Corresponding author:

Diana L. Six
Department of Ecosystem and Conservation Sciences
The University of Montana
Missoula, MT 59812
Diana.six@cfc.umt.edu
Tel: 406.531.1813

**Temperature effects on growth of fungal symbionts of the western pine beetle,
*Dendroctonus brevicomis***

Joseph C. Dysthe, Ryan Bracewell, and Diana L. Six
*Department of Ecosystem and Conservation Sciences,
The University of Montana, Missoula, MT 59812*

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ABSTRACT:

Differences in temperature ranges and optima among poikilothermic partners in symbioses can have profound effects on their interactions and stability. In this study, we investigated how the two mutualist mycangial fungi (*Ceratocystiopsis brevicomi* and *Entomocorticium* sp. B) associated with the western pine beetle, *D. brevicomis*, respond to temperature *in vitro*. Little variability in growth rate at the various temperatures tested occurred among isolates of *C. brevicomi* either within or among sites. In contrast, *E. sp. B* exhibited highly variable responses

to mid-range temperatures among sites and within some sites, and, unlike *C. brevicomi*, grew not at all or only very poorly at the highest and lowest temperatures tested. This variability affected both optimal temperature and maximum growth rate. The high variability in response to some temperatures among isolates of *E. sp. B* in some populations indicates that the ability to capture spatial and nutritional resources can vary greatly within this species which may have considerable impact on the outcome of both inter- and intra-specific competition among the fungi within trees and the short- and long-term dynamics of the fungi with the host beetle.

Introduction

Several species of bark beetles (Coleoptera: Curculionidae, Scolytinae) in the genus *Dendroctonus* are associated with mutualistic fungi that provide nutrient supplementation to their host insects (Six and Paine, 1998, Ayres et al. 2000, Bleiker and Six 2007, Cooke et al. 2010). These fungi are transported from tree to tree by adult beetles in specialized structures (mycangia) maintaining the continuity of the association among generations (Whitney and Farris 1970, Six 2003). *Dendroctonus*-mycangial fungus symbioses typically involve two, or less commonly, three fungal partners specific to each beetle host (Lee et al. 2005, Rice and Langor 2009, Six 2012). The various fungal partners exert differential effects on the host insect's fitness with one typically superior to the other(s) (Goldhammer et al. 1990, Coppedge et al. 1995, Bleiker and Six 2007). The relative prevalence of each fungus is, therefore, predicted to affect the host beetle's population dynamics (Six and Bentz 2007).

The factors that affect the relative prevalence of mycangial fungi within and among populations of a host beetle remain poorly studied. However, recent work indicates that temperature plays a major role (Hofstetter et al. 2006, Six and Bentz 2007, Rice et al. 2008,

Addison et al. 2013). This should not be surprising as fungi are poikilotherms, with their growth rates and sporulation highly dependent upon temperature. The effect of temperature on *Dendroctonus*-mycangial fungus symbioses has only been studied in detail in one system. The relative prevalence of the two fungi associated with developing and dispersing *D. ponderosae* (mountain pine beetle) (a univoltine beetle) is highly dependent upon temperature (Six and Bentz 2007, Addison et al. 2013). One partner, *Grosmannia clavigera*, grows best and dominates under cooler conditions, while the opposite is true for the other partner, *Ophiostoma montium*. These differences lead to dissimilar rates of growth and resource capture by the fungi in the host tree over the larval development period as well as differential rates of dissemination when brood adults disperse (Six and Bentz 2007). It also results in some beetle populations being dominated by one fungus, although both are typically present (Six and Bentz 2007). The temperature dependent nature of this symbiosis is predicted to provide a mechanism for the maintenance of its multipartite structure (Addison et al. 2013); temperature fluctuations within and among years constraining any one fungus from completely dominating over time. It also implies that global warming has the potential to decouple the symbiosis as one or both fungi are marginalized or lost as conditions become too warm to support their growth and/or reproduction (Addison et al. 2013).

Temperature also likely plays an important role in the dynamics of other *Dendroctonus*-fungus symbioses. For example, the two mycangial fungi associated with *D. frontalis* (southern pine beetle) (a multi-voltine species with as many as seven generations a year) fluctuate in prevalence among generations over the course of a year in response to temperature (Hofstetter et al. 2006). An understanding of the effects of temperature on the symbionts is critical to understand how *Dendroctonus*-fungus symbioses have remained stable over evolutionary time,

how environmental conditions influence short- and long-term fluctuations in symbiont prevalence, and hence beetle fitness and population dynamics, and how climate change may affect the continued persistence of these symbioses.

In this study, we investigated how the two mycangial fungi associated with *D. brevicomis* (western pine beetle) respond to temperature *in vitro*. This system is similar to the *D. ponderosae*-fungus system in that two mycangial fungi are associated with the beetle across its geographic range (Bracewell and Six 2014). However, it differs in several important ways. Instead of feeding entirely within the phloem layer as larvae as does *D. ponderosae*, *D. brevicomis* feeds in phloem only briefly before moving into the outer bark. In addition, the mycangia of *D. ponderosae* are located on the mouthparts and are functional in both sexes (Whitney and Farris 1970), while the mycangia of *D. brevicomis* are located on the anterior pronotum and are functional only in females (Whitney and Cobb 1972, Paine and Birch 1983). This indicates an independent origin of the structures and symbiosis with fungi. The fungi associated with the two beetles are also quite different. The fungi associated with *D. ponderosae* (*G. clavigera* and *O. montium*) are bionecrotrophic blue-staining ascomycetes in the Ophiostomatales. In contrast, *D. brevicomis* is associated with a non-staining saprotrophic ascomycete in the Ophiostomatales, *Ceratocystiopsis brevicomi*, and a saprotrophic basidiomycete, *Entomocorticium* sp. B, in the Corticiales (Hsiau and Harrington 1997, 2003).

