 10 sites (1.6%). Hyper-methylation was found at 14 or 12 sites (2.2 or 1.9 %) in DNA from unstressed (E-) or (E+) plants (Fig. 5.3).

5.5.2 Methylation and demethylation events

If the DNA methylation patterns of two treatments are compared, a given site can be monomorphic, polymorphic or uninformative (Fig. 5.2B). Monomorphic sites can be unmethylated (type I), semi-methylated (type II) or methylated (type III; Fig. 5.2A and B). Hyper-methylated (type IV) sites are only detectable as part of polymorphic or uninformative CCGG sequences. Polymorphic sites can undergo either methylation or demethylation. Transitions from type I to type II, III or IV are classified as methylation events, as are changes from type II to type III or IV. Conversely, type II, III or IV sites that become type I sites, or type III or IV sites that change to type II have undergone demethylation. Shifts between hyper-methylation (type IV) and type III are uninformative.

When methylation states were compared between DNA extracted from drought-stressed, (E-) and (E+) seedlings, 84.8% of sites (529 sites out of a total of 624) were observed to be non-polymorphic, while 8.2% (51 sites), 5.3% (33 sites) and 1.8% (11 sites) underwent methylation, demethylation and uninformative changes in methylation state, respectively (Fig. 5.4). Within non-polymorphic sites, 260, 202 and 67 CCGG sequences were unmethylated, semi-methylated and methylated, respectively. Among methylation events, 36, 8 and 7 changed from unmethylated to either semi-methylated or methylated, while 7 sites went from semi-methylated to hyper-methylated. There were 12, and 15 instances in which semi-methylated or methylated sites became unmethylated and six situations where hyper-methylated CCGG sequences were demethylated to a semi-methylated state. Uninformative events included three transitions between a hyper-methylated and a methylated state, seven sites at which the reverse occurred and one sequence that was hyper-methylated in both treatments.

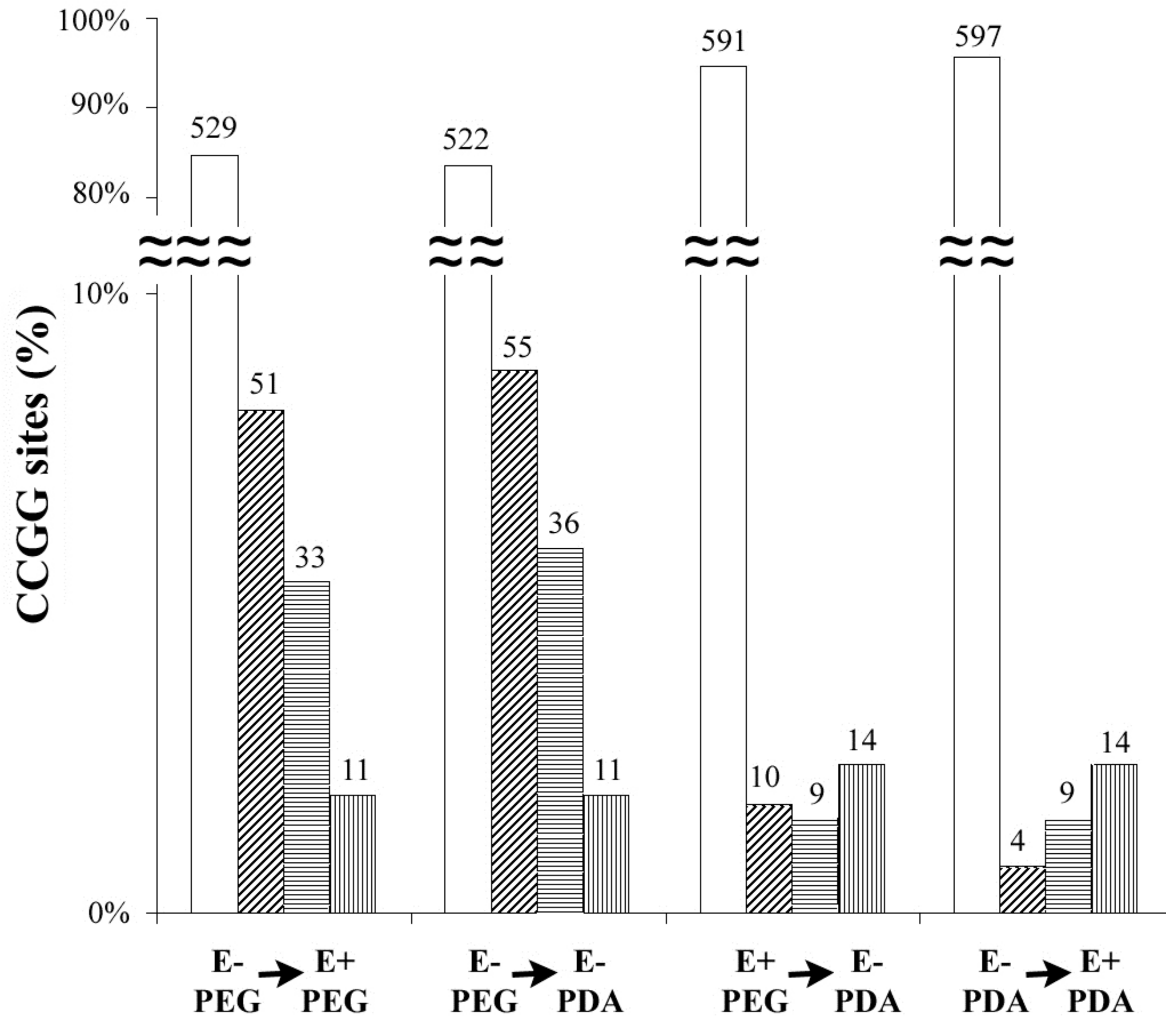


Fig. 5.4 Methylation and demethylation events between treatments. Changes in genomic DNA methylation patterns between pairs of treatments are represented in terms of frequency (numbers above bars) and percent occurrence of nonpolymorphic (empty bars □), methylation (diagonal cross-hatching ◪), demethylation (horizontal cross-hatching ⊟) and uninformative (vertical cross-hatching ◫) events. The four treatments were: endophyte-free (E-) and subjected to drought stress (potato dextrose agar [PDA] amended with 8% polyethylene glycol [PEG]); SMCD 2206 colonized (E+) and drought-stressed; uncolonized (E-) and stress-free; SMCD 2206 treated (E+) and unstressed.

When the methylation patterns of DNA from drought-stressed, (E-) seedlings were compared to those of (E-) seedlings germinated under control no change in methylation state was found at 83.7%, corresponding to 522 of 624 CCGG sites (Fig. 5.4). In addition, 55 (8.8% of sites), 36 (5.8%) and 11 sites (1.8%) experienced methylation, demethylation or uninformative methylation changes, respectively (Fig. 5.4). Sites that did not change between the two treatments included 254 unmethylated, 202 semi-methylated and 66 methylated entities. One or more methyl groups were added to unmethylated sites, giving rise to 42 semi-methylated and eight methylated sequences. Semi-methylated CCGG sequences became hyper-methylated on seven occasions. One hyper-methylated, 14 methylated and 14 semi-methylated sites were demethylated to produce unmethylated sites. Demethylation also gave rise to semi-methylated sites from one methylated and six hyper-methylated locations. Hyper-methylated sites became methylated in two locations and remained hyper-methylated in one instance, while 8 methylated sites became hyper-methylated.

Non-polymorphic sites made up 94.7% of sites (591 out of 624) discernible in DNA from drought-stressed (E+) seedlings and their unstressed (E-) counterparts (Fig. 5.4). Of the remaining 33 sites, 10 underwent methylation (1.6% of sites), nine experienced demethylation (1.4% of sites) and 14 were not informative (2.2% of sites). Sites that were not polymorphic consisted of 277 unmethylated, 238 semi-methylated and 76 methylated CCGG sequences. All 10 observed methylations took the form of unmethylated sites becoming semi-methylated. Methyl groups were shed from six semi-methylated sites, producing unmethylated sites. In addition, three hyper-methylated sites were demethylated to a semi-methylated state. Finally, two methylated sites became hyper-methylated and 12 sites remained hyper-methylated.

When DNA from stress-free (E-) seedlings was compared to that of (E+) plants germinated in the same conditions, 597 out of 624 sites (95.7%) were not polymorphic (Fig 5.4). However, 27 polymorphic sequences were observed. Of these, methylations accounted for 0.6% (four sites) of all sites. Demethylations made up 1.4% (nine sites) and uninformative changes occurred at 14 sites (2.2% of sites; Fig. 5.4). Of the 597 monomorphic sites, 279 were unmethylated, 243 semi-methylated and 75 methylated. Methylation events consisted of four unmethylated sites becoming semi-methylated. Loss of

methylation took the form of eight semi-methylated and one methylated site becoming unmethylated. Uninformative events included two hyper-methylated locations becoming methylated and 12 hyper-methylated sites remaining hyper-methylated respectively.

5.5.3 Sequence analysis of polymorphic fragments

Sequence data was obtained from five of the most prominent polymorphic bands (Table 5.3). E-values indicate the probability that the percent similarity values occurred by chance. Hence, smaller e-values indicate a more meaningful similarity. One of these sequences had 100% similarity to a cytochrome p450 expressed sequence tag (EST) from wheat, with an e-value of 0.00. Two sequences were 95% (with an e-value of 6×10^{-62}) and 93% (with an e-value of 8×10^{-64}) similar to Copia and Gyspy type retrotransposons from wheat, respectively. Another of the sequences displayed 98% similarity to a CACTA type wheat transposon. The associated e-value was 3×10^{-43}. Finally, the shortest of sequences had no similarity to sequences in publicly available databases.

5.6 Discussion

The fungal endophyte SMCD 2206 is able to confer drought tolerance to germinating wheat seedlings (Chapter 3). Given that drought stress diminishes wheat yield by up to 50% or more (Duggan et al. 2000; Bagci et al. 2007), methods for offsetting these losses are of practical significance. Increased wheat stress resistance is measurable in terms of improved seed germination (Chapter 3) or mycovitalism (Vujanovic and Vujanovic 2007). Endophyte-free seedlings subjected to drought stress experienced more detrimental effects (such as reduced or slower germination) because of stress than their inoculated counterparts did. The former tended to have lower levels of genomic DNA methylation (Fig. 5.3). This is consistent with the impact of drought on DNA methylation levels in rice (Wang et al. 2011a) as well as with the epigenetic changes induced in plants by other abiotic stress factors such as cold, heavy metals, aluminum toxicity, and salt (Lizal and Relichova 2001; Alina et al. 2004; Choi and Sano 2007; Lu et al. 2007; Zhong et al. 2009; Wang et al. 2011b; Li et al. 2012). However, this study is novel in that it deals with the ability of fungal endophytes to modulate plant epigenetic responses to stress. Increased DNA methylation inhibits gene expression (Harrisson et al. 1971; Christman et al. 1977; Hepburn et al. 1983;

Table 5.3 Methylation state, characteristics and sequence similarities of five polymorphic bands isolated from methyl-sensitive amplified polymorphism (MSAP) polyacrylamide gels. The symbols "E-" and "E+" denote DNA extracted from SMCD 2206 treated and untreated plants, respectively. The Roman numerals "I", "II" and "III" indicate that a band was unmethylated, semi-methylated or methylated, respectively.

Primer pairs	Treatment and Methylation state				Fragment size (bp)	Accession numbers of similar DNA sequences	E-value	% identity	Similar proteins
	E- PEG	E+ PEG	E- PDA	E+ PDA					
E2 / HM2, band 25	I	II	II	II	788	GR302786 EST WRIC_402 cDNA library of a compatible interaction between stripe rust (*Puccinia striiformis*) and wheat *Triticum aestivum* cDNA 5' similar to cytochrome P450 like_TBP, mRNA sequence (PlantGDB)	0.00	100%	BAD26579.1 cytochrome P450 like_TBP [*Citrullus lanatus*]
E2 / HM2, band 32	II	I	I	I	639	FN564434 PLN *Triticum aestivum* chromosome 3B-specific BAC library, contig ctg0954b (NCBI nucleotide collection (nr/nt))	6 E-62	95%	Aligns with retrotransposon, Copia type
E3 / HM2, band 47	I	II	II	II	188	NONE	N/A	N/A	NONE
E4 / HM4, band 23	III	I	I	I	725	FN564430 PLN *Triticum aestivum* chromosome 3B-specific BAC library, contig ctg0464b (NCBI nucleotide collection (nr/nt))	3 E-43	98%	Aligns with transposon, CACTA type
E5 / HM3, band 44	III	I	I	I	205	FN564428.1 *Triticum aestivum* chromosome 3B-specific BAC library, contig ctg0091b (NCBI nucleotide collection (nr/nt))	8 E-64	93%	Aligns with retrotransposon, Gypsy type

Finnegan et al. 1998). Thus, demethylation in seedlings exposed to stress implies that elevated gene expression is associated with abiotic stress. Although the overall trend is towards loss of methylation with increasing plant stress (Fig. 5.3 and 5.4) the reverse process took place between inoculated and uninoculated drought stressed seedlings at 7.9% of sites (Fig. 5.4). This allows us to infer that a process more complex than genome-wide elevated gene expression is taking place in response to stress. Instead, certain genes may be up regulated, while others are down regulated.

