THE LODGEPOLE × JACK PINE HYBRID ZONE IN ALBERTA, CANADA: A STEPPING STONE FOR THE MOUNTAIN PINE BEETLE ON ITS JOURNEY EAST ACROSS THE BOREAL FOREST?

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Abstract - Historical data show that outbreaks of the tree killing mountain pine beetle are often preceded by periods of drought. Global climate change impacts drought frequency and severity and is implicated in the range expansion of the mountain pine beetle into formerly unsuitable habitats. Its expanded range has recently reached the lodgepole × jack pine hybrid zone in central Alberta, Canada, which could act as a transition from its historical lodgepole pine host to a jack pine host present in the boreal forest. This field study tested the effects of water limitation on chemical defences of mature trees against mountain pine beetle-associated microorganisms and on beetle brood success in lodgepole × jack pine hybrid trees. Tree chemical defences as measured by monoterpene emission from tree boles and monoterpene concentration in needles were greater in trees that experienced water deficit compared to well-watered trees. Myrcene was identified as specific defensive compound, since it significantly increased upon inoculation with dead mountain pine beetles. Beetles reared in bolts from trees that experienced water deficit emerged with a higher fat content, demonstrating for the first time experimentally that drought conditions benefit mountain pine beetles. Further, our study demonstrated that volatile chemical emission from tree boles and phloem chemistry place the hybrid tree chemotype in-between lodgepole pine and jack pine, which might facilitate the host shift from lodgepole pine to jack pine.

Key Words – Mountain pine beetle, range expansion, drought, tree defences, beetle condition.

INTRODUCTION

Global climate change has allowed a vast number of plant and animal species to extend their range into formerly unsuitable habitats (Parmesan 1996; Parmesan 2006; Stange and Ayres 2001), including the mountain pine beetle (hereafter MPB), *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculinoidae, Scolytinae) (Cudmore et al. 2010; Cullingham et al. 2011). The MPB is the most destructive insect pest of pine forests in western North America (Safranyik and Carroll 2006). During the most recent outbreak, the MPB has killed an estimated 18.1 million ha of mainly lodgepole pine (*Pinus contorta* Dougl. ex. Loud.) forests in British Columbia (BC, www.for.gov.bc.ca). The MPB is native to western North America and its historical range within Canada is in central BC, west of the Rocky Mountains (Safranyik et al. 2010). The unprecedented source population that established in BC in the late 1990's promoted the dispersal of beetles eastward into the neighbouring province of Alberta where MPB initiated a massive invasion of the north-eastern slopes of the Rocky Mountains in 2002 (Safranyik et al. 2010). In Alberta, MPB has continued to expand its range eastward and attacked mature pine trees in an area, where lodgepole pine and a closely related pine species, jack pine (*Pinus banksiana* Lamb), overlap and hybridize (Mirov 1956). Within this lodgepole × jack pine hybrid zone, MPB has attacked both hybrid and genetically-pure jack pine trees (Cullingham et al. 2011). Jack pine is the major pine species in the boreal forest of Canada and extends from northern Alberta to eastern Canada and to the Great Lakes Region of the United States. Despite the fact that jack pine represents a novel host for MPB, it can support beetle colonization and development, as well as growth of the MPB-associated fungal symbionts (Cerezke 1995; Rice et al. 2007a).

The impact of global climate change on drought frequency and severity (Breshears et al. 2009) may facilitate further range expansion of MPB as drought has been linked to

78 eruptive population dynamics of several insect species (Mattson and Haack 1987), including
79 MPB (Alfaro et al. 2010). Drought is suspected to increase the susceptibility of forests to
80 insect herbivores through a decrease in the expression of tree defences (Thomson and
81 Shrimpton 1984). One climate-suitability model, originally developed in 1975 (Safranyik et
82 al. 1975) and modified in 2004 (Carroll et al. 2004), includes water deficit as a factor that
83 benefits beetle fitness under the assumption that water deficit reduces tree resistance
84 (Safranyik et al. 2010). However, the effect of drought-stressed host trees on the colonization
85 and development of MPB has never been tested experimentally.
86 Tree resistance in conifers to bark beetle attacks is primarily based on chemically-
87 mediated tree defences and can correlate with monoterpene content of individual trees
88 (Gollob 1980; Schiebe et al. 2011; Sturgeon 1979; Zhao et al. 2011). Monoterpenes are a
89 major constituent of conifer resin along with sesquiterpenes and diterpenoid resin acids
90 (Keeling and Bohlmann 2006; Bohlmann 2012) that act as both physical and chemical
91 barriers against bark beetles. However, mass attacks triggered by MPB aggregation
92 pheromones and inoculation of phloem and xylem with its fungal symbionts can rapidly
93 deplete host tree defences (Raffa and Berryman 1983; Boone et al. 2011). Female MPB
94 produce the aggregation pheromone that is needed for mass attack by hydroxylating α-pinene,
95 an abundant monoterpene in the Pinaceae, to *trans*-verbenol, which attracts both sexes of
96 MPB (Pitman et al. 1968; Blomquist et al. 2010). During the subsequent colonization
97 process, arriving males produce *exo*-brevicomin which attracts more females until the ideal
98 attack density is reached, at which time both male and female MPB emit anti-aggregation
99 pheromones to prevent further colonization of the host tree (Rudinsky et al. 1974; Ryker and
100 Libbey 1982). α-Pinene and other monoterpenes such as 3-carene, terpinolene and myrcene
101 also synergize the response of MPB to its aggregation pheromones (Borden et al. 2008), thus

MPB has evolved to exploit the primary chemical defences of its host tree to its own advantage.

MPB is associated with several symbionts including the fungi *Grosmannia clavigera*, *Ophiostoma montium* and *Leptographium longiclavatum* (Lee et al. 2005; Rice and Langor 2008; Six and Klepzig 2004; Khadempour et al. 2012), bacterial symbionts and yeasts (Adams et al. 2008). The fungi help the beetle to deplete tree defences (Reid et al. 1967; Rice et al. 2007b), reduce sap flow (Yamaoka et al. 1990), and serve as a nutrition source for MPB larvae (Adams and Six 2007; Bleiker and Six 2007) and teneral adults (Whitney 1971; Paine et al. 1997). Some of the bacteria might have the potential to help MPB to overcome tree defences (Adams et al. 2013). Alternatively, bacteria and yeasts can affect the distribution of symbiotic fungi in the host tree, which subsequently might influence MPB fitness (Adams et al. 2008). Because of the close association between MPB and its fungal symbionts, artificial inoculation of trees with those fungi is often used to simulate beetle attack as a proxy to study tree defences (Lieutier et al. 2009; Boone et al. 2011; Wang et al. 2013).

Due to the proven impact of drought on host plant-herbivorous insect interactions, we further tested the hypothesis that tree defence stimulation will vary between well-watered trees and those that experience water deficit. In order to test this hypothesis, we conducted a field experiment at the front of the eastward range expansion of MPB in Alberta, Canada within the lodgepole × jack pine hybrid zone with the following objectives: (1) to develop a chemical profile of volatile organic compounds (VOCs; primarily monoterpenes) released from the bole of lodgepole × jack pine hybrids; (2) to evaluate variation of volatile chemical profiles of different water (water-deficit vs. well-watered) and biological treatments that stimulate tree defence; (3) to determine whether the monoterpene content of phloem and needle tissue is affected by the water treatments; and (4) to assess whether water and biological treatments applied to trees affect MPB brood success.