Patterns of DNA methylation in seedlings exposed to drought and colonized by SMCD 2206 more closely resemble those of their non-stressed counterparts (with or without endophyte treatment) than endophyte-free, drought-stressed seedlings (Fig. 5.3 and 5.4). This is true in terms of the percent of unmethylated, semi-methylated and methylated sites (Fig. 5.3) as well as the percent of CCGG sequences that are monomorphic between mycobiont-colonized, drought-stressed seedlings and uncolonized stress-free control plants (Fig. 5.4). Specifically, while SMCD 2206-treated drought stressed plants share methylation patterns with the control at 91.3% of sites, uncolonized, moisture-deprived seedlings are only 78.6% similar to the control. In addition, the pattern of methylation and demethylation was quite divergent between endophyte-free and endophyte-colonized seedlings germinated on media containing 8% PEG (Fig. 5.4). Thus, it appears that SMCD 2206 protection of wheat seedlings from the most severely detrimental impacts of drought stress may coincide with alterations in plant gene expression to mimic that exhibited by non-stressed seedlings.

The DNA sequences extracted from five of the most conspicuous polymorphic bands were similar to a cytochrome p450 expressed sequence tag (EST), as well as two retrotransposons and one transposon in wheat (Table 5.3). The fact that expression levels of a cytochrome p450 gene appears to be epigenetically modulated is consistent with reports in the literature linking cytochrome p450s to drought responses in plants. For example, Lu et al. (2010) identified several cytochrome p450 genes as contributing to tolerance for drought in maize. Consistently, Kondo et al. (2010) showed that an increase in abscisic acid (ABA) resulted from the application of a cytochrome p450 inhibitor to drought stressed citrus trees. The band with high sequence similarity to cytochrome p450 is unmethylated in endophyte-free, drought stressed seedlings, but semi-methylated in all other treatments. This suggests that increased stress is related to high expression levels of cytochrome p450.

Because cytochrome p450s are enzymes involved in oxidation reactions in plants (Zimmerlin et al. 1992; Helvig et al. 1996; reviewed in Coon 2005) and that oxidation can accompany stress, these results are logical. One specific reaction catalyzed by plant cytochrome p450s (belonging to the CYP707A family) is the catabolism of ABA via the ABA 8'-hydroxylation pathway (Kushiro et al. 2004; Saito et al. 2004). Consistent with my results, both Kushiro et al. (2004) and Saito et al. (2004) found that CYP707A cytochrome p450 expression was upregulated under drought. Thus, my results imply that SMCD 2206 mediated drought tolerance coincides with an increase in the expression of a cytochrome p450 gene and, potentially, reduction of oxidative stress and/or modulation of ABA levels. However, plant cytochrome p450s also catalyze a wide range of other oxidation reactions (reviewed in Coon 2005). My data do not clearly point to a specific cytochrome p450. Hence, future exploration of the gene and/or protein expression levels of cytochrome p450s are warranted in drought stressed wheat, with and without SMCD 2206.

Mobile genomic elements (TEs) such as transposons and retrotransposons are associated with increased epigenetic diversity in plants, frequently expressed in response to stress (McClintock 1984; Peterson 1985; reviewed in Grandbastien 2004; Mirouze and Paszkowski 2011; McCue et al. 2012). Elevated methylation of DNA is linked to reduced transposon activity and mobility, hence increasing genome stability (Martin et al. 1989; Wang et al. 1996). The differential methylation of a DNA sequence similar to a Gypsy type retrotransposon (Table 5.3) is comparable to the findings Mason et al. (2008) in which the methylations status and expression levels of an MSAP fragment with sequence similarity to a TE of the same classification was altered in tomatoes subjected to virus infection compared to mock-inoculated controls. The similarity of three of the five polymorphic bands excised and sequenced to TEs suggests that endophyte colonization has an impact of these elements of the wheat genome. All three TEs underwent demethylation when plant stress levels decreased (Table 5.3), suggesting that stress in the absence of endophyte inoculation could correspond to changes in TE mobility.

The mechanism(s) by which fungal endophytes are able to bring about host DNA methylation changes have yet to be elucidated. Demethylation can be active or passive. Active demethylation takes place independent of DNA replication, whereas passive demethylation involves methyl groups being lost during replication (Jost et al. 2001; Kress

et al. 2001; Zhu et al. 2007). Because DNA replication is often reduced in plant tissues challenged with environmental stress, DNA demethylation likely occurs via active processes (Steward et al. 2002). The DNA glycosylase, belonging to the DEMETER (DME) family, is involved in active demethylation in *Arabidopsis thaliana* (Agius et al. 2006; Gehring et al. 2006; Morales-Ruiz et al. 2006; Penterman et al. 2007). A member of the DME family, REPRESSOR OF SILENCING 1 (ROS1), is a 5-methylcytosine DNA glycolase/lyase, which is expressed ubiquitously in plant tissues (Zhu et al. 2007). It is possible that SMCD 2206 is able to influence the activity or specificity of ROS1.

The ability of SMCD 2206 to alter the epigenetics of its host through DNA methylation or demethylation could offer insight into how other mycobionts, such as *Piriformospora indica* (Wal1er et al. 2005; Fakhro et al. 2010), *Trichoderma hamatum* (Bae et al. 2009), *Curvularia protuberata* (Márquez et al. 2007) and other SMCD organisms (Chapters 3 and 4), are able to interact with host plants and enhance stress tolerance. The endophyte *P. indica* has been shown to “reprogram” its host, thereby increasing reactive oxygen species (ROS) scavenging (Waller et al. 2005; Baltruschat et al. 2008). This “reprogramming” could occur via fungus-mediated changes in plant DNA methylation. Future research in this area might explore the sequence and putative functions of the genes encoded by the sequences whose methylation state is altered in endophyte-colonized plants.

When the results of the current study are considered in conjunction with those presented in Chapter 3, in which SMCD 2206 enhanced wheat seed germination by altering the parameters of the hydrothermal model of germination (Gummerson 1986; Bradford 2002; Köchy and Tielbörger 2007), it seems reasonable to hypothesize that there is a connection between epigenetic control of plant genes and the hydrothermal parameters of seed germination. Given that successful seed germination is an indispensable pre-requisite for all future plant development stages (Baskin and Baskin 2004; Finch-Savage and Leubner-Metzger 2006), a more in-depth understanding of mycovitalism, defined as endophyte-mediated improvements in seed germination, is of great interest. Mycovitalism could prove valuable in the enhancement of crop performance in drought stressed environments (Vujanovic 2007), which are likely to become more problematic as climatic change progresses (IPCC 2007).

Given that Waller et al. (2005), Márquez et al. (2007) and Rodríguez et al. (2008) have all documented the capacity of endophytic fungi to promote abiotic stress tolerance in mature plants, investigation of the epigenetic status of adult plants is merited. In addition, exploration of the DNA methylation patterns in a second generation of endophyte-free wheat whose parents were colonized by SMCD 2206 seems worthwhile in light of evidence that epigenetic modifications to plant DNA methylation are heritable to subsequence generations (Kakutani et al. 1999; Vaughn et al. 2007; Johannes et al. 2009), even in the absence of the stress (Verhoeven et al. 2010).

5.7 Conclusions

In Chapter 3, I found that the fungal endophyte SMCD 2206 was able to enhance germination of wheat seeds exposed to drought stress. The data presented in this chapter shows that colonization with SMCD 2206 coincides with altered DNA methylation patterns of drought stressed wheat seedlings such that they resemble those of unstressed plants. This suggests that SMCD 2206 colonization may epigenetically alter wheat to function as though drought is absent. Future research on the specific genes or mobile genomic elements being regulated in wheat could facilitate a greater understanding of plant-endophyte interactions and lead to more effective use of endophytic fungi in agriculture. Based on the findings of this study, initial target genes could include those encoding enzymes involved in oxidative reactions, such a cytochrome p450s.

5.8 Connection to the next study

The DNA methylation work presented in Chapter 5 suggests that colonization with the fungal endophyte SMCD 2206 coincides with epigenetic alterations in wheat. Because epigenetic changes can be passed on from one generation of plants to the next, I wished to gain insight into the stress tolerance of a second, endophyte-free generation of wheat whose parents were inoculated with SMCD 2206.

6.0 EPIGENETIC INHERITANCE OF ENDOPHYTE-DERIVED HEAT AND DROUGHT TOLERANCE TO A SECOND ENDOPHYTE-FREE GENERATION OF WHEAT

6.1 Abstract

Heat and drought are major problems for world agricultural production that are predicted to increase in frequency and severity due to climate change. Thus, research into methods for offsetting yield losses due to these factors is imperative. Because endophytic fungi are able to increase plant tolerance for abiotic stress and deleterious environmental conditions result in heritable epigenetic changes in plants, leading to stress adaptation, I hypothesized that a fungal endophyte would induce intergenerational epigenetic modifications, leading to elevated heat and drought resistance. I compared the performance of endophyte-free second generation (F_2) heat or drought stressed wheat – whose first generation (F_1) parents were inoculated with endophyte strain SMCD 2206 and exposed to stress – to uncolonized F_1 plants grown under the same conditions, hypothesizing that the former would outperform the latter. Plants were evaluated in terms of photosynthetic stress (PS), average seed weight (ASW), total seed weight (TSW) and water use efficiency (WUE). Under heat stress, F_2 wheat with endophyte-treated and heat-stressed parents performed best – closely followed by inoculated F_1 wheat – outdoing the F_1 uncolonized heat-challenged plants. Endophyte-treated F_1 plants were more tolerant of drought than any of the F_2 plants. However, F_2 plants exhibited greater drought resistance than the endophyte-free F_1 control. Epigenetic inheritance of endosymbiont-induced heat or drought tolerance is hitherto undocumented and offers unique insights into endophyte-plant interactions. It also has the potential alleviate the problem of wheat yield loses and to accelerate adaptation to abiotic stress in sustainable agriculture.

6.2 Introduction

Adverse environmental conditions, such as high temperatures and lack of moisture, are likely to worsen as climatic change intensifies (IPCC 2007), posing an ever-increasing problem for agricultural production. From a pragmatic human perspective, this difficulty is particularly acute when it results in reduced yield in staple food crops such as wheat. Thus, developing appropriate methods for ameliorating the negative impacts of these abiotic stressors is of both scientific and practical interest. The worldwide demand for wheat is projected to increase while the availability of arable land and favourable climatic conditions is likely to decrease (Rosegrant et al. 2001). Currently, agricultural and scientific enhancements of plant adaptation to biotic, and, to a lesser degree, abiotic stress are being achieved largely through plant breeding, bringing about incremental improvements in yield (Richards 2006; Graybosch and Peterson 2009). However, wheat breeding tends to focus on increasing yield under optimal conditions or on disease resistance (Reynolds and Borlaug 2006), and has made only limited progress in improving grain production under heat or drought conditions (Ceccarelli and Grando 1996). Hence, other approaches, such as those studied and presented in this paper, have the potential to complement and enhance gains made in plant breeding, pointing a way out of the quandary outline above.

One such alternate strategy is the use of symbiotic microorganisms, such as endophytic fungi, to enhance plants' innate ability to adapt to stress. Recently, a range of fungal endophytes have been shown to be capable of increasing plant tolerance for abiotic stress (Márquez et al. 2007; Rodriguez et al. 2008; Sun et al. 2010; Khan et al. 2012a and b; Chapter 3). In particular, the Ascomycoteous mycobiont available in the Saskatchewan Microbial Collection Database (SMCD) and assigned the name SMCD 2206 improves wheat performance under heat or drought. This stress resistance occurs in germinating seeds *in vitro* (Chapter 3), likely through mycovitalism, or myco-mediated augmentation of seed germination parameters (Vujanovic and Vujanovic 2007). While the mechanisms by which fungal endophytes interact with their hosts are incompletely understood, studies involving the Basidiomycoteous endophyte *Piriformospora indica* suggest that mycobionts can reprogram host plants by influencing stress-induced epigenetic modifications (Waller et al. 2005 and 2008; Baltruschat et al. 2008), variations in gene expression without an alteration in underlining nucleotide sequence. The possibility that a similar process may be involved

between plants and Ascomycoteous endosymbionts, such as SMCD 2206, merits further investigation.

Tolerance for abiotic stressors can be passed on to subsequent generations of plants (reviewed by Boyko and Kovalchuk 2008). Hence, it is of interest to ascertain if the same is true of endophyte-derived stress tolerance. Evaluation of the ability of a second, endophyte-free generation of plants to withstand heat or drought would be of considerable theoretical interest, with potential real-world benefits. It could uncover evidence of a novel mode of plant-endophyte interaction, involving mycobiont-induced heritable epigenetic modifications in host plants. Furthermore, it would facilitate sustainable agriculture in that the seeds produced by heat or drought stressed endophyte-colonized plants could be saved and planted in environments likely to experience high temperature and/or moisture deficit. Because SMCD 2206 differs from some Ascomycoteous endophytes (Siegel et al. 1984) in that it does not colonize the reproductive organs of its host and is therefore not present in second-generation plants. Thus, any impacts of parental stress and/or endophyte-colonization on the uninoculated offspring of colonized plants are reasonably attributable to inherited epigenetic modifications rather than the vertical transmission of the fungal endosymbiont. While other studies examine inheritance of stress tolerance (Akimoto et al. 2007; Lang-Mladek et al. 2010) or epigenetic changes induced by biotic or abiotic stress in plants (Molinier et al. 2006; Verhoeven et al. 2010), this research is unique in exploring the impact of endophytic fungi on inter-generational, epigenetic adaptation to environmental perturbations.