METHODS AND MATERIALS

To understand the potential importance of water limitation to tree defence response against invading organisms, we conducted a field study in the summer of 2009 at a site 25km north-west of Whitecourt, Alberta, Canada (54°13.595' N, 116°03.148' W). In this area, the range of lodgepole and jack pine overlap and the species hybridize (Mirov 1956). Forty mature putative lodgepole × jack pine hybrid trees with an average diameter at breast height (DBH) of 23.9cm ± 2.52 SD were selected to investigate our objectives.

Water Treatments. Selected trees were randomly assigned to one of two water treatment groups: well-watered and water-deficit trees (n= 20 for each treatment group). For a continuous water supply, a slow release watering bag setup (treegator®, Spectrum Products Inc., Youngsville, NC, U.S.A.) with a water capacity of 160L was attached to the well-watered trees. The bags were filled with water from the nearby Athabasca River on a biweekly-basis from 5 June - 20 August, 2009. The soil at the base of the water-deficit trees was covered with a tarpaulin (size: 12' × 14' (3.66m × 4.27m); G. Hjukstrom Limited, Surrey, B.C., Canada) to limit ambient water supply. Soil water content (SWC) around each tree, as well as at three randomly selected spots in the field site, was monitored using time domain reflectometry (TDR) (Hillel 1998). The apparent dielectric constant of the soil was measured using stainless steel probes at depths of 30cm, 60cm, and 90cm with a Tektronix 1502B (Beaverton, Oregon, USA) and related to water content using an empirical equation for mineral soils (Robinson et al. 2003). Soil water content data for trees that experienced the different water treatments was analysed using a nested ANOVA with probe length (30, 60, 90 cm) and time (0 day, 1 day, 2, 5, and 7 weeks) during the season specified as random factors.

The differences in SWC between water treatments were analysed using subsequent pairwise comparisons with Bonferroni correction. All statistical analyses were conducted using SPSS 19.0 for Windows (IBM Corporation, Armonk, New York, USA), unless otherwise stated.

Biological Treatments. Five weeks after the water treatments were initiated, 5 trees in both water treatment groups were additionally exposed to one of four biological treatments: (1) no induction control, (2) mechanical wounding only, (3) mechanical wounding followed by fungal inoculation with *Grosmannia clavigera,* or (4) mechanical wounding followed by inoculation with mashed beetles alternated with *G. clavigera* inoculation. Inoculation with live beetles was not permitted in this geographic area at the time of the experiment, thus we used mashed beetles and associated fungal symbionts to simulate MPB attacks. All trees, with the exception of the control trees, were wounded ten times with a cork borer (1cm diameter) evenly spaced around the bole at breast height. Fungal inoculations were conducted with *G. clavigera* by placing a malt extract agar plug with active fungal mycelium into the wound with the mycelium facing the sapwood. For the mashed beetle treatment, freshly emerged MPBs were killed by freezing them over night at -20°C the day before inoculation. This approach would leave only the cold-hardy symbionts of the MPB. Late instar larvae, the overwintering stage, can withstand winter temperatures close to -40°C (Safranyik and Carroll 2006), so their fungal symbionts should also able to tolerate similar low temperatures. *G. clavigera* and *Leptographium longiclavatum*, resist freezing at -20°C for a 3-month period (Rice et al. 2008). Frozen beetles were tranported to the field site on ice, and 20 MPBs per each inoculation point were mashed and placed in every other wound of designated trees; the remaining wounds were inoculated with *G. clavigera*. This allowed a direct comparison between the resulting lesions caused by the two treatments within the same tree.

176 *Volatile Collection and GC Analysis.* To determine tree chemical response to the water and
177 biological treatments, we collected VOCs emitted from the bole of each tree at the following
178 time points: one day before application of biological treatments, four days, two, three, five,
179 seven and nine weeks post biological treatments. To enable volatile collection, two strips of
180 1cm thick foam material (Quilting foam, Fabricland, Edmonton, AB) were attached to each
181 tree: one 15cm above and one 15cm below the biological treatment application site on the
182 tree bole. An oven bag (LOOK®, 45×55cm) made of inert material was cut open and wrapped
183 around the tree covering both pieces of foam and secured. An adsorbent tube (Porapak Q (OD
184 6mm, length 110mm; absorbent: front layer 150mg, back up layer 75mg; separated by glass
185 wool) SKC Inc., Pennsylvania, USA) was inserted underneath the foam into the space
186 covered by the oven bag and attached to a pump secured to the tree with Velcro. Bole
187 volatiles were collected for 1h at a flow rate of 1L/min before biological treatment
188 application. Emission rates of VOCs were greatly enhanced after biological treatment
189 applications, and therefore collection time was reduced to 15min. After VOC collection, the
190 sorbent tubes were capped and stored on ice and transferred to a freezer in the lab at -40°C
191 before extraction. Porapak Q tubes were extracted with 1mL of dichloromethane (Sigma-
192 Aldrich, St. Louis, Missouri, USA) spiked with 0.01% (v/v) tridecane (Sigma-Aldrich, St.
193 Louis, Missouri, USA) as surrogate standard and subsequently stored at -40°C before GC
194 analysis.
195 Extracted VOC samples (1μL) were injected in an Agilent 7890A Gas Chromatograph
196 (Agilent Technologies, Santa Clara, California, USA) with an HP Innowax column (I.D.
197 0.32mm, length 30m; Agilent Technologies), helium carrier gas flow at 1.8mL/min,
198 temperature 50°C for 2min, increased to 160°C by 5°C per min and then ramped up to 250°C
199 by 20°C. Peaks were identified using the following standards: Borneol, pulegone, α-
200 terpinene, γ-terpinene, α-terpineol (Sigma-Aldrich, St. Louis, Missouri, USA), camphor, (+)-

3-carene, α-humulene, terpinolene, α- and β-thujone, (-)-α-pinene, (-)-β-pinene, (*S*)-(-)-limonene, sabinene hydrate, myrcene, (-)-camphene, *p*-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, *cis*-ocimene (SAFC Supply Solutions, St. Louis, Missouri, USA), β-phellandrene (Glidco Inc., Jacksonville, Florida, USA). Calibration with these standards allowed for analysis of quantitative differences of volatile samples at different time points among treatments. The total monoterpene emission was log(x+1) transformed to meet the assumptions of normality. A two-way repeated measures ANOVA was conducted to account for repeated measurements of volatile emission from the same trees over the course of the experiment. The last two time points were omitted from the analysis because they did not meet the assumption of homogeneity and transformation did not resolve the matter. Therefore, the composition of monoterpene emissions at all time points as impacted by water and biological treatments of mature trees was analyzed using a canonical redundancy analysis with the rdaTest package (Legendre and Durand 2010) in R (R development core team, 2011). RDA axes were tested for significance by permutations with the vegan package (Oksanen et al. 2010). Explanatory variables included water and biological treatments, as well as the following tree variables: DBH, phloem thickness, and age. Temperature and humidity from a nearby Environment Canada weather station for the days of volatile collection were also used as explanatory variables. The quantities of all individual monoterpene released at all time points were the response variables.

Tissue Samples and GC/MS Analysis. At the end of the experiment, nine weeks post biological treatment application, all experimental trees were harvested and lesion dimensions from inoculated trees were recorded. Phloem tissue was sampled from the area between two lesions and needles were collected from mid-crown of the tree, shock frozen in liquid nitrogen and transferred onto dry ice before storage at -40°C in the lab prior to extraction.

226 Tissue was ground in liquid nitrogen, and 100mg of the tissue was transferred to a 1.5mL
227 microcentrifuge tube and extracted as described in Lusebrink et al. (2011). Extracts were
228 transferred into amber GC vials (Agilent Technologies) and stored at -40°C before Gas
229 Chromatograph/Mass Spectrometer (GC/MS) analysis.
230 Extracts of tissue samples (3µL) were injected at a split ratio of 20:1 in an Agilent
231 7890A/5062C GC/MS (Agilent Technologies; the MS was not available during VOC
232 collection) with a HP-Chiral-20B column (I.D. 0.25mm, length 30m; Agilent Technologies),
233 helium carrier gas flow at 1.1mL/min, temperature 75°C for 15min, increased to 230°C by
234 5°C. Calibration with the standards used in GC-analysis and additionally: 4-Allylanisole
235 (also named estragole), (+)-α-pinene, (+)-β-pinene, (*R*)-(+)-limonene (Fluka, Sigma-Aldrich,
236 Buchs, Switzerland), and α-phellandrene (SAFC Supply Solutions, St. Louis, Missouri, USA)
237 allowed the quantification of tissue chemical content, as well as the analysis of differences in
238 stereoisomer composition of the differently treated trees.
239 Total carbon (dry combustion) and total nitrogen (Dumas combustion method) of
240 phloem samples were determined by the Natural Resources Analytical Laboratory
241 (Department of Renewable Resources, University of Alberta, Edmonton, Canada) using an
242 ECS 4010 Elemental Combustion System CHNS-O (Costech Analytical Technologies Inc.,
243 Valencia, California, USA).
244 Lesion lengths from the variously inoculated trees were compared using a two-way
245 nested ANOVA with lesion type (fungal and MPB mash caused lesion) specified as a random
246 factor nested within biological treatment. The total and individual amounts of monoterpenes
247 extracted from phloem did not fulfil the assumptions of normality or homogeneity even after
248 transformation. Therefore the data were analysed using non-parametric Kruskal-Wallis
249 followed by Mann-Whitney U tests as a post-hoc procedure. Additionally, we conducted a
250 principal component analysis (PCA) based on the ratios of the individual monoterpenes. The

phloem chemistry data obtained in the current study were compared to those of pure lodgepole and jack pine trees retrieved in the same way from a related field study conducted in 2010 and not reported here. The quantities of monoterpenes extracted from needles met the assumptions of an ANOVA after log (x+1) transformation. A MANOVA was conducted on the needle data that included individual and total monoterpenes as response variables.

Total nitrogen and carbon content of the phloem from the variously treated trees were compared with a two-way ANOVA followed by Tukey's HSD as post-hoc test in order to determine the differences among biological treatments.

Beetle Condition in Experimentally Manipulated Trees. To test the effect of water and biological treatments on the condition of MPB, a 50cm bolt was harvested from just above the inoculation site of each inoculated tree. Bolts were cut from untreated control trees at the same height. All bolts were transported to the laboratory where both ends of each bolt were covered in paraffin wax to avoid desiccation. They were stored in a walk in growth chamber (22°C, 50% humidity, 16h light/ 8h dark). Each bolt was inoculated with four pairs of live female and male MPB. One female MPB was introduced to each of 4, 1.5mL microcentrifuge tubes attached to the lower portion of the bolt. When it had burrowed in, a male MPB was added. Beetles were replaced if introduction was not successful. Bolts inoculated with beetles were kept in the growth chamber for 4-5 weeks to allow early larval instar development and then were subjected to a cold period at 4°C and constant darkness to emulate winter conditions. Following a 3 month cold period, bolts were transferred into rearing bins in the growth chamber to allow the offspring of the mating pairs to complete their development. Emerging adult beetles were measured for fresh weight, size (pronotum width and total body length) and sex and then were killed and stored in a freezer. Dead beetles were oven dried for 24h at 60°C before extraction with petroleum ether using a 250mL soxhlet apparatus to

276 determine fat content as percentage of removed dry weight. Fat content of emerged beetles
277 was compared among treatments with a 2-way ANOVA followed by Tukey's HSD as post-
278 hoc test. After all beetles emerged, the number of larval galleries was also assessed for all
279 bolts. Age of harvested trees was determined by scanning a cross section harvested from the
280 base of each tree on a flatbed scanner followed by analysis with WinDENDRO™ (Regent
281 Instruments Inc., Quebec, Canada).
282
283 RESULTS
284
285 *Soil Water Content.* Soil water content differed significantly with water treatment applied to
286 trees ($F_{(2,6.217)}$= 11.674, P= 0.008; Fig. 1), but not with soil depth ($F_{(6,\ 36.010)}$= 1.558, P=
287 0.188). Soil water content was lower across all sample depths in the soil surrounding water-
288 deficit trees compared to well-watered trees; the latter did not differ from SWC in the rest of
289 the field site.
290
291 *Volatile Emission.* As compared to VOCs emitted from pure species (Jost et al. 2008;
292 Lusebrink et al. 2011; Pureswaran et al. 2004; Rhoades 1990), those emitted from the hybrid
293 trees in the current study represent a mixture of pure lodgepole and jack pine monoterpene
294 profiles (Table 1).
295 Total monoterpene emission was significantly influenced by both water ($F_{(1,32)}$=
296 5.250, P= 0.029; Fig. 2a) and biological ($F_{(3,32)}$= 62.434, P< 0.001; Fig. 2b) treatments. Post-
297 hoc tests with Bonferroni correction revealed that trees that experienced a water deficit
298 released more total monoterpenes than well-watered trees (Fig. 2a).Trees that were inoculated
299 with the fungus *G. clavigera*, MPB mash or were mechanically wounded, emitted
300 significantly greater amounts of monoterpenes than untreated control trees (P< 0.001). The

emission of volatiles from trees inoculated with fungus at 10 points around the bole did not differ significantly from trees inoculated with fungus and MPB mash, each at 5 points around the bole. The level of monoterpene emission from mechanically wounded trees without inoculum was significantly lower than fungal- inoculated trees ($P< 0.001$) but similar to trees treated with MPB mash and fungus together (Fig. 2b). Emission of monoterpenes from trees also varied over time ($F_{(3.026,96.828)}= 78.592$, $P< 0.001$), with a sharp increase in emission following biological treatment applications (Fig. 2c). There was also a significant interaction between sampling time point and biological treatments ($F_{(9.078,96.828)}= 14.041$, $P< 0.001$; Fig. 2d). Monoterpene emission among treated trees was similar shortly after treatment application. The emission went down over time in MPB-mash/fungus-inoculated and wounded trees, whereas fungal-inoculated trees continued to emit monoterpenes at high levels until the end of the 3rd week. Even in fungal-inoculated trees, the VOC emission sharply declined by the 5th week following inoculation.