6.3 Hypothesises and objectives

I hypothesized that endophyte-colonization of wheat would enhance epigenetic adaptation to heat or drought stress and that such adaptations would be passed on to subsequent generations of plants. Photochemical capacity of photosystem II is a measure of plant photosynthetic stress (PS) and accurately reflects drought stress in wheat (Paknejad et al. 2007). Clearly, production of a large, good-quality grain yield is the primary objective in growing wheat and hence a critical measure of performance. Carbon isotope discrimination (Δ) is both an indicator of water use efficiency (WUE) and positively correlated with grain yield (Richards 2006; Dong et al. 2011). When considered in concert, PS, grain yield and

WUE give an accurate and complete picture of wheat performance under abiotic stress. To test my hypothesis, I grew the offspring of endophyte-colonized and endophyte-free stressed plants, as well as those of endophyte-colonized and endophyte-free non-stressed plants to maturity under stressed and stress-free conditions. I evaluated the performance of colonized and uncolonized first generation (F_1) plants and endophyte-free second generation (F_2) plants under heat, drought and control (non-stress) conditions. Plant performance was measured in terms of PS, average seed weight (ASW), total seed weight (TSW), and Δ in the case of drought stressed plants.

6.4 Materials and methods

6.4.1 Experimental design

The overall experimental design is summarized in Figure 6.1. Two six-month experiments were carried out. The first experiment used parental (P_1) seeds to produce a first generation of plants (F_1). Experiment two used the F_1 seeds produced in experiment one to grow a second generation (F_2). Half of the F_1 plants were inoculated with the fungal endophyte SMCD 2206 (E+) and half were not (E-). All F_2 seeds were uninoculated (E-). In both experiments, pots containing seeds were placed in either a phytotron *Conviron* PGR15 growth chamber (Controlled Environments Ltd., Winnipeg, MB) for heat stress and the associated stress-free control, or in a greenhouse for drought stress and corresponding well-watered control treatments.

Experiment one consisted of 10 pots per treatment. In both the phytotron and the greenhouse, the four treatments were: (1) stressed (E+) plants, (2) stressed (E-) plants, (3) unstressed (E+) plants and (4) unstressed (E-) plants. In experiment two, there were five pots in each treatment, except for F_1 greenhouse-grown plants, for which there were 10 pots per treatment. The sample size was smaller in experiment two than in experiment one due to the larger number of treatments, which limited the space available for each treatment. The treatments used in both the phytotron and the greenhouse were stressed and stress-free for each of the four treatments employed in experiment one, leading to a total of eight treatments. In addition, the four treatments outlined for the first experiment were repeated in

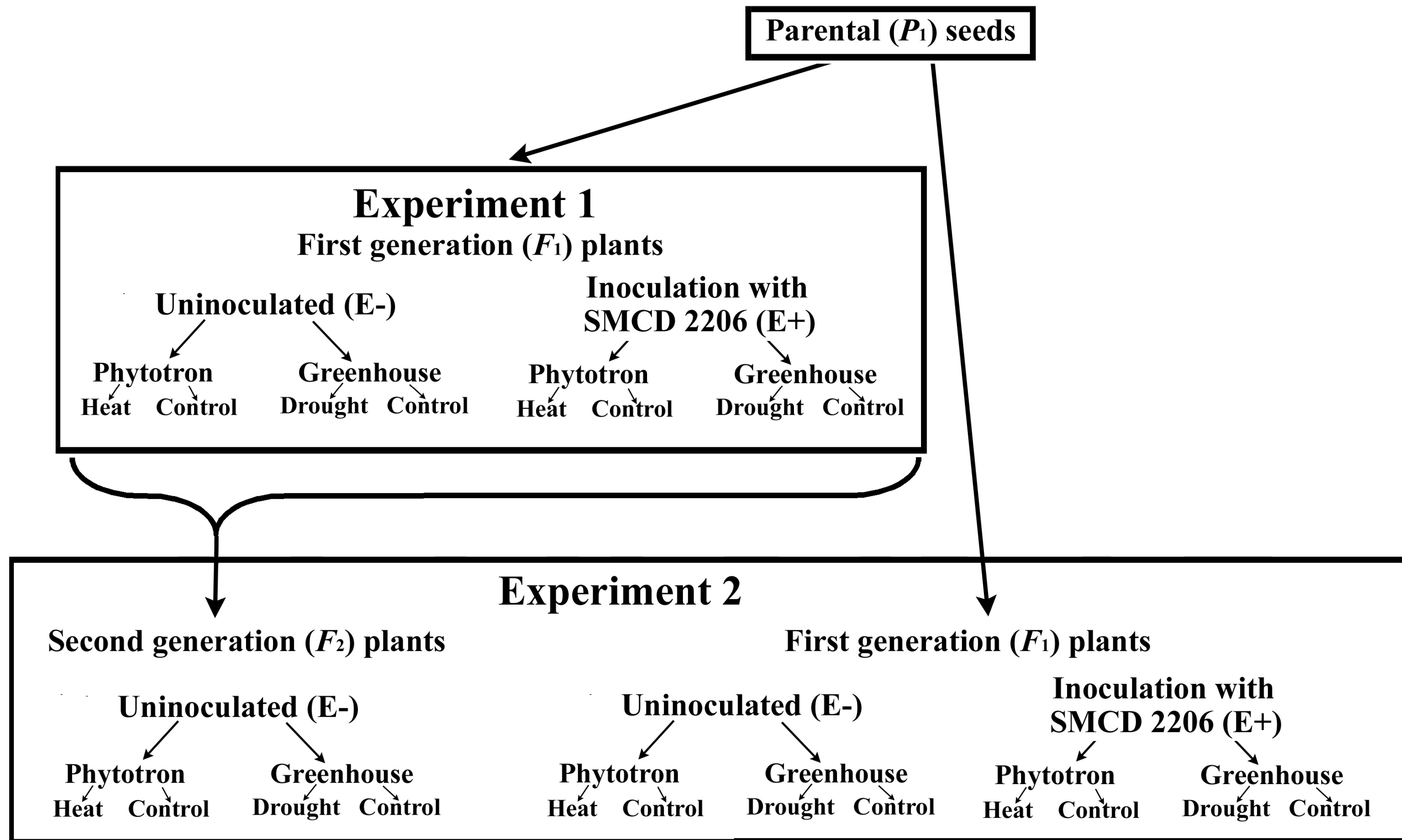


Fig. 6.1 Experimental design.

the second experiment. The treatments in the phytotron and greenhouse experiments are shown graphically in Figures 6.2 and 6.3, respectively. The pots were arranged in a randomized block design. The locations of pots were changed approximately every 14 d. Data on all parameters were obtained from experiment two, with the exception carbon isotope discrimination (Δ) values reported for F_1 plants. The Δ values were measured from experiment one.

6.4.2 Plant and fungal material

As described in Chapter 3, Paterson Grain produced the P_1 seeds of AC Avonlea durum wheat. These seeds were surface-sterilized using 95% ethanol, sterile distilled water and 5% sodium hypochlorite. The mitosporic Ascomycoteous fungal isolate employed in this study was isolated from Saskatchewan field-grown durum wheat (*Triticum turgidum* L.) roots. This fungus was isolated and characterized by Drs. Vujanovic and Germida and deposited in the Saskatchewan Microbial Collection Database (SMCD) and referred to as SMCD 2206. This mycobiont has been shown to be compatible with wheat and capable of elevating heat and drought stress tolerance *in vitro* (Chapter 3). The fungal organism was cultivated at 23 °C in darkness on potato dextrose agar (PDA) for a minimum of 3 d before experimental use.

Wheat seeds were inoculated with SMCD 2206 in pots using a method adapted from Abdellatif et al. (2010). Briefly, a 5 mm^2 agar plug was excised from the margin of a growing fungal colony and deposited fungal-side down in the middle of a pot filled with autoclaved Sunshine mix 4. Five seeds were arranged around this plug and submerged under 3.5-4.0 cm of the same potting blend. After F_1 seedlings emerged in experiment one, the surface of the potting mix was covered in a 5 mm layer of 1 mm diameter plastic beads (The Science Source Co., 299 Atlantic Highway, Waldoboro, ME, 04572, USA) with the aim of impeding the spread of SMCD 2206 to the endophyte-free pots.

6.4.3 Heat stress

Temperature stress was applied in a *Conviron* controlled environment reach-in growth chamber, measuring 1.22 x 2.44 m and located in the phytotron at University of Saskatchewan, College of Agriculture and Bioresources. Seedlings that were to be heat stressed were grown under non-stress (control) temperature conditions for the first 10 to 14 d after planting, or until they reached Zadoks stage 12 (Zadoks et al. 1974). These control

conditions consisted of 8 h at 16 ºC and 1 h at 20 ºC (both in darkness), followed by 4 h at 20 ºC, 6 h at 24 ºC, 4 h at 20 ºC (all while exposed to light) and 1 h in darkness at 20 ºC. Over a 7 d period, seedlings were acclimated to heat stress using a light and temperature regime consisting of 10 h at 18 ºC (in darkness), succeeded by 14 h of light exposure at 20, 22, 24, 26, 28 and 30 ºC for 1 h each. Plants were then held for 2 h at a peak temperature of 32 ºC. Next, the temperature was ramped down step-wise by 2 ºC each hour. After acclimation, full heat stress was applied. This treatment consisted of 10 h at 20 ºC (in darkness), and 14 h in daylight at 25 and 30 ºC (2 h each), followed by 6 h at a maximum temperature of 36 ºC. Subsequently, the temperature was lowered to 30 ºC, and then to 25 ºC (2 h each). The conditions applied to control and heat stressed plants were intended to resemble those of a typical and extremely hot Canadian Prairie summer growing season, respectively (Madsood et al. 2005; Grant et al. 2009). A combination of 60W incandescent and T12VHO 4200K fluorescent bulbs generated light with a mean intensity of 393 μmol m^2 s^{-1} (Baird et al. 2010). Potential effects of variability in the chamber environment were minimized through utilization of a randomized block design and via the frequent movement of plants. Relative humidities were set for 50% during light exposure and 100% during darkness. Heat stressed and control plants in the phytotron were water to 100% water holding capacity (WHC) every 2 d.

6.4.4 Drought stress

The College of Agriculture and Bioresources Greenhouse was used for drought application. Relative humidity varied between 45 and 100%, with the lower and higher values generally being recording in the late afternoon and overnight, respectively. Daily temperature highs reached 20 to 35 ºC (with daily maxima in the high 20s and low 30s being most common) and dropped to 16 to 20 ºC overnight. Temperatures tended to vary less between day and night in the second experiment, as compared to the first. Also, relative humidities were generally lower in the second experiment. Light was provided by either sunlight alone, or in combination with 1000 watt high-pressure sodium light bulbs, hung from the roof approximately 2 m over the plants.

In both experiments one and two, drought stressed and control plants were grown at 40% and 100% water capacity (WHC), respectively. These moisture levels were consistent with the drought stress and control moisture levels applied by Gunes et al. (2007) and Zhao

et al. (2007), respectively, to greenhouse-grown wheat. The plants to be subjected to drought were grown under control conditions (100% WHC) for the first 10 to 14 d after planting, until they attained Zadoks stage 12 (Zadoks et al. 1974). Next, these plants were grown at 70% WHC for 7 d in order to permit them to acclimate to moisture deficit. Subsequent to acclimation, the drought stressed plants were maintained at 40% WHC.

During experiment one, a randomly selected sub-set of pots in each treatment (consisting of at least 30 to 50% of all pots in the treatment), plus the soil, plants and water it contained, were weighed every 1 to 5 d (with waterings 2 d apart being the most common) and an average weight was calculated. This average weight was used to calculate the amount of water to be added. Hence, on most days, the same amount of water was added to each drought stressed pot. On a few days some pots had not lost enough water to bring them below 40% WHC. On such days, all drought stressed pots were weighed and watered until the target weight was reached. In the second experiment, all pots subjected to drought – rather than the subset used in the initial experiment – were weighed every 1 to 5 d (with waterings separated by 2 d being the most common) and water was added until the pot reached the desired weight.

6.4.5 Photosynthetic stress

Photosynthetic stress (PS) was evaluated in plants subjected to heat, drought or control conditions which had attained Zadoks stages 31 to 37 (Zadoks et al. 1974). A plastic clip was applied to the second-youngest leaf on the five tallest stems in each pot for 20 min, inducing dark adaptation. Following this, a portable OPTI-SCIENCES OS-30P Chlorophyll Fluorometer (8 Winn Avenue, Hudson, NH, 03051, USA) was employed to measure PS based on maximal photochemical efficiency. A decrease in this trait is indicative of greater damage to photosystem II (Farquhar et al. 1989b). The ratio $F\text{v}/F\text{m}$ – where $F\text{m}$ and $F\text{v}$ denote maximum and variable dark-adapted fluorescence, respectively – is used to calculate maximal photochemical efficiency. $F\text{v}$ is calculated from the equation

$$F\text{v} = F\text{m} - F_0, \qquad \text{(Equation 6.1)}$$

in which F_0 represents dark-adapted fluorescence. Increased abiotic stress is linked to decreased maximal photochemical efficiency (Karavata and Manetas 1999).

6.4.6 Total seed weight and average seed weight

Spikes were manually harvested once they reached maturity. In a laboratory setting, the seeds were extracted from the spikes, air dried (Andoh et al. 2001) and weighed on a Mettler Toledo PG802-S laboratory balance. The seeds produced by all plants in each pot were pooled and weighed. An average was calculated between pots within each treatment to give a total seed weight (TSW) per pot. Average seed weight (ASW) was based on the mean weight per 10 seeds. Each group of 10 seeds was selected at random from all pots within a treatment. When produced under drought or control conditions, ASW values were calculated from 30 or more groups of 10 seeds. All seeds arising from heat stressed wheat were randomly divided into groups of 10 seeds and weighed. This was due to the reduced number of seeds yielded by wheat exposed to temperature stress.