A canonical redundancy analysis (RDA) tested the influence of water and biological treatments on monoterpene emissions from the variously treated trees. The RDA triplot illustrates the relationship between the explanatory variables and the emission of individual monoterpenes from mature hybrid pine trees (Fig. 3). The correlation triplot could be considered as one dimensional because the first axis of the RDA was significant ($P= 0.001$) but the second axis was only moderately significant ($P= 0.092$) and explained less than 1% of the variation in the RDA. Emission of α-pinene, 3-carene, β-pinene and β-phellandrene was mainly correlated with fungal inoculation and water deficit, as well as with high ambient humidity and temperature.

Lesion Length. Lesions caused by inoculation with MPB-mash were shorter than those caused by fungal inoculation ($F_{(1,25)}= 16.246$, $P< 0.001$; Fig. 4).

Tissue Extracts. One of the trees in the water deficit × fungal inoculation treatment was naturally attacked by MPB during our experiment and was removed from the analysis. The phloem chemical profile of the ten control trees consisted of: β -Phellandrene (45.7%±6.68 SE), (+)-α-pinene (14.3%±3.09 SE), (*S*)-(-)-limonene (9.4%±4.47SE), 3-carene (9.3%±2.05SE), (-)-α-pinene (5.6%±1.55SE), (-)-β-pinene (5.0%±0.95SE), myrcene (3.2%±0.31SE), and 7.5% other monoterpenes, which contributed less than 2.5% each to the chemical profile. The separation factor (α= retention time$_2$/retention time$_1$) for α-pinene was α=1.03, for β-pinene, α=1.03, and for limonene, α=1.02.

There was no difference in total monoterpene concentration of extracted phloem tissue based on water treatments of the tree. Further analyses were conducted on trees pooled from both water treatments, which showed that the biological treatments significantly affected the amount of myrcene ($H(3)$= 8.864, P= 0.031; Fig. 5a) and 3-carene ($H(3)$= 8.130, P= 0.043; Fig. 5b) in the phloem. Phloem tissue from trees treated with the MPB-mash/fungus treatment contained significantly more myrcene than mechanically-wounded and untreated control trees. 3-carene was higher in the phloem of MPB-mash/fungus-treated trees than wounded trees but did not differ statistically from that found in the phloem of control or fungal-inoculated trees. Fungal-inoculated trees had an intermediate amount of both compounds (Fig. 5a, b). Likewise, biological treatments caused a marginal increase in terpinolene and borneol in the phloem ($H(3)$= 7.632, P= 0.054 and $H(3)$= 7.568, P= 0.056).

Total monoterpenes occurred in higher concentrations in the needles of trees that experienced a water deficit ($F_{(1,37)}$= 6.437, P= 0.016, Fig. 5c). This significant difference was caused by elevated levels of major individual monoterpenes, such as (-)- and (+)-α-pinene (F=7.660, P= 0.009 and F=6.126, P= 0.018), myrcene (F=8.640, P= 0.006), 3-carene (F=6.063, P= 0.019), (-)-β-pinene (F=4.632, P= 0.038) and bornyl acetate (F=9.113, P=

0.005). The water treatment had no influence on the amount of β-phellandrene (P= 0.237), the signature compound of lodgepole pine.

The principal component analysis (PCA) conducted on the phloem chemistry data separated lodgepole and jack pines from each other (Fig. 5d). The hybrid trees analysed in the current study, clustered in-between the two pure species. Some of the hybrid trees showed more similarity with lodgepole pine than jack pine.

Elemental Analysis. Total carbon content of phloem tissue from the variously treated trees was not influenced by either the water or biological treatments. In contrast, total nitrogen was significantly affected by the biological treatments ($F_{(3,32)}$= 5.693, P= 0.003, Fig. 6) and was higher in tissues from fungal-inoculated trees (P= 0.003) than in control or MPB mash/fungus trees. Wounded trees had an intermediate level of nitrogen in the phloem (Fig. 6). The phloem of watered trees contained marginally higher amounts of N than trees that experienced a water deficit ($F_{(1,32)}$= 3.573, P= 0.068).

Beetle Condition in Experimentally Manipulated Trees. Overall 329 viable adult beetles, 210 females and 119 males, emerged from 39 bolts, 67 of them on the same day. Neither biological nor water treatment affected the number of beetles that emerged, their fresh weight, size or the number of larval galleries. However, the fat content of female beetles reared in bolts from the variously treated trees was significantly affected by the water treatment applied to standing trees ($F_{(1,202)}$= 11.185, P= 0.001; Fig. 7). Female beetles, which are the pioneering sex in MPB, had a higher fat content when they emerged from water-deficit trees (23.86% ± 8.47 SD) compared to the well-watered trees (19.53% ± 9.60 SD). As expected, male beetles contained less fat than females (15.86% ± 7.79 SD) and their fat content was not influenced by biological or water treatments.

DISCUSSION

The stem volatiles emitted from the experimental trees in the current study represent a mixture of pure lodgepole and jack pine monoterpene profiles, although the chirality of these compounds was not measured (Jost et al. 2008; Lusebrink et al. 2011; Pureswaran et al. 2004; Rhoades 1990). A recent genotyping study of lodgepole, jack pine and their hybrids revealed that the ancestry of hybrid trees in central Alberta is biased towards lodgepole pine (Cullingham et al. 2012). The phloem chemical composition of the majority of the hybrid trees tested in this study appears closer to that of pure lodgepole than pure jack pine. MPB shares a long co-evolutionary history with lodgepole pine (Kelley and Farrell 1998) and thus has adapted to exploit the secondary chemistry of this host species (Keeling and Bohlmann 2006; Boone et al. 2011). The most recent establishment of MPB in hybrid trees and a few jack pine trees within the hybrid zone (Cullingham et al. 2011) might facilitate subsequent colonization of jack pine trees in the eastern boreal forest. The phloem chemistry of hybrid trees resembles a mixture of both pure species and may therefore provide the perfect stepping stone to enable further range expansion of MPB. Preference for chemical similarity during a host shift occurs in several beetle-plant relationships (Becerra 1997; Futuyma et al. 1995), including one other *Dendroctonus* species, *D. valens* (Erbilgin et al. 2007).