6.4.7 Carbon isotope discrimination

In plants, carbon isotope discrimination (Δ) is positively liked to stomatal conductance (Khan et al. 2007) and negatively correlated with water used efficiency (WUE; Farquhar and Richards 1984). In turn, WUE is usually calculated by dividing plant biomass accrued or seed yield by water used (Sinclair et al. 1984; Hochman et al. 2009). Seeds were prepared for Δ analysis by being dried at 70 °C for a minimum of 4 d and before being finely powdered with a Spex SamplePrep 8000D Mixer/Mill®. From each treatment, three 3.5 ± 0.4 mg thoroughly mixed samples were analysed in a Europa 20:20 continuous flow isotope ratio mass spectrometer linked to a Robo-Prep elemental analyser. Finely powdered field green pea (^{13}CN.PEAGRN), which had been calibrated with International Atomic Energy Association (IAEA), was used as a standard and interspersed between every eight experimental samples. As put forward by Farquhar et al. (1989a) carbon isotope composition ($\delta^{13}C$) was calculated using the equation

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000 \qquad \text{(Equation 6.2)}$$

in which R_{sample} and $R_{standard}$ represented the ratio of ^{13}C to ^{12}C in the sample and in the Peedee belemite carbonate formation standard, respectively. The results of equation 6.2 were expressed in units of per mil (‰). To determine Δ, the equation

$$\Delta = (\delta_a - \delta_p)/(1 + \delta_p), \qquad \text{(Equation 6.3)}$$

was employed. In equation 6.3, δ_a and δ_p indicate the carbon isotope composition of the atmosphere and the plant sample, respectively. The value of δ_a was assumed to be –8‰ (Johnson et al. 1990).

6.4.8 Statistical analysis

In order to compare endophyte-colonized (E+) F_1 plants and their endophyte-free (E-) F_1 offspring in each treatment (heat, drought and controls) to their (E-) F_1 counterparts in terms of each parameter assessed – PS, Δ, ASW and TSW per pot – an analysis of variance (ANOVA) was carried out. A post-hoc Fischer's least significant difference (LSD) test followed each ANOVA. P-values less than alpha levels of 0.05 or 0.01 were considered significant or highly significant, respectively. The software package SPSS Inc 2011 was used for all statistical tests.

6.5 Results

Data is presented for plants grown in the phytotron, followed by data obtained from greenhouse-grown plants. For each stress and trait, values were compared to the F_1 endophyte-free (E-) control. All F_2 plants are (E-). The overall results are summarized in Table 6.1. It should be noted that F_1 plants that were (E-) and subjected to heat produced so few viable seeds that they gave rise to no offspring, meaning that no data were available from F_2 plants with heat stressed (E-) F_1 parents.

6.5.1 Phytotron results

6.5.1.1 Photosynthetic stress

In the phytotron, SMCD 2206-colonized (E+) F_1 plants challenged by high temperatures experienced less photosynthetic stress (PS) than the uninoculated (E-), heat stressed F_1 control ($p \leq 0.01$; Fig. 6.2A). The same was true of F_2 heat stressed plants whose parents were (E-) and subjected to elevated temperatures ($p \leq 0.01$; Fig. 6.2A and B). The PS detected in phytotron-grown F_1 and F_2 plants exposed to an optimal temperature regime did not differ from the control, regardless of treatment of (E-) / (E+) parental status ($p > 0.05$; Fig. 6.2A and B).

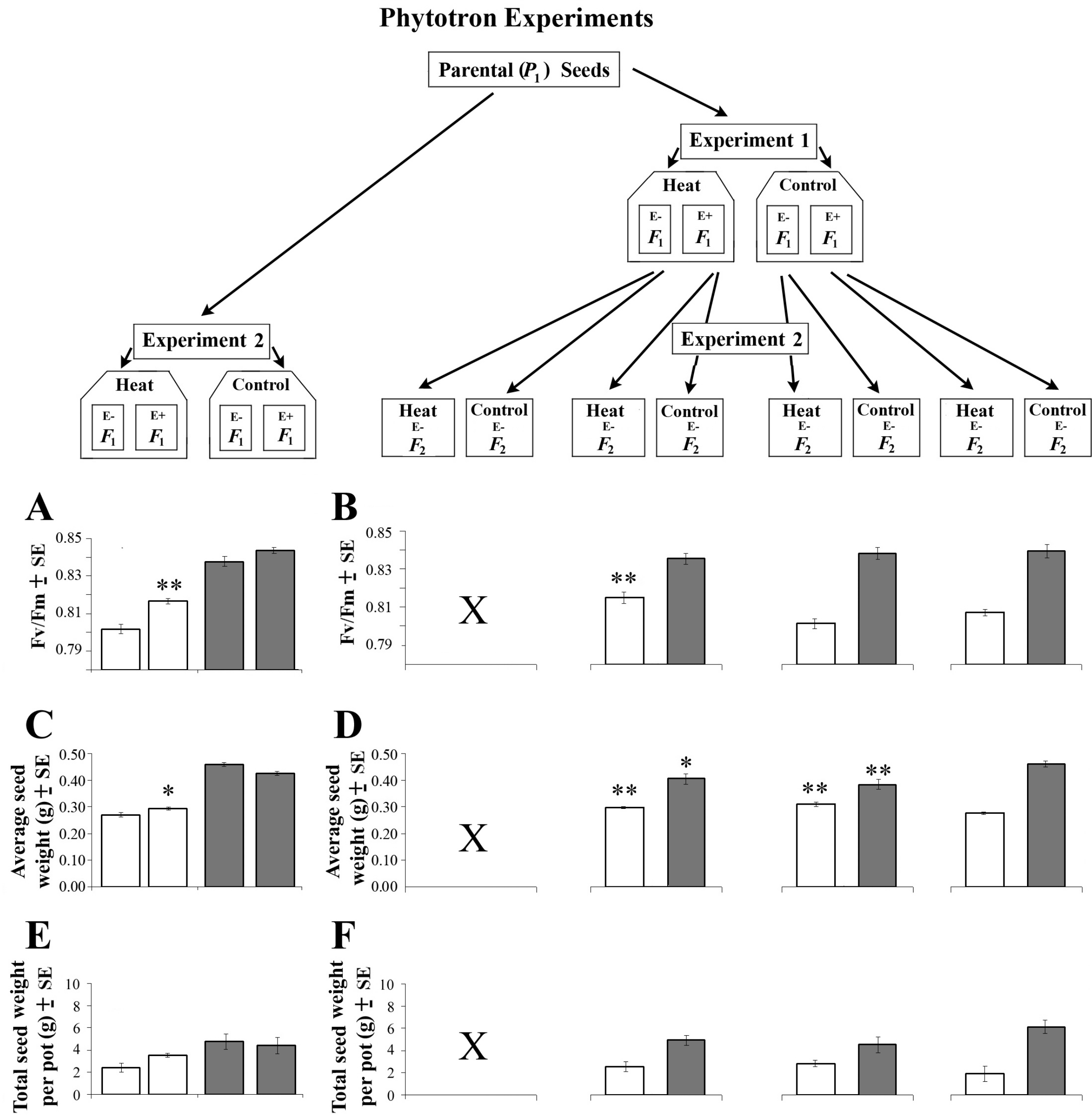


Figure 6.2 Maximal photochemical efficiency (*F*v/*F*m ratio), which is inversely related to photosynthetic stress (PS) (A and B), average seed weight (ASW) per 10 seeds produced (C and D) and total seed weight (TSW) per pot (E and F) produced by F_1 (A and C and E) and F_2 (B and D and F) plants grown under heat stress (white bars [□]) and control conditions (grey bars [■]) in the phytotron in experiment two. The symbols “E-” and “E+” indicate the absence and presence of endophyte colonization, respectively. Statistical comparisons were made only within heat stressed or control plants (bars of the same color), not between heat stressed and control plants (bars of different colors). Bars labeled with one or two asterix (*) are significantly, or highly significantly, different from (E-) F_1 plants grown under the same conditions ($p \leq 0.05$ or $p \leq 0.01$, respectively; ANOVA, followed by post-hoc LSD test). Error bars represent standard error (SE) of the mean.

6.5.1.2 Average seed weight

Heat stressed F_1 plants inoculated with SMCD 2206 (E+) produced seeds with a higher mean weight per 10 seeds, or average seed weight (ASW) than their endophyte-free (E-) counterparts ($p \leq 0.05$; Fig. 6.2C). In addition, F_2 plants subjected to heat stress in the phytotron whose parents were either (E+) heat stressed, or (E-) and not heat stressed had a greater ASW than the (E-) F_1 control ($p \leq 0.01$; Fig. 6.2C and D). However, the ASW did not differ between (E-) F_1 controls and heat stressed F_2 plants whose parents were (E-) but not exposed to high temperatures ($p > 0.05$; Fig. 6.2C and D). In the absence of temperature stress, the ASW associated with (E+) F_1 plants did not differ from their (E-) counterparts ($p > 0.05$; Fig. 6.2C). When grown in the phytotron under non-stress temperatures, F_2 plants arising from (E+) parents did not differ from the (E-) F_1 control in terms of ASW ($p > 0.05$; Fig. 6.2C and D). In contrast, F_2 plants whose parents were (E+) and grown under heat stress, or were (E-) and grown in stress-free conditions, gave rise to seeds with a lower ASW than the F_1 control ($p \leq 0.05$ and $p \leq 0.01$, respectively; Fig. 6.2C and D).

6.5.1.3 Total seed weight

Under heat stress in the phytotron, the total seed weight (TSW) per pot was 44% greater for (E+) F_1 plants than for the (E-) F_1 control ($p = 0.11$; Fig. 6.2E). F_2 plants whose parents were subjected to each of the following three treatments: (E+) and heat stressed; (E-) and grown under control temperatures; or (E+) and grown under control temperatures, had TSWs which did not differ from that of the $F1$ (E-) heat stressed control ($p > 0.30$; Fig. 6.2E and F). In the absence of heat stress, the TWS per pot of phytotron-grown (E+) F_1 plants was not different from that of the (E-) F_1 control ($p > 0.30$; Fig. 6.2E). Unstressed F_2 plants whose parents were heat stressed and (E+) produced a TSW per pot the same as that of the (E-) $F1$ control ($p > 0.30$; Fig. 6.2E and F). The TSW per pot of F_2 wheat whose parents were (E+) and stress-free was no different from that of the (E-) F_1 control ($p > 0.30$; Fig. 6.2E and F). F_2 plants whose (E+) parents grew in the absence of stress had a 38% greater TSW per pot than the (E-) F_1 control ($p = 0.15$; Fig. 6.2E and F).

6.5.2 Greenhouse results

6.5.2.1 Photosynthetic stress

In the greenhouse, F_1 drought stressed (E+) plants displayed less PS than their (E-) counterparts ($p \leq 0.01$; Fig. 6.3A). Similarly, all treatments of F_2 plants suffered less severe PS than did the F_1 control ($p \leq 0.01$; Fig. 6.3A and B). Under well-watered conditions, PS did not differ between (E+) and (E-) F_1 plants, nor between (E-) F_1 plants and any of the F_2 treatments ($p \leq 0.01$; Fig. 6.3A and B).

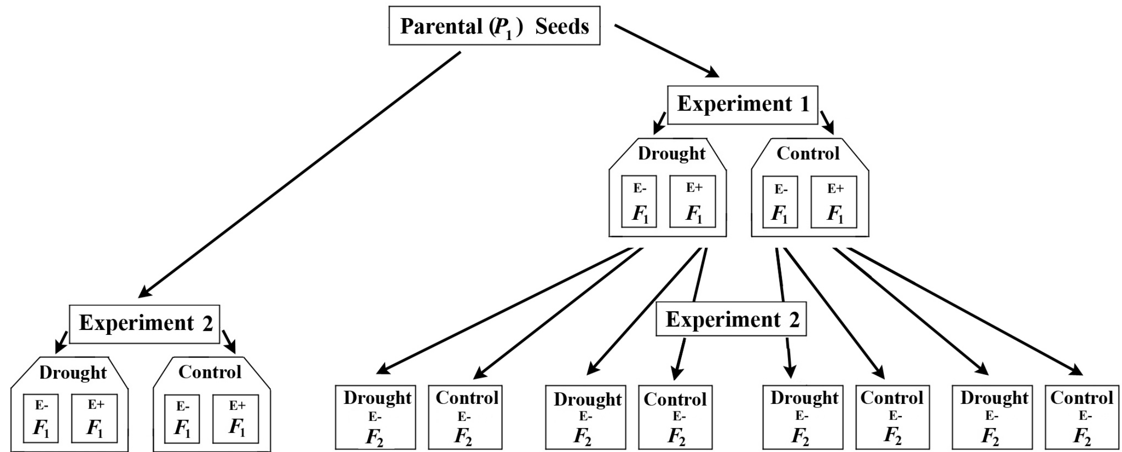
6.5.2.2 Average seed weight

The ASW of (E+) F_1 wheat grown in the greenhouse under drought stress was greater than (E-) F_1 plants ($p \leq 0.01$; Fig. 6.3C). Similarly, greenhouse-grown, well-watered F_1 plants had a greater ASW when (E+) than when (E-) ($p \leq 0.01$; Fig. 6.3C). In contrast, drought stressed F_2 plants, arising from (E+) or (E-) parental plants subjected to drought produced seeds with a lower ASW than the (E-) F_1 control (both $p \leq 0.01$; Fig. 6.3C and D). The same is true of F_2 plants whose (E-) F_1 parents were grown in the absence of drought ($p \leq 0.01$; Fig. 6.3C and D). The ASW did not differ between F_2 plants whose parents were (E+) and well-watered and the (E-) F_1 control ($p > 0.05$; Fig. 6.3C and D). Drought-free F_2 plants did not differ from the (E-) F_1 control in terms of ASW, regardless of the treatment of the maternal plants ($p > 0.05$ for all; Fig. 6.3C and D).

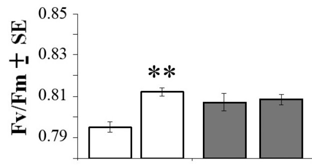
6.5.2.3 Total seed weight

In the greenhouse, none of the drought stressed plants had TSWs that differed significantly from those of the (E-) F_1 control plants. Similarly, the TSWs of well-watered plants were not significantly different from the (E-) F_1 control plants, with the exception of F_2 plants whose parents were drought stressed and (E+) ($p \leq 0.05$; Fig. 6.3 E and F). However, non-statistically significant differences were observed. Drought stressed (E+) F_1 plants had a TSW 23% greater than that of the (E-) F_1 control ($p = 0.15$; Fig. 6.3E). Well-watered (E+) F_1 wheat yielded a TSW per pot 32% greater than their (E-) counterparts ($p = 0.10$; Fig. 6.3E). The TSW per pot of drought challenged F_2 plants were lowest – at 72% of the TSW per pot of drought stressed, (E-) F_1 plants – if F_1 parental plants were both (E-) and subjected to drought ($p = 0.15$; Fig. 6.3E and F). Drought stressed F_2 plants whose

Greenhouse Experiments



A



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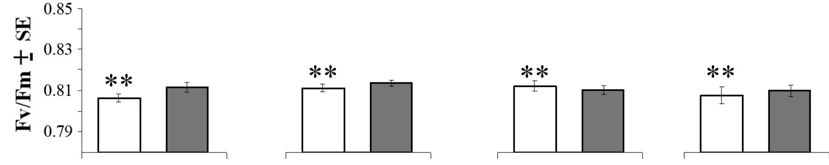

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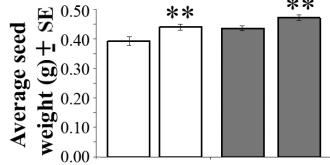

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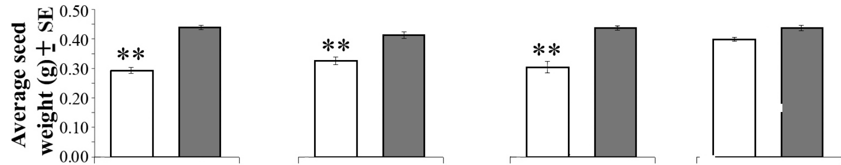
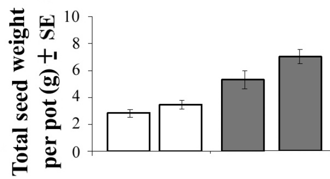


E



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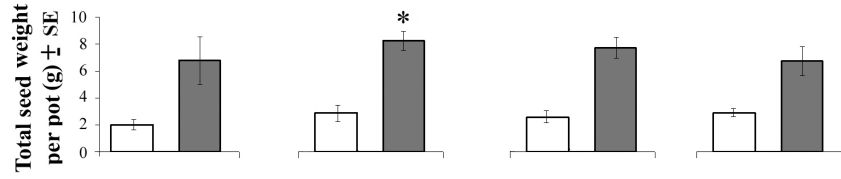


Experiment 1

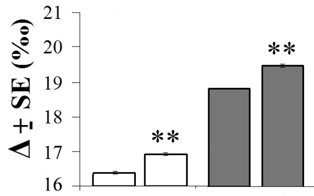
Drought: E- F_1, E+ F_1

Control: E- F_1, E+ F_1

Experiment 2

Drought E- F_2 | Control E- F_2 | Drought E- F_2 | Control E- F_2 | Drought E- F_2 | Control E- F_2 | Drought E- F_2 | Control E- F_2

G



H

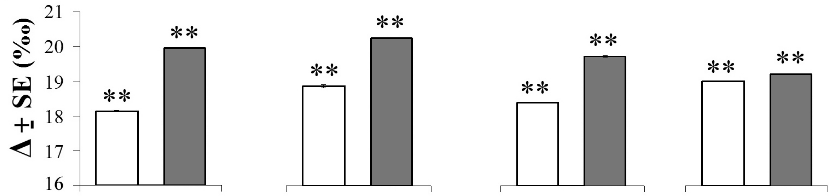


Figure 6.3 Greenhouse results. Maximal photochemical efficiency (*F*v/*F*m ratio), which is inversely related to photosynthetic stress (PS) (A and B), average seed weight (ASW) per 10 seeds produced (C and D) and total seed weight (TSW) per pot (E and F) produced by F_1 (A, C, E and G) and F_2 (B, D, F and H) plants grown under drought stress (white bars [□]) and control conditions (grey bars [■]) in the greenhouse in experiment two. Carbon isotope discrimination (Δ) values (G and H) from seeds produced by F_1 plants grown under drought stress (white bars [□])) and control conditions (grey bars [■]) in the greenhouse in experiment one. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Statistical comparisons were made only within drought stressed or control plants (bars of the same color), not between drought stressed and control plants (bars of different colors). Bars labeled with two asterix (*) are highly significantly different from (E-) F_1 plants grown under the same conditions ($p \leq 0.01$; ANOVA, followed by post-hoc LSD test). Error bars represent standard error (SE) of the mean.

parents were (E+) and exposed to drought, or whose parents were well-watered and (E-) or (E+), had a TSW per pot no different from the F_1 (E-) drought stressed control ($p > 0.30$ for all; Fig. 6.3E and F). Similarly, F_2 plants whose parents were drought stressed and (E-); drought stressed and (E+); well-watered and (E-); or well-watered and (E+), had TSWs per pot that were 27% ($p = 0.25$), 55% ($p \leq 0.05$), 44% ($p = 0.07$) and 26% ($p = 0.27$) greater, respectively, than the F_1 (E-), well-watered control (Fig. 6.3E and F).

6.5.2.4 Carbon isotope discrimination

Greenhouse-grown F_1 drought stressed plants had higher carbon isotope discrimination (Δ) values if they were (E+) than did their (E-) counterparts ($p \leq 0.01$; Fig. 6.3G). When F_1 plants were amply watered, (E+) wheat had a higher Δ than (E+) controls ($p \leq 0.01$; Fig. 6.3G). All F_2 plants subjected to drought had increased Δ relative to the (E-) drought-challenged F_1 plants ($p \leq 0.01$; Fig. 6.3G and H). Similarly, the Δ values of all well-watered F_2 plants, regardless of the treatment of their parents, were higher than the no endophyte F_1 control ($p \leq 0.01$; Fig. 6.3G and H).

6.5.3 Plant phenotype

Heat or drought stressed (E+) F_1 plants subjected to heat (Fig. 6.4A or C) or drought (Fig. 6.4C) showed marked differences in phenotype from their (E-) counterparts. Under heat stress F_2 plants whose F_1 parents were (E+) and subjected to heat or control temperatures displayed a more vigorous phenotype than plants with (E-) parents (Fig. 6.4B). The physical appearance of drought challenged F_2 plants arising from well-watered or drought stressed F_1 (E+) plants was distinct from that of F_2 plants descended from (E-) F_1 wheat (Fig. 6.4D).

6.6 Discussion

Food security is a critical issue facing the world in the 21st century (FAO 2008). Due to growing global human population, Foresight (2011) predicts that food production will need to double by the year 2050. However, this is likely to present a major challenge as drought stressed environments are likely to become ever more problematic as climatic change progresses (IPCC 2007; Gornall et al. 2010). The fact that drought dramatically reduces wheat grain harvests (Cannell et al. 1984; Whalley et al. 2006; Li et al. 2011) is of particular concern because wheat is a staple food crop. Improving wheat drought resistance

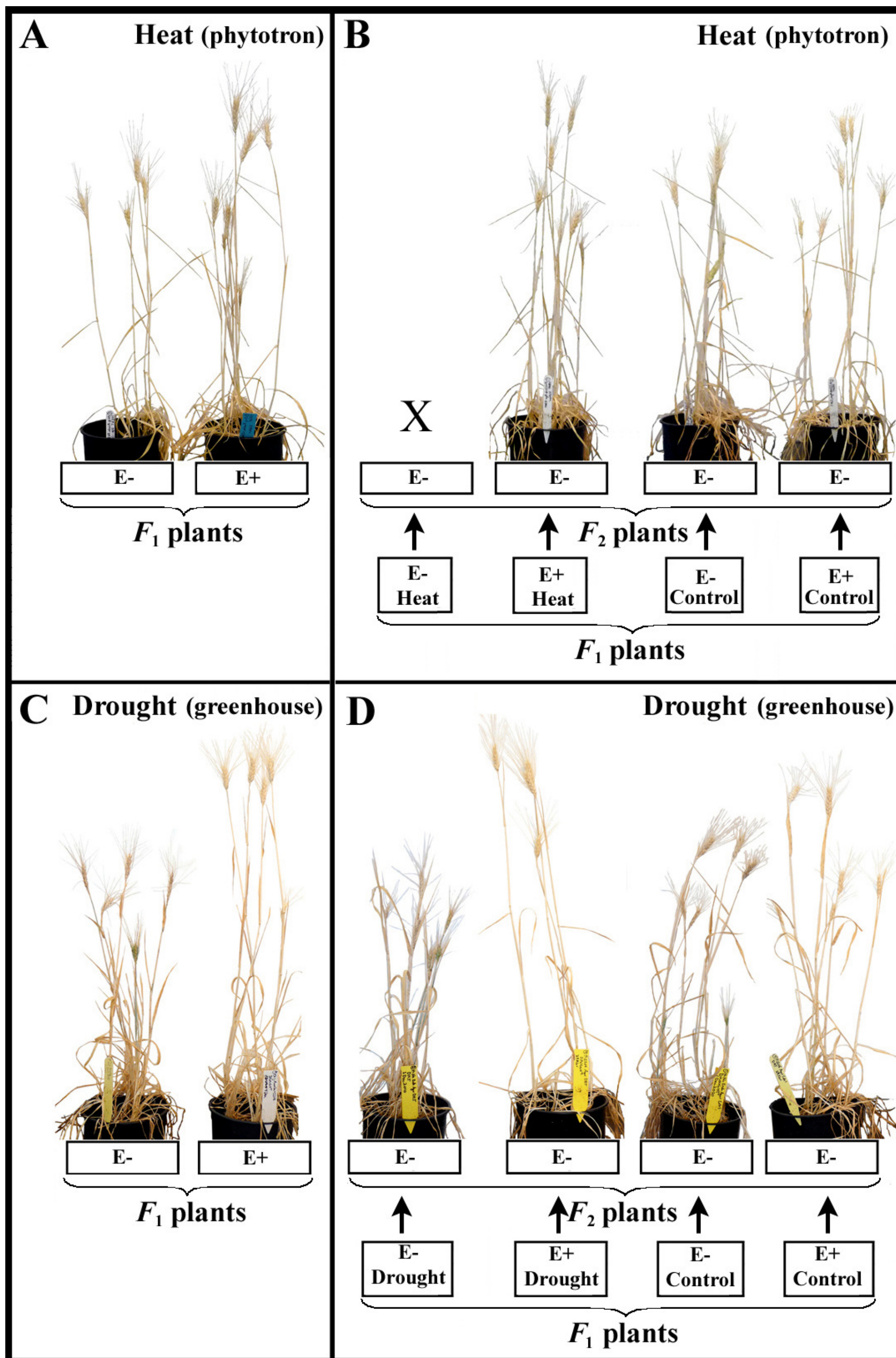


Figure 6.4. Effect of the fungal endophyte on appearance of F_1 (**A** and **C**) and F_2 (**B** and **D**) plants grown under heat (**A** and **B**) or drought (**C** and **D**) stress. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively.

could diminish reliance on irrigation. Reduced irrigation has numerous benefits, which include water conservation (Kang et al. 2008), lower agricultural costs (in terms of both energy and financial input) and decreased risk of soil salinity and erosion (reviewed in Wu and Sardo 2010). Although less well documented than the negative consequences of drought, heat stress has a similar, or even more severe, impact on wheat germination (Chapter 3) and yield (Porter and Semenov 2005; Semenov and Shewry 2011). Hence, there is a need for research and development of sustainable agricultural practices that can improve wheat tolerance for and yield under either elevated temperature or moisture deficit stress.

The use of indigenous, naturally occurring fungal endophytes, such as SMCD 2206, offers a promising solution to the problem of sustainable cropping in stress-prone environments. This mycobiont is a particularly good candidate because it is able to confer abiotic stress tolerance to germinating wheat seedlings (Chapter 3). This increased stress resistance is measurable in terms of improved energy of germination (EG) and percent seed germination (Chapter 3) or mycovitalism (Vujanovic and Vujanovic 2007). This last finding suggests that SMCD 2206 may be capable of inducing heritable epigenetic changes in its host. However, when F_1 seeds produced by plants subjected to abiotic stress are germinated under unstressed conditions it is possible that any improvements observed are attributable to gains in average seed weight (ASW), rather than epigenetic alterations. Notably, the results presented in the current study imply that something more than elevated ASW was at play. Hence, symbiotic relationships – such as the interactions between SMCD 2206 and wheat – could prove valuable in the enhancement of crop performance in drought stressed environments.