Drought conditions can influence insect-plant interactions (Mattson and Haack 1987) and may influence the rapidity of range expansion by MPB (Alfaro et al. 2010). Soil water content was significantly lower around water-deficit trees as compared to well-watered trees, showing that water treatments altered water availability to plants. Water limitation and the biological treatments of plant defence stimulation affect the emission of stem volatiles in the current field study on mature lodgepole × jack pine hybrids. Monoterpene emission from the

401 stem of host trees is hypothesized to be more relevant for MPB host-finding and colonization
402 behaviour than emission from foliage, since beetles mostly constrain their flight to the lower
403 bole of their pine hosts (Seybold et al. 2006). Water-deficit and fungal inoculation correlated
404 with the emission of α-pinene and 3-carene from the stem of lodgepole × jack pine hybrid
405 trees in this study. Similar results were found in a previous study with pine seedlings
406 (Lusebrink et al. 2011). Both compounds act as kairomones (Borden et al. 2008) to the MPB
407 through synergy with the aggregation pheromone and may make the emitting host tree more
408 attractive to aggregating beetles.

409 Plants exposed to mild and moderate droughts are expected to shift carbon allocation
410 toward the production of secondary metabolites, like monoterpenes (Monson et al. 1995).
411 This hypothesis is supported by the chemical analysis of the current needles in which needles
412 from water-deficit trees contain a higher monoterpene concentration than needles from well-
413 watered trees. The results are in accordance with previous studies that show that drought
414 stress increases the concentration of monoterpenes in the needles of several conifers,
415 including Scots pine (*Pinus sylvestris*; Turtola et al. 2003), Aleppo pine (*Pinus halepensis*;
416 Llusia and Penuelas 1998), ponderosa pine (*Pinus ponderosa*; Johnson et al. 1997), and
417 Norway spruce (*Picea abies*; Kainulainen et al. 1992). A study on loblolly pine in Louisiana
418 (Lombardero et al. 2000) also concluded that drought is more likely to increase than decrease
419 tree defences. However, under severe drought conditions carbon assimilation, and therefore
420 the production (Herms and Mattson 1992) and emission (Blanch et al. 2007) of secondary
421 metabolites, such as monoterpenes, is predicted to decline.

422 The length of necrotic lesions in the phloem in response to fungal inoculation is a
423 commonly used measure of tree resistance or fungal virulence (Krokene et al. 2008; Rice et
424 al. 2007b). Resistant trees show a more efficient defence response which restricts fungal
425 growth more swiftly inside shorter lesions than susceptible trees (Krokene and Solheim 1998;

426 Raffa and Berryman 1983), and therefore longer lesions may indicate better performance of
427 the fungus (Lieutier et al. 2004; Masuya et al. 2003; Rice et al. 2007b). Unlike water
428 treatment, inoculation type had an impact on lesion formation in our study. In the MPB-
429 mash/fungus treatment, inoculation with mashed MPB and *G. clavigera* were alternated
430 around the bole of the same tree in order to directly compare the lesions created by
431 inoculation with all cold hardy MPB-associated microorganisms (e.g. Rice et al. 2008,
432 Adams et al. 2008) or by *G. clavigera* alone. MPB-mash causes smaller lesions than
433 inoculation with *G. clavigera* suggesting that the fungus alone is more virulent than the mash,
434 most likely because of a higher inoculum load. Tree defence response was more efficient
435 against the MPB mash and this response was specific. The monoterpene myrcene is evoked
436 at significantly higher levels in the mash treatment compared to controls, which indicates that
437 myrcene is important for tree defence against microbes associated with the MPB (Bonello
438 and Blodgett 2003). 3-carene was also present in phloem surrounding lesions created by
439 inoculation with the MPB-mash/fungus treatment, though not at significantly higher
440 concentrations than in wounded trees. 3-Carene, in particular, might play an important role in
441 tree defence in mature lodgepole (Ott et al. 2011) and jack pines (Raffa and Smalley 1995),
442 as its concentration in the necrotic lesion tissue increases upon inoculation with bark beetle-
443 associated fungi. Ironically, MPB uses both these defensive compounds: myrcene and 3-
444 carene as kairomones for host location, since both synergize beetle response to the
445 aggregation pheromone *trans*-verbenol (Borden et al. 2008). Similarly, the pine shoot beetle,
446 *Tomicus piniperda*, identifies susceptible trees based on increased defensive compounds of its
447 host tree (Byers et al. 1985).
448 Even though high monoterpene levels are toxic to many herbivores (Langenheim
449 1994), the MPB has evolved to overcome these defences through aggregation and mass attack
450 (Pitman et al. 1968). The MPB-associated fungal symbionts help the beetle to detoxify

phloem monoterpenes via fungal metabolism and may also use some monoterpenes as a carbon source (DiGuistini et al. 2011; Wang et al. 2013). The symbionts also benefit the MPB by enhancing phloem nutrition (Bleiker and Six 2007; Goodsman et al. 2012). The hyphae of fungal symbionts of *Dendroctonus* species can increase nitrogen levels in brood galleries of bark beetle larvae through redistribution of nitrogen from the sapwood and distant phloem (Ayres et al. 2000; Bleiker and Six 2007). In the current study, phloem nitrogen levels are significantly higher in fungal-inoculated trees compared to the control and MPB-mash/fungus treatments. Likewise, *Entomocorticium* sp., one of the mycangial fungi associated with the southern pine beetles, *Dendroctonus frontalis*, concentrates nitrogen around larval galleries (Ayres et al. 2000). Consequently, *D. frontalis* associated with *Entomocorticium* sp. develop into larger adult beetles with higher fat content (Coppedge et al. 1995).

In this study, female beetles that emerged from bolts of trees that received the water-deficit treatment had a higher fat content than beetles reared in bolts from well-watered trees. This is the first experimental evidence that MPB directly benefits from water-deficit conditions. Higher fat content in bark beetles is expected to positively influence dispersal, colonisation, and reproductive success (Graf et al. 2012). Atkins (1966, 1975) found that Douglas-fir beetles, *Dendroctonus pseudotsugae*, behave differently depending on their fat content: 1) beetles with a high fat content (above 20%) have the tendency to disperse and respond less to host volatiles; 2) beetles with intermediate fat content (11-20%) respond immediately to host volatiles and are good flyers; and 3) beetles with low fat content (10% and below) fail to fly. Female MPB from the water-deficit treatment have a fat content that is higher than 20%, whereas the beetles from the well-watered treatment contain less than 20% fat. If there is a similar relationship between fat content and behaviour in MPB, water-deficit would enhance dispersal and therefore range expansion; but additional studies are need to

476 establish a link between fat content and MPB behaviour. Bark beetles can benefit from the
477 effects of drought on host trees through: elevated nutrient levels, increased emission of plant
478 volatile attractants, reduced oleoresin exudation pressure, and improved conditions for their
479 symbionts (Mattson and Haack 1987). Since we artificially introduced and reared beetles in
480 bolts, volatile attractants and resin pressure can be excluded as possible reasons for the link
481 between high fat content and water-deficit. Nitrogen levels were not the cause of this result,
482 since phloem nitrogen levels are higher in well-watered than water-deficit trees. Other
483 nutrients may play an important role and further research on the benefits of drought on bark
484 beetle performance is needed.

485 The range expansion of the MPB and the accompanying colonization of jack pine as a
486 new host could alter beetle pheromone production and their ability to mass attack trees as
487 tree-produced α-pinene is the precursor of the MPB aggregation pheromone *trans*-verbenol
488 (Blomquist et al. 2010). The enantiomeric composition of terpenoid bark beetle pheromones
489 depends on the stereochemistry of the precursor, the enantiomeric-specificity of the
490 synthesizing enzymes, and enantiomeric-specific olfactory receptors (Byers 1989). In MPB (-
491)-*trans*-verbenol elicits a significantly higher response than (+)-*trans*-verbenol (Whitehead et
492 al. 1989), for which the (-)-isomer of α-pinene most likely acts as a precursor (Vaněk et al.
493 2005). Unfortunately, there are barely any studies on the chirality of chemical profiles of
494 mature lodgepole or jack pine. Pureswaran et al. (2004) reported that the bole of mature
495 lodgepole pine emits 67.7% of (-)-α-pinene. We found that the phloem of mature hybrids
496 contains 36.8% (-)-α-pinene and 63.2% (+)-α-pinene, which might have a negative impact on
497 pheromone production and attractiveness. Furthermore, jack pine occurs on extremely well-
498 drained, nutrient-poor soils (Vidacović 1991; Kenkel et al. 1997), and the response to drought
499 in jack pine might differ from that observed in lodgepole × jack pine hybrids in the current

study. Therefore, further research on the effect of drought on tree defence response of pure jack pines and the chemically-mediated interactions with MPB needs to be conducted.

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742 ZHAO, T., KROKENE, P., HU, J., CHRISTIANSEN, E., BJÖRKLUND, N., LÅNGSTRÖM, B., SOLHEIM,
743 H., and BORG-KARLSON, A.-K. 2011. Induced terpene accumulation in Norway spruce inhibits bark beetle
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745 **Table 1** Percentages of selected monoterpenes as part of the entire monoterpene profile
746 emitted or extracted from the boles of mature lodgepole, jack and lodgepole × jack pine trees
747 or entire seedlings determined in this and earlier studies.

Species	α-Pinene	β-Pinene	3-Carene	Limonene	β-Phellandrene	Source	Reference
lodgepole	8	9	7	3	49	bole VOCs	Rhoades 1990
pine	17	35	n.a.	6	30	bole VOCs	Jost et al. 2008
	5	16	6	6	50	bole extracts	Pureswaran et al. 2004
	7	23	7	4	27	seedling VOCs	Lusebrink et al. 2011
jack pine	91	7	n.a.	1	1	bole VOCs	Jost et al. 2008
	27	21	21	9	1	seedling VOCs	Lusebrink et al. 2011
hybrids	80	10	n.a.	5	1	bole VOCs	Jost et al. 2008
	46	8	17	3	16	bole VOCs	this study
	21	7	11	9	42	bole extracts	this study

n.a.= data not available

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Figure legends

Fig. 1 Soil water content (mean ± 95% CI) at three different soil depths (30, 60, and 90cm) over the time course of the experiment. Bars with non-overlapping error bars are significantly different from each other

Fig. 2 Bole monoterpene emission (mean ± SE) from mature lodgepole × jack pine hybrids as a result of different (a) water treatments (b) biological treatments (c) sampling time points (d) interaction of sampling time point and biological treatments. Different lowercase letters indicate a statistically significant difference ($P< 0.05$, repeated measures ANOVA)

Fig. 3 Canonical redundancy analysis (RDA) triplot (scaling 2) illustrating the influence of water and biological treatments as well as tree characteristics (age, phloem thickness, and DBH) and climate variables (temperature and humidity) on volatile emission of individual monoterpenes in mature lodgepole × jack pine hybrids

Fig. 4 Length of lesions (mean ± 95% CI) caused by inoculation with different biological treatments under water-deficit (light grey bars) and well-watered (dark grey bars) conditions. Bars with non-overlapping error bars are significantly different from each other across both panels

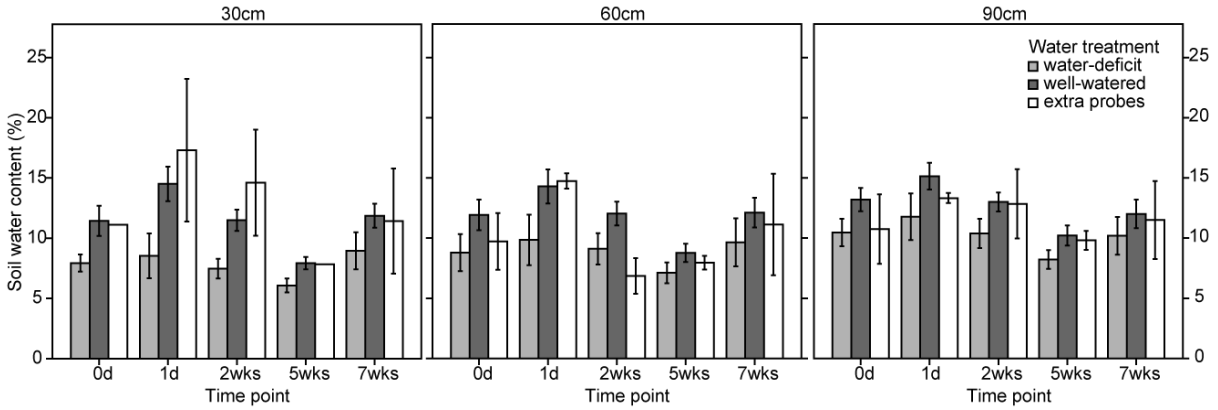
Fig. 5 Myrcene (a) and 3-carene (b) concentrations (mean ± SE) in the phloem of mature lodgepole × jack pine hybrids in response to biological treatments. Bars marked with different lowercase letters indicate a statistically significant difference ($P< 0.0125$, Mann-Whitney U test with Bonferroni correction). Water treatments affected total monoterpene

content (mean ± SE) of needles of mature lodgepole × jack pine hybrids (c) ($P< 0.05$, ANOVA). Principal component analysis plot showing the separation of lodgepole and jack pine based on their phloem chemistry and the position of lodgepole × jack pine hybrids as intermediate (d)

Fig. 6 Total nitrogen content (mean ± SE) of the phloem of mature hybrid trees in response to biological treatments. Bars marked with different lowercase letters indicate a statistically significant difference ($P< 0.05$, ANOVA)

Fig. 7 Fat content (mean ± 95% CI) of beetles reared in bolts from experimental trees receiving different water treatments. Bars marked with different lowercase letters indicate a statistically significant difference ($P< 0.05$, ANOVA)