The results presented in this study are consistent with a growing body of evidence highlighting the ability of fungal endophytes to improve the performance of plants subjected to abiotic stressors, such as heat or drought. The fungi involved in these symbiotic relationships include Glomeromycota – which form arbuscular mycorrhizal (AM) associations with plants – as well as Ascomycota and Basidiomycota. Although most frequently associated with improved plant nutrient uptake (reviewed in Miransari et al. 2011), AM are also able to enhance plant drought tolerance. For example, both Al-Karaki et al. (2004) and Solaiman et al. (2010) found that wheat yield was improved by inoculation with AM in drought conditions. Endosymbiotic Ascomycota can be classified as being

members of the relatively extensively studied category of clavicipitaceous endophytes, which include *Neotyphodium* spp. and *Epichloë* spp. (Bacon and White 2000) as well as less clearly understood non- clavicipitaceous endophytes. The fungus SMCD 2206 belongs to this latter group. Both clavicipitaceous (Kannadan and Rudgers 2008; Kane 2011) and non-clavicipitaceous Ascomycoteous endophytes (Márquez et al. 2007; Rodriguez et al. 2009; Zhang et al. 2011; Chapter 3) improve plant performance under abiotic stress. Basidiomycota, such as *Piriformospora indica*, also form endophytic associations with plants and elevate plant resistance to abiotic stressors, such as drought and salinity (Waller et al. 2005; Sun et al. 2010). Although members of Glomeromycota, Ascomycota and Basidiomycota all increase tolerance for abiotic stress in their hosts, only limited investigations have explored whether similar mechanisms are involved in the interactions between plant hosts and each phylum of fungi. In addition, information on the capacity of endophytic fungi to induce heritable changes in their hosts has been unavailable, prior to the current study.

Because drought and heat resistance are complex traits, it is useful to examine the impact of these stressors on a variety of wheat characteristics, in addition to grain yield, or total seed weight (TSW). Such an approach has been used in other studies. For example, Li et al. (2011) assessed a wide range of agronomic traits in drought stressed and non-stressed wheat and related this data back to grain yield. It is valuable to consider parameters other than yield – given that grain yield requires sufficient time for the plants to complete their entire life cycle – while data on other traits can be collected much earlier. The parameters PS (Yang et al. 2002), WUE (Richards 2006; Dong et al. 2011), plant height (Khan et al. 2010), time to physiological maturity, grain volume and grain protein content (Li et al. 2011) have been shown to be good predictors of wheat grain yield under heat or drought stress. In addition, in the current study, assessment of characteristics besides TSW per pot adds value and reliability to the assessment of wheat stress tolerance in that the conclusions that could be drawn based on TSW per pot alone do not necessarily match with those merited by a consideration of all the parameters. For example, the TSW per pot produced by heat stressed plants did not show a statistically significant improvement between F_1 endophyte-treated plants and that of the F_1 endophyte-free control or between any of the F_2 plants, regardless of the treatment of their parents, relative to the same F_1 control (Fig. 6.2E and F). These results

are summarized in Table 6.1A. In contrast, when PS and ASW are also taken into account, three of the treatments described above improved heat tolerance over the F_1 no endophyte control (Table 6.1A; Fig. 6.2). In addition, the smaller sample size associated with TSW per pot data made it difficult to determine the statistical significance of differences between treatments. Changes in TSW of up to 44% are greater than, or equal to, those reported in the literature in association with important agricultural interventions such as irrigation of wheat (Whalley et al. 2006). Thus, is seems advisable to consider a range of other plant traits, especially when such parameters are consistent with the trends, albeit statistically insignificant ones, observed in the TSW per pot data.

Plants have the capacity to adapt to, and be altered by, environmental stress. Both abiotic (Bray and West 2005; Molinier et al. 2005) and biotic (Lucht et al. 2002) stressors may have a mutagenic impact on plant genomes. Notably, some of these modifications are heritable (Molinier et al. 2006). On a more subtle level, epigenetic changes are also triggered by and promote tolerance for stress (Boyko et al. 2007). Plant epigenetic alterations occur via a variety of mechanisms, including DNA methylation (Holliday 1989; Razin and Cedar 1992; Henderson and Jacobsen 2007), histone remodeling and chromatin rearrangement (Ben-Porath and Cedar 2001). It is well established that abiotic environmental stress leads to trans-generational genomic changes (Kovalchuk et al. 2000; Ries et al. 2000). Johannes et al. (2011) postulated that genetic and epigenetic inheritance are two points on a gradient ranging from more enduring to relatively transient variability. The heritable, yet unstable, nature of epigenetic modifications induced by stress in plants has been shown (Lang-Mladek et al. 2010; McCue et al. 2012). For example, Lang-Mladek et al. (2010) used *Arabidopsis thaliana* to demonstrate that stress-induced epigenetic alternations in expression of a reporter gene may be passed on to several generations of unstressed progeny. However, the epigenetic signatures of stress were lost with either the passing of three generations or seed aging. In addition, the stress related epigenetic changes were less pronounced in even the first non-stressed daughter generation than in the stressed parental plants. Future studies could assess whether SMCD 2206-conferred epigenetic modifications fade over multiple stress-free generations in a similar manner.

Table 6.1 Summary of the positive, neutral and negative impact of the fungal endophyte SMCD 2206 on each of the traits tested under heat in the phytotron (A), control conditions in the phytotron (B), drought in the greenhouse (C) or control conditions in the greenhouse (D). The significance of positive or negative impacts are classified as either significant "↑" ($p \leq 0.05$) or highly significant "↑↑" ($p \leq 0.01$). Significance was assessed using ANOVA, followed by a post-hoc LSD test.

A **Heat** (phytotron)

	Treatment (relative to F_1 (E-) control)				
		Endophyte-free 2nd generation (F_2)			
		Treatments of F_1 parents			
Trait	**(E+) Heat**	**(E-) Heat**	**(E+) Heat**	**(E-) Control**	**(E+) Control**
Photosynthetic stress	↑↑ ($p \leq 0.01$)	n/a	↑↑ ($p \leq 0.01$)	($p > 0.05$)	($p > 0.05$)
Average seed weight	↑ ($p \leq 0.05$)	n/a	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	($p > 0.05$)
Total seed weight / pot	($p > 0.05$)	n/a	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)
Overall	**Positive (+3)**	n/a	**Positive (+4)**	**Positive (+2)**	**Neutral**

B **Control** (phytotron)

	Treatment (relative to F_1 (E-) control)				
		Endophyte-free 2nd generation (F_2)			
		Treatment of F_1 parents			
Trait	**(E+) Control**	**(E-) Heat**	**(E+) Heat**	**(E-) Control**	**(E+) Control**
Photosynthetic stress	($p > 0.05$)	n/a	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)
Average seed weight	($p > 0.05$)	n/a	↓ ($p \leq 0.01$)	↓↓ ($p \leq 0.01$)	($p > 0.05$)
Total seed weight / pot	($p > 0.05$)	n/a	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)
Overall	**Neutral**	n/a	**Negative (-1)**	**Negative (-2)**	**Neutral**

C Drought (greenhouse)

Trait	Treatment (relative to F_1 (E-) control)				
		Endophyte-free 2nd generation (F_2)			
		Treatment of F_1 parents			
	(E+) Drought	(E-) Drought	(E+) Drought	(E-) Control	(E+) Control
Photosynthetic stress	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)
Average seed weight	↑↑ ($p \leq 0.01$)	↓↓ ($p \leq 0.01$)	↓↓ ($p \leq 0.01$)	↓↓ ($p \leq 0.01$)	($p > 0.05$)
Total seed weight / pot	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)
Overall	**Positive (+4)**	**Neutral**	**Neutral**	**Neutral**	**Positive (+2)**

Trait	(E+) Drought	(E-) Drought	(E+) Drought	(E-) Control	(E+) Control
Carbon isotope discrimination ($\Delta^{13}C$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)
Overall	**Positive (+2)**	**Positive (+2)**	**Positive (+2)**	**Positive (+2)**	**Positive (+2)**

D Control (greenhouse)

Trait	Treatment (relative to F_1 (E-) control)				
		Endophyte-free 2nd generation (F_2)			
		Treatment of F_1 parents			
	(E+) Control	(E-) Drought	(E+) Drought	(E-) Control	(E+) Control
Photosynthetic stress	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)
Average seed weight	↑↑ ($p \leq 0.01$)	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)	($p > 0.05$)
Total seed weight / pot	($p > 0.05$)	($p > 0.05$)	↑↑ ($p \leq 0.01$)	($p > 0.05$)	($p > 0.05$)
Overall	**Positive (+2)**	**Neutral**	**Positive (+2)**	**Neutral**	**Neutral**

Trait	(E+) Control	(E-) Drought	(E+) Drought	(E-) Control	(E+) Control
Carbon isotope discrimination ($\Delta^{13}C$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)	↑↑ ($p \leq 0.01$)
Overall	**Positive (+2)**	**Positive (+2)**	**Positive (+4)**	**Positive (+2)**	**Positive (+2)**

The findings described above are consistent with the observation that the protective impact of endophyte inoculation under drought stress was less pronounced, but still present to some degree, in endophyte-free progeny of stressed and mycobiont-colonized wheat (Table 6.1C; Fig. 6.2 and 6.3). Among F_1 plants grown under a moisture deficit, treatment with SMCD 2206 leads to a highly significant improvement in WUE, PS and ASW and a neutral impact on TSW per pot. Values for Δ from endophyte treated F_1 plants were also higher than those of endophyte-free F_1 plants to highly significant degree (closer to those of well-watered plants). The preceding augmentations amount to an overall highly positive impact – highly significant improvement on three out of four traits assessed – relative to the endophyte-free, drought stressed F_1 control (Table 6.1C). Drought-challenged F_2 plants arising from endosymbiont-treated and drought stressed parents also outperformed the same control, though to a lesser extent – leading to a highly significant enhancement of two of the four traits, and a highly significant decrease in one trait (Table 6.1C). This score differs by only one from that of F_2 plants whose F_1 parents were endophyte-free and subjected to drought. In contrast to drought resistance, heat tolerance is strongly passed on to uncolonized F_2 plants (Table 6.1A; Fig. 6.2). This is consistent with the findings of Chapter 3 that the ability of mycobionts, including SMCD 2206, to confer heat tolerance is greater than the capacity of these same organisms to increase drought resistance. The heat treatment used may have been more severe or extreme than the drought applied. For example, the ASW of heat stressed uninoculated F_1 plants was only 60% as great as the ASW of the control (Fig. 6.2C). In contrast, drought stressed F_1 plants had an ASW that was 91% of the control ASW (Fig. 6.3C). As discussed in Chapter 3, protective effects of SMCD 2206 on wheat may be greater under more adverse conditions.

Methylation of DNA is a common mechanism behind epigenetic variation and inheritance (Boyko and Kovalchuk 2008) and is involved in plant responses to drought (Labra et al. 2002; Wang et al. 2011) and salt (Lu et al. 2007; Zhong et al. 2009; Khan et al. 2012a) stress. Thus, exploration of the DNA methylation patterns of inoculated and endophyte-free wheat exposed to heat or drought and control conditions could be valuable. If such investigations found mycobiont-associated DNA methylation alterations in F_1 plants, studies on potential inheritance of any of these methylation signatures to a second F_2

generation of endophyte-free wheat, whose parents were colonized by SMCD 2206, seem worthwhile. This is especially true in light of evidence that modifications of plant DNA methylation patterns are sometimes transmitted through mitosis, meiosis and gametogenesis (Saze 2008) and are heritable to subsequent generations (Vaughn et al. 2007; Johannes et al. 2009), even in the absence of the stress (Verhoeven et al. 2010). A resultant clearer picture of the interplay between endophytic fungi and their plant hosts could complement both my findings and those of Waller et al. (2005) and Baltruschat et al. (2008) in which the endophyte *P. indica* reprograms barley to enhance salt tolerance. Clearly, a more complete understanding of the interplay between plants and endophytes is likely to facilitate the more effective and appropriate deployment of fungal endophytes in agriculture.

Future research could explore the impact(s) of SMCD 2206 on wheat grown in the field and on biomass and straw production. The latter could contribute to sustainable agriculture through its utility as a soil amendment and in animal husbandry. As measures of stress tolerance used in this study, such as Δ under drought stress, have been linked to increased biomass in wheat (Zhang et al. 2010), the possibility that SMCD 2206 could augment wheat biomass seems worth investigating. The ability of the mycobiont to promote stress tolerance in wheat in the field has clear value as real-world wheat cropping takes place in a field environment, rather than in a greenhouse of phytotron growth chamber. The results of such field trails are uncertain because field-grown wheat must contend with greater soil microbial diversity, climatic and soil type variability, pests and diseases as well as competition from weeds than its greenhouse-grown counterparts. Given that field trials have successfully demonstrated the ability of mycorrhizal fungi to increase wheat yield under drought conditions (Solaiman et al. 2010), it is reasonable to hypothesize that indigenous endophytic fungi, such as SMCD 2206, might be similarly effective.