787 Figure 1



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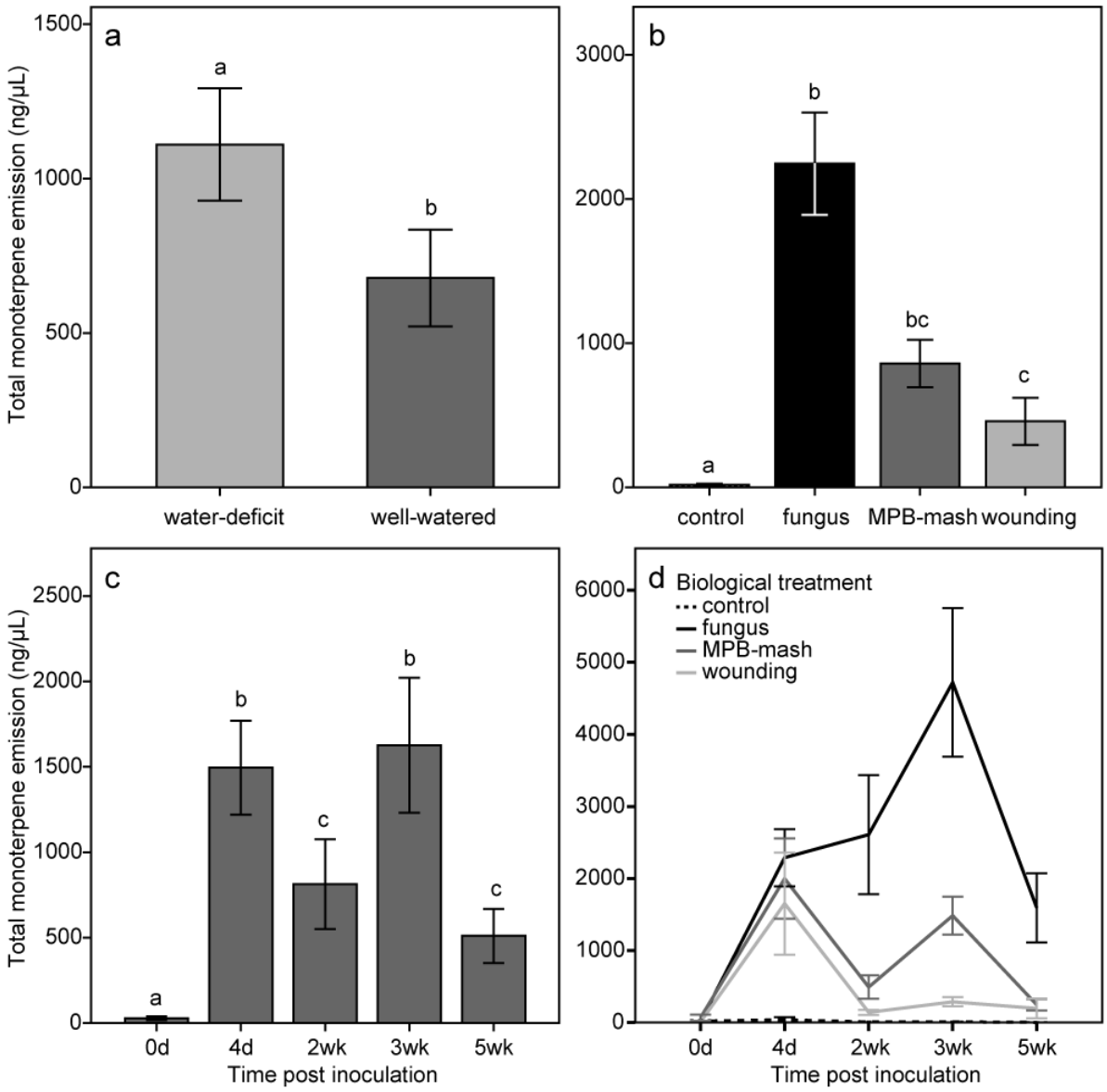

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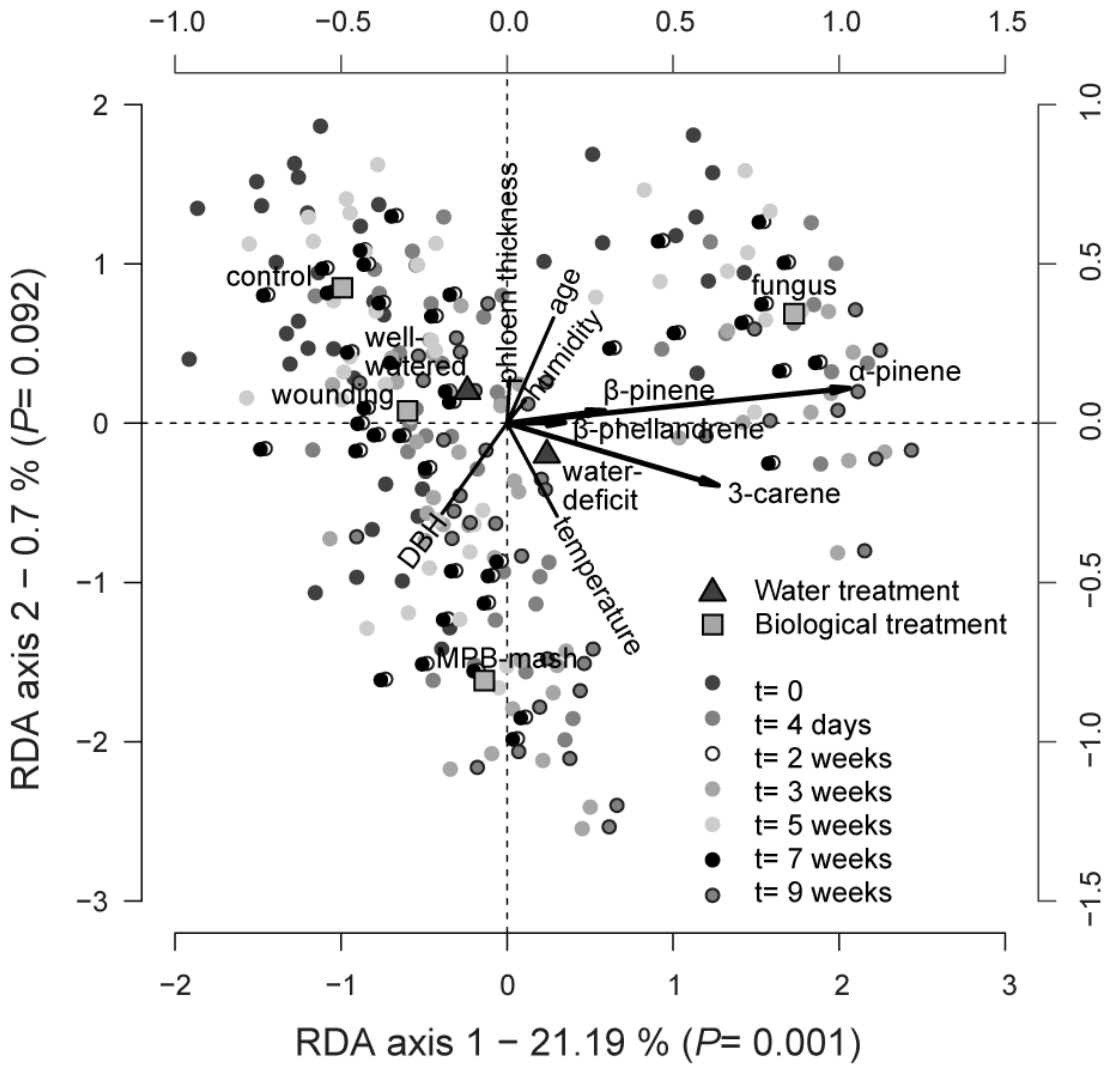