6.7 Conclusions

In a world faced with climate change, limited water resources and human population growth, sustainable wheat production in environments prone to abiotic stress is a major problem. Fortunately, wheat inoculated with the indigenous endophytic fungus SMCD 2206 exhibited elevated tolerance for heat and drought stress, offering a potential avenue for meeting this challenge. Endophyte-conferred heat, and, to a lesser extent, drought resistance,

was inherited by mycobiont-free progeny. Trans-generational mycomediated stress tolerance, if found in field-grown wheat, combined with further elucidation of the role(s) epigenetic modifications and inheritance play in plant-endophyte interactions, could lead to more effective utilization of fungal endophytes in agricultural production.

7.0 GENERAL DISCUSSION

Due to growing human populations and climatic change, an increase in crop production from marginal environments is needed. Indigenous microorganisms, such as the fungal endophytes SMCD 2204, 2206, 2208, 2210, 2214 and 2215 offer an adaptable and environmentally responsible way of meeting this need. Endophytic fungi can be put to better use if they are more completely understood. The mechanisms by which endophytic fungi interact with their hosts are incompletely known. The aim of this study was to shed more light on theoretical and practical aspects of plant mycovitality (Vujanovic and Vujanovic 2007) and, thus, the SMCD strains were tested on seeds under abiotic (heat and drought) stress conditions. The results acquired bring new insight into the mode of mycobiont interactions with host plants, aside from their functional and structural characterization reported in association with wheat-root system (Abdellatif et al. 2009).

The three isolates SMCD 2206, 2210 and 2215 were able to improve wheat heat and drought tolerance in terms of seed mycovitality *in vitro*, performance of adult plants in the pot studies, grain yield and germination of F_1 seeds produced. Both *in vitro* and greenhouse of phytotron studies, heat stress was more detrimental to wheat performance than drought. Isolate SMCD 2206 induced epigenetic modifications in wheat through changing DNA methylation patterns. These epigenetic modifications brought about altered gene expression and increased transposable element activity. Notably, the work presented in this thesis is an extension of work performed using other fungal endophytes in that it explores the performance of a second, endophyte-free generation (F_2) of wheat. This was done both in terms of seed germination under control conditions (Chapter 4) and of mature plants under stress and stress-free conditions (Chapter 6).

The putative epigenetic impact SMCD 2206 had on wheat is both similar to and different from that which the root-colonizing Basidiomycete *Piriformospora indica* and the below- and above-ground colonizing (Class 2) endophytes studied by the Rodriguez and

Redman groups appear to have on their hosts. Rodriguez et al. (2009a) observed that endophytic *Alternaria* sp. have an epigenetic impact on host plants by promoting sexual reproduction of symbiotic plants over that of non-symbiotic plants. However, no molecular or mechanistic studies on DNA methylation or histone modifications have been done on *Alternaria*, *Fusarium* or *Curvularia* sp. In addition, caution is merited when comparing SMCD endophytes with *Alternaria*, *Fusarium* or *Curvularia* sp. because the latter are facultative pathogens of some plant species. For example, *Curvularia protuberata* can function as an endophyte, conferring dramatic heat tolerance to its hosts (Márquez et al. 2007), or as a plant pathogen (Sisterna and Dal Bello 1998). Furthermore, *P. indica* reprograms barley by altering levels of antioxidant enzymes such as ascorbate, dehydroascorbate, dehydroascorbate reductase (Waller et al. 2005; Baltruschat et al. 2008), superoxide dismutase and catalase (Baltruschat et al. 2008; Sun et al. 2010). Increased production of these enzymes may be due to epigenetic processes. However, in the work done by Waller et al. (2005) where enzyme levels were measured in the early stages of endophyte colonization and in the absence of stress, it seems more likely that higher enzyme levels are linked to the roles dehydroascorbate reductase appears to play in maintain a mutualistic relationship between *P. indica* and its hosts (Vadassery et al. 2009). In contrast to Waller et al. (2005), Sun et al. (2010) show that endophyte colonized and drought stressed plants produce more superoxide dismutase and catalase than did endophyte-free and drought stressed plants. The fact that the same trend was not found in drought free plants implies epigenetic involvement in enhanced drought tolerance, rather than activation of plant-fungus communication or plant defenses. The evidence in favour of epigenetic involvement in the interactions between *P. indica* and its hosts is further strengthened by the upregulation of drought-related genes in *P. indica* colonized plants subjected to drought (Sherameti et al. 2008; Sun et al. 2010).

A more complete picture of the genes involved, beyond the cytochrome p450 implicated in Chapter 5, could be obtained through analysis of microarray, transcriptome, proteomic and/or metabolomic profiles of stressed plants, with and without colonization by a phytoprotective fungal endophyte. Analyses of leaf transcriptomes and metabolites have shed light on the interactions of *P. indica* with its host under pathogen attack (Molitor et al. 2011). The expression levels under various growth conditions of putative genes pointed to

by the above approaches could be further characterized using real-time PCR. In addition, determining if SMCD 2210 and 2215 induce similar epigenetic or gene expression changes in wheat subjected to abiotic stress as SMCD 2206 could help whether endophyte-induced epigenetic changes are widespread or highly specific to the endophyte, host, site or environmental conditions.

Future studies could also focus on the interactions of SMCD 2206, 2210 and 2215 with other plant-associated microbes. Root-colonizing Ascomycota and Basidiomycota endophytes co-occur with mycorrhizae in a variety of environments, such as the Tatra mountains in Poland (Zubek et al. 2009), a heavy metal contaminated site in Slovenia (Regvar et al. 2009) and the Canadian Prairies (Perez-Naranjo 2009). Hence it is possible that inoculation of wheat, or other host plants, with SMCD 2206, 2210 or 2215 in conjunction with other beneficial microorganisms could provide greater benefits than a single inoculant. Facilitation (meaning that colonization by a given endophyte increases the likelihood of colonization by another endophyte or group of endophytes) between endophytic fungi has been reported in maize (Pan and May 2009). However, it is also conceivable that plants colonized by an SMCD endophyte might be less able to form symbiotic associations with advantageous microbes such as other fungal endophytes, endophytic bacteria or plant growth promoting rhizobacteria. This latter potentiality could be mediated by microbe-microbe competition for space and nutrients, mycoparasitism, activation of host defenses or antagonism involving the production of antifungal or antibacterial compounds (reviewed in Saunders et al. 2010). Such antagonistic interactions between potentially beneficial microorganisms could even lead to SMCD endophytes being deleterious in some field environments. In order to assess the likelihood of such an unintended outcome, *in vitro* co-inoculations with multiple symbiotic microorganisms, followed by co-inoculations in pot studies are merited. The host range of SMCD 2206, 2210 and 2215 should also be explored, both to determine if these organisms have the potential to switch to a pathogenic lifestyle on other plants and to evaluated their likelihood of promoting weed growth at the expense of the desired crops. A greater understanding of the interactions of SMCD 2206, 2210 and 2215 with other agriculturally relevant microbes and plants is likely facilitate safe and effective use of these fungi in agriculturally applications and to mitigate risks.

8.0 GENERAL CONCLUSIONS

The fungal endophytes SMCD 2206, 2210 and 2215 were able to improve wheat performance under heat or drought stress both at the seedling stage (mycovitality) and in mature plants in pot studies. The evidence presented in this thesis indicates that SMCD 2206 influences wheat DNA methylation patterns under moisture stress, suggesting that this mycobiont epigenetically modulates its host. The fact that improved heat or drought tolerance persists to some extent in a second, uninoculated generation of wheat suggests that these epigenetic alterations are partially heritable.

The identification of these three promising endosymbiotic fungi offers agricultural potential. In order to most effectively tap into this bioresource, further investigations into areas such as the methylation of state of endophyte-free F_2 plants; a more complete characterization of genomic elements epigenetically modulated in endophyte treated wheat exposed to stress; effectiveness and appropriateness of various inoculation methods and reasons for the (in)effectiveness; impact of these mycobiont in field-growth wheat; interactions of these endophytes with other microorganisms and potential host plants; and additional mechanism(s) involved in plant-endophyte interactions are merited.

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10.0 APPENDIX

10.1 Complete set of methyl-sensitive amplyfied polymorphism (MSAP) gels

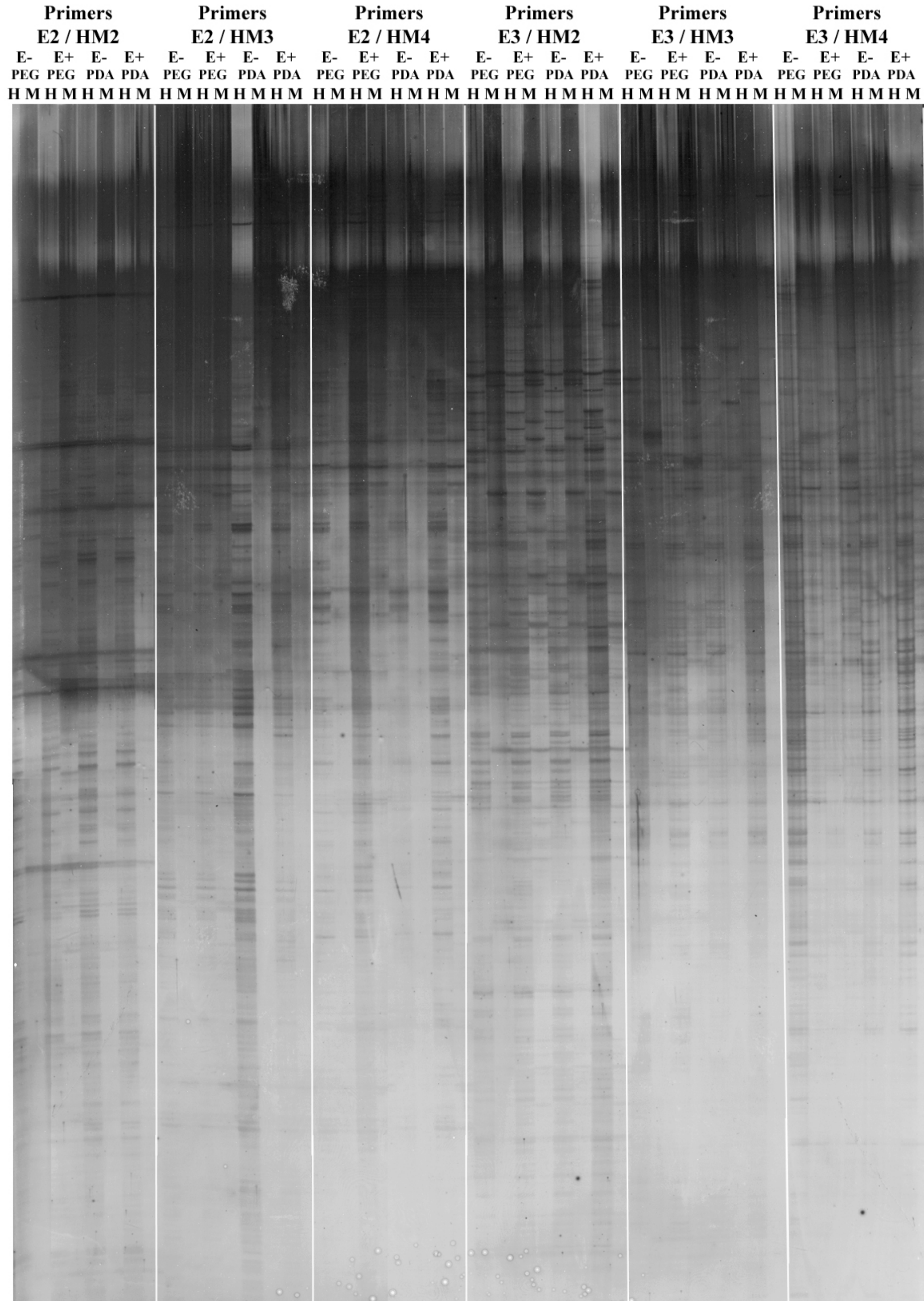

Fig. 10.1 Methyl sensitive amplified polymorphism (MSAP) gel banding patterns from primers E2/HM2, E2/HM3, E2/HM4, E3/HM2, E3/HM3 and E3/HM4.