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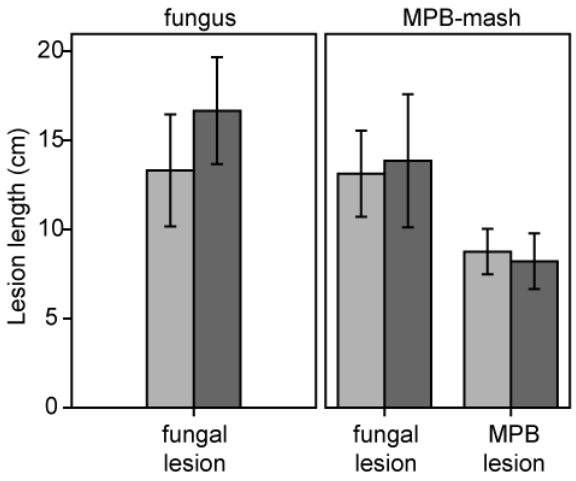

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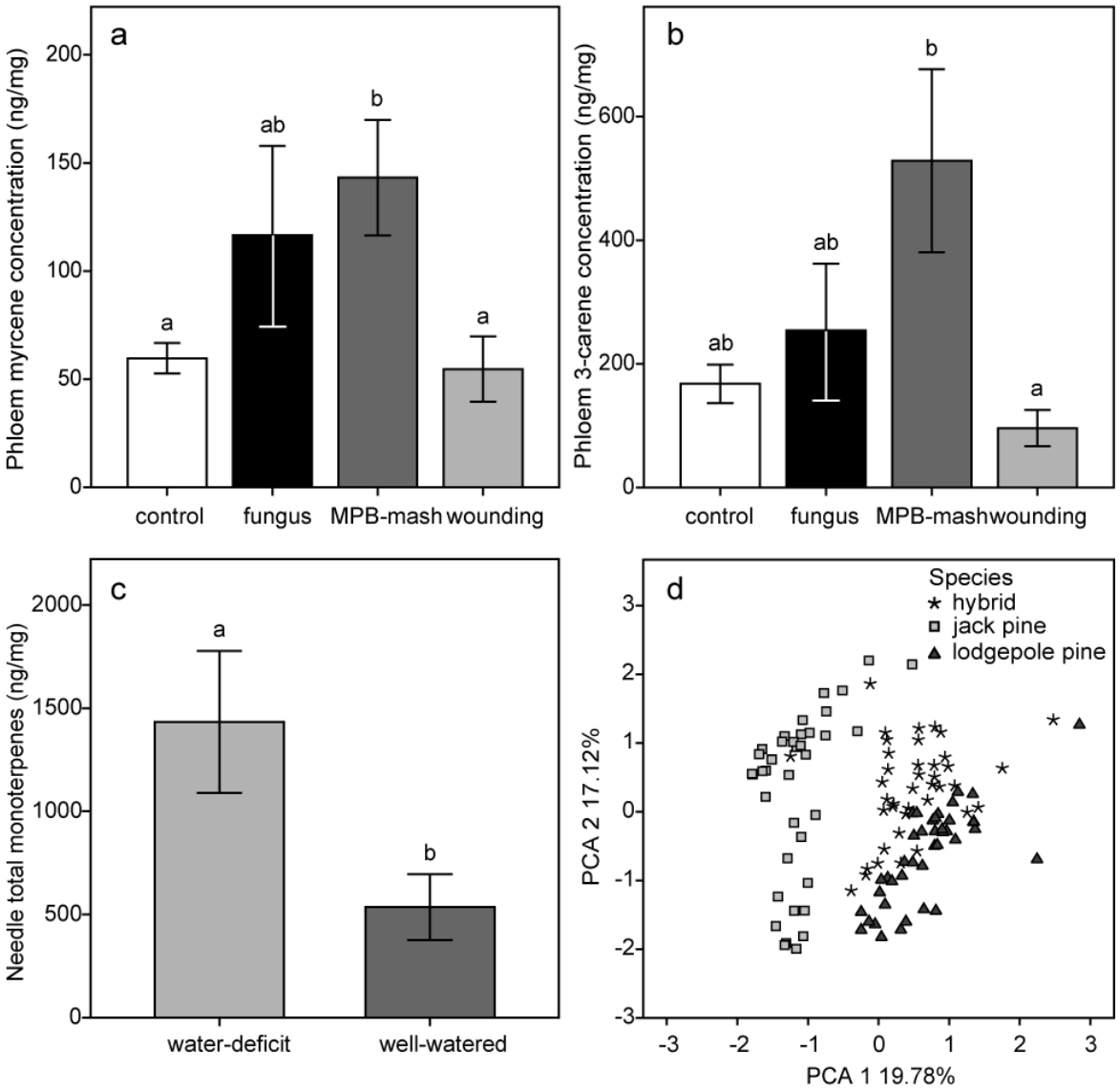
799 Figure 5



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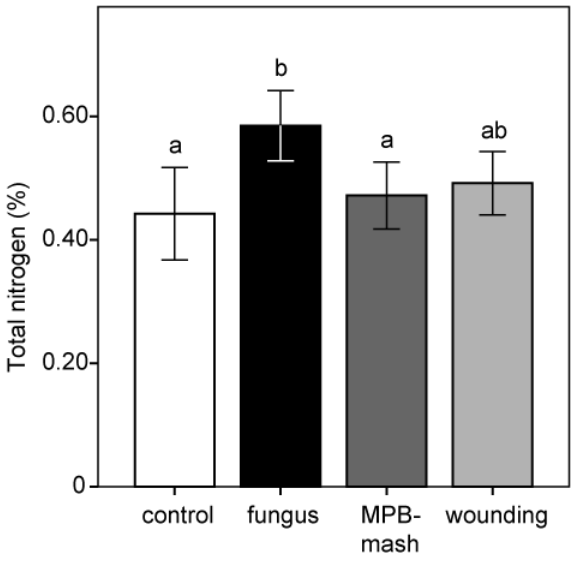

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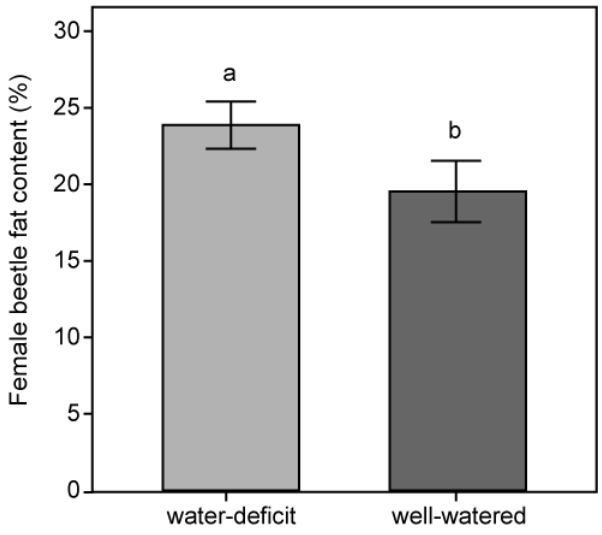
805 Figure 7



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