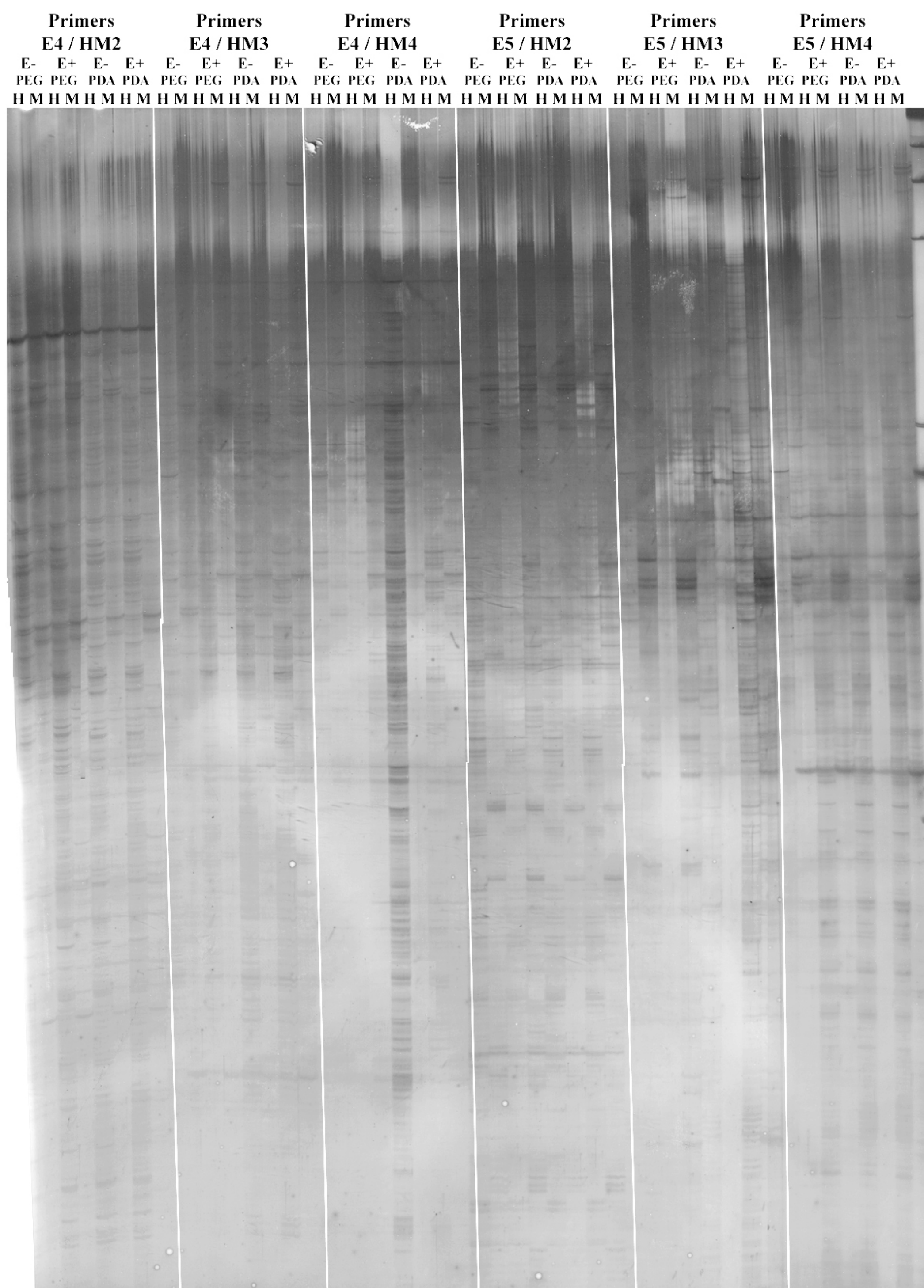

Fig. 10.2 Methyl sensitive amplified polymorphism (MSAP) gel banding patterns from primers E4/HM2, E4/HM3, E4/HM4, E5/HM2, E5/HM3 and E5/HM4.

10.2 Optimal inoculation methods are crucial for unlocking the potential of fungal endophyte-plant interactions

10.2.1 Abstract

Endophytic fungi, including SMCD 2206, can protect their plant hosts from the deleterious effects of abiotic stressors, such as heat or drought. However, for the benefits of such phytoprotective mycobionts to be realized, optimal inoculation strategies must be found. The need for investigation into microbial formulation will become particularly acute as research progresses from *in vitro* laboratory studies, to the greenhouse, field, and ultimately, large-scale production and/or widespread use. I compared the relative effectiveness of a soil-based inoculation method to a peat seed treatment, carrying either a lower or higher load of colony forming units (CFU). Efficacy was evaluated by monitoring the performance, in terms of photosynthetic stress, average seed weight (ASW), total seed weight (TSW) and germination of seeds produced, of treated and uncolonized (control) wheat under heat, drought and non-stressed conditions. I found that the soil-based approach led to a greater, and more consistent, level of abiotic stress tolerance in wheat.

10.2.2 Hypothesis and objectives

In this study, I hypothesized that a soil-based inoculation method will permit the mycobiont SMCD 2206 to confer the same level of heat and drought tolerance as will a peat seed treatment. My objective for this study was to assess the level of heat or drought resistance in wheat inoculated via one of the following methods: 1) soil-base method, 2) a peat seed coating containing a lower density of SMCD 2206 or 3) a peat treatment impregnated with a higher density of SMCD 2206.

10.2.3 Materials and methods

The plant and fungal material used have been described in the material and methods sections of Chapters 3, 4, 5 and 6. Photosynthetic stress, average seed weight (ASW), total seed weight (TSW) and germination of seed produced were measured and statistically analyzed as outlined in Chapters 4 and 6. Heat and drought stress were induced as described for experiment one in Chapter 6.

10.2.3.1 Inoculation techniques

The fungal endophyte SMCD 2206 is compatible with wheat and was applied to wheat seeds prior to germination according to either a soil-based or seed-based method. The soil-based inoculation procedure was based on the *in vitro* method described in Abdellatif et al. (2010). Briefly, five surface-sterilized seeds were placed at a distance equivalent to 48 h hyphal growth from a 5 mm^2-agar plug, placed hyphal side down in the centre of a 2 L plastic pot filled with autoclaved Sunshine mix 4 potting soil (SunGro Horticulture Canada Ltd., 200 Burrard Street, Suite 1200, Vancouver, British Columbia, V7X 1T2, Canada) and then covered in approximately 2 cm of the same potting mix.

The seed treatment involved coating surface-sterilized wheat seeds in sterile peat containing either 10^4 (low dose) or 10^6 (high dose) colony forming units (CFU) of SMCD 2206 per seed. This treatment was achieved using an adaptation of a protocol used by the Gustafson Lab (University of Saskatchewan) soybean seed treatment method. The fungal isolate SMCD 2206 was grown in 50 mL aliquots of Potato Dextrose Broth (PDB), with shaking at 200 r.p.m. at room temperature for either 3 d (low dose) or 6 d (high dose). Next, 5.0 g of peat powder, which had been autoclaved to ensure sterility, was added to each 50 mL of SMCD 2206 and air dried under sterile conditions in the laminar flow hood. Subsequently, 1.0 g of each of the preparations of dried peat containing SMCD 2206 (low

and high dose) were added to sterile ziploc bags, and mixed, via vigorous shaking, with 103 surface-sterilized wheat seeds and 2.0 mL autoclaved distilled H_2O, resulting in seeds coated in peat, just saturated in water. The seeds were either planted immediately, or stored at 4 ºC for not more than 48 hrs prior to planting. The number of CFUs per seed was assessed, via serial dilution, for at least three seeds from each treatment. An endophyte-free control, in which 103 seeds were coated, as described above, with 2.0 g of sterile dried peat and PDB, plus 2.0 mL, was also used. The absence of microbial contamination was confirmed for a minimum of three no endophyte control seeds through serial dilution.

10.2.4 Results

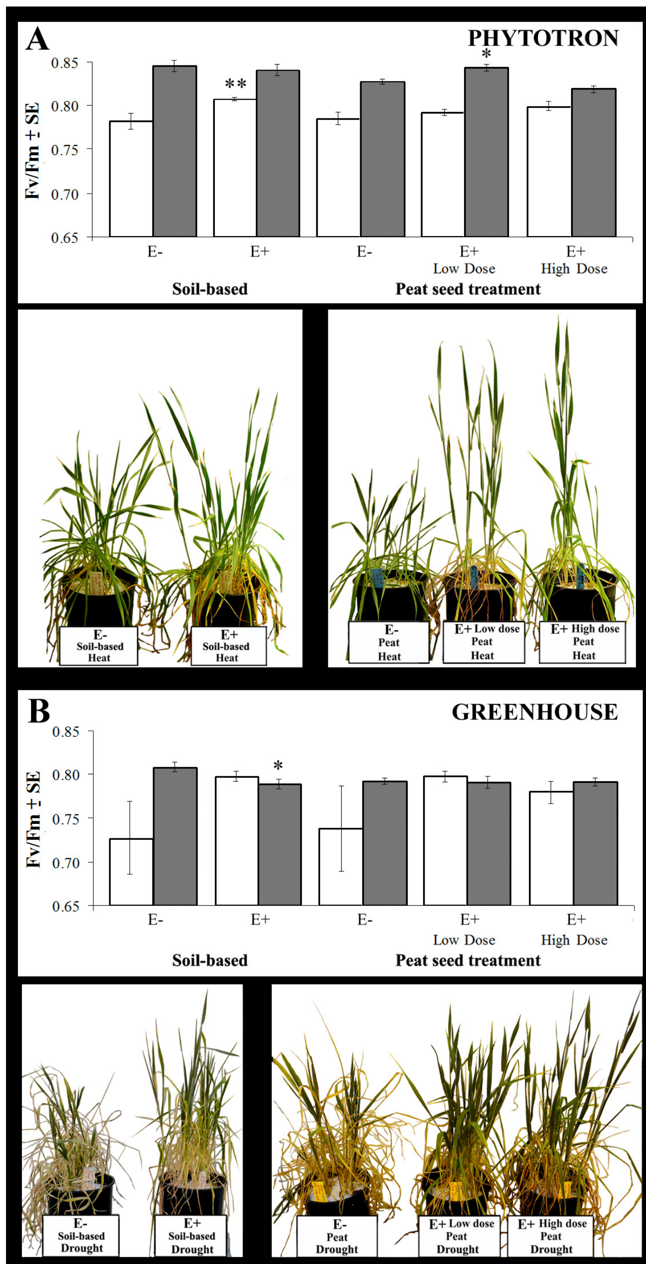

Fig. 10.3 Photosynthetic stress, in terms of maximal photochemical efficiency (*F*v/*F*m ratio), measured in one month old plants and appearance of representative plants. An increase the *F*v/*F*m ratio indicates decreased stress. Photosynthetic stress of plants grown in the phytotron (A), with white bars (□) and grey bars (■) representing heat stressed and control plants, respectively, and greenhouse (B), with white bars (□) and grey bars (■) representing drought stressed and control plants, respectively. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Bars labelled with one or two asterix (*) are significantly, or highly significantly, different from the no endophyte control ($p \leq 0.05$ or $p \leq 0.01$, respectively; ANOVA, followed by post-hoc LSD test). Error bars represent standard error (SE) of the mean.

Figure 10.4 Average seed weight and images of all seeds produced in the phytotron (A) and greenhouse (B). White bars (□) correspond to heat stress in the phytotron and drought stress in the greenhouse. Grey bars (■) represents the stress-free controls. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Bars labelled with one or two asterix (*) are significantly, or highly significantly, different from the no endophyte control ($p \leq 0.05$ or $p \leq 0.01$, respectively; ANOVA, followed by post-hoc LSD test). Error bars represent standard error (SE) of the mean.

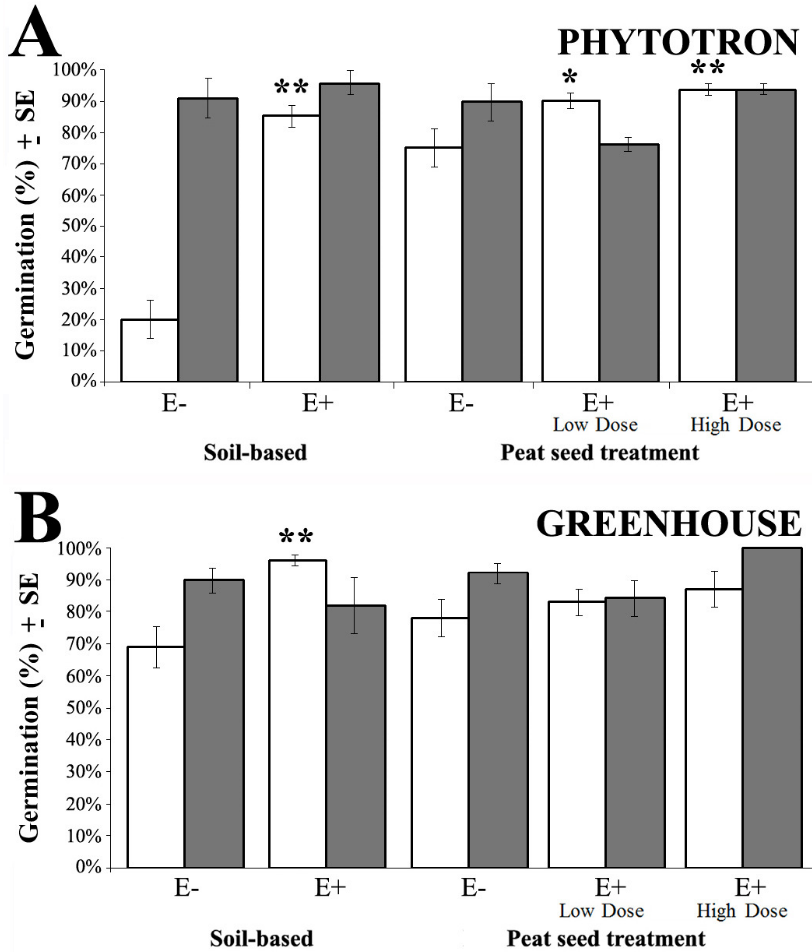

Fig. 10.5 Germination of F_1 seeds produced in the phytotron (A) and greenhouse (B). White bars (□) represent seeds produced under heat (phytotron) or drought stress (greenhouse); Grey bars (■) signify seeds which developed in unstressed (control) conditions. The symbols "E-" and "E+" indicate the absence and presence of endophyte colonization, respectively. Bars labelled with one or two asterix (*) are significantly, or highly significantly, different from the no endophyte control ($p \leq 0.05$ or $p \leq 0.01$, respectively; ANOVA, followed by post-hoc LSD test). Error bars are standard error (SE) of the mean.

10.2.5 Conclusions

When soil-based inoculation methods were used, the fungal endophyte SMCD 2206 was effective in contributing to wheat tolerance for heat or drought stress. Peat seed treatments were less effective than soil-based inoculation, but still provided some degree of heat and drought resistance to wheat.