Only one paper to date has looked at growth rates of the fungi associated with the western pine beetle. Davis et al. (2010) compared growth of isolates of the two mycangial fungi from a population of the beetle in California with isolates from a population in Arizona. Growth rates were determined from radial growth measurements, which may be problematic with *E. sp. B* given the erratic rather than uniform growth margins it produces in culture. The high end of

temperature tolerances for the fungi was also not tested; however, it was at higher temperatures that differences in the growth rates of the two fungi began to become apparent.

Our main objective was to investigate whether the two mycangial fungi associated with *D. brevicomis* differ in response to temperature which may influence their prevalence and thus their interaction with the beetle in nature. Furthermore, we were interested in determining if there was any evidence of geographic variation in response between isolates from different parts of the western pine beetle's range. Fungi from different populations likely encounter different biotic and abiotic conditions which may shape genetic variation and fungal response (Mueller et al. 2011). For our study, we build on Davis et al. (2010) and use a larger number of isolates from a broader geographic range of sites. We measured area rather than radial growth to more accurately capture growth rates and explicitly test if responses to temperature by the two symbiotic fungi vary among populations.

Materials and methods

Fungal isolates

Ceratocystiopsis brevicomi and *E. sp. B* isolates used in our study originated from isolations from the mycangia of western pine beetle in Bracewell and Six (2014). Four isolates of *C. brevicomi* and *E. sp. B* from each of four geographically distinct sites (SB, SierraII, LA, SU) extending along a north-south latitudinal gradient from British Columbia to southern California were selected for use in growth studies (Fig 1). Methods used to initially culture fungi from beetles and information about the beetle collection method and location are described in Bracewell and Six (2014). Cultures of *C. brevicomi* were purified using single spore isolations by removing a small mass of spores from each culture and placing them into 1.5 ml

microcentrifuge tubes containing 150 µl of sterile water. The tubes were agitated for 1 min to separate the spores and the suspension was then poured onto the surface of 2 % malt extract agar (MEA) in individual Petri dishes. Once spores germinated, single spores were lifted from the culture medium surface and placed individually onto new medium for growth. *Entomocorticium* sp. B does not readily produce spores in culture; therefore, single hyphae were removed from the growing edge of a culture and transferred to new medium.

Temperature effects on growth of E. sp. B and C. brevicomi

In growth rate studies it is important that cultures are in an actively growing state. When a fungus is inoculated onto new medium, there is initially a lag period when growth is absent or occurs only slowly (Lilly and Barnett, 1951). The length of this lag phase is affected by the age and vigor of the existing culture and the conditions under which it has been stored. Subcultures from old or dormant cultures typically exhibit long and variable length lag phases that can add considerable error to growth rate estimates. In contrast, actively growing cultures tend to have very short, more uniform, lag phases. To ensure our cultures were in an active growth phase, they were grown for 3 wk on MEA prior to use in the experiment. Mycelial plugs (5 mm in diameter) were removed from the edge of each of these cultures and placed mycelium side down on the center of Petri dishes (90 mm diameter) containing MEA. Three replicates of each isolate were placed in incubators at 10, 15, 22, 28, and 34°C and allowed to grow until hyphae met the edge of the plate or up to 68 d. These temperatures were chosen as they span the range of temperatures encountered by the western pine beetle during the part of the season when growth occurs (Miller and Keen 1960, Bracewell et al. 2014), and correspond to temperatures tested in other studies (Davis et al. 2010, Addison et al. 2013).

Growth was measured by tracing the edge of the area colonized by each fungus on the bottom of the Petri dish with a permanent marker. The two fungi grew at different rates, so the period between tracings varied. The first tracings of *C. brevicomi* cultures were conducted 4 d after inoculation and after 6 d for *E. sp. B*. Isolates were then traced every 2, 4, or 6 d depending upon how rapidly they grew. After 68 d, a Nikon D40 digital camera was used to photograph the series of tracings on each plate. Digital images were then analyzed to calculate the area colonized between traces using ImageJ (Abràmoff et al. 2004). To calculate area between tracings, the color saturation threshold was set to recognize only the lines traced in black permanent marker on the bottom of each Petri dish. Then, the magic wand tool was used to highlight the area between traces and ImageJ was used to calculate the area.

Statistical analyses

All statistical analyses were conducted using the software package R (R Development Core Team, version 3.0.1). Daily growth rates were estimated for each replicate by conducting linear regressions of area occupied by mycelium versus time. Daily growth rate estimates were not normally distributed and occasionally included zeros, so all growth rates estimates were increased by a value of one and then log transformed. To test for differences in growth rates of the two species of fungi within sites, linear mixed-effect models (LMMs) were constructed using the lmer function in R package lme4 (Bates, 2010). This method was chosen because it allows for the inclusion of random effects and nested random effects (Bolker et. al, 2009). LMMs for each site were constructed with growth rate as the response variable, and temperature, species, and the interaction of temperature and species, as explanatory variables. Isolate and replicate nested within isolate were included as random effects in the model. To test for differences in growth rate among isolates of a fungal species among sites, LMMs were performed for each

species with growth rate as the response variable and temperature, site, and the interaction of temperature and site as explanatory variables. Isolate and replicate nested within isolate were included as random effects in the model. Least-squares and the difference in least-squares means were calculated using `lsmeans` and `diffsmeans`, respectively, from the `lmerTest` package. Analysis of variance (ANOVA) tests were performed on each LMM using `AOV` function and all post hoc pairwise comparisons were calculated using Tukey's Honestly Significant Difference tests.

Results

A total of 480 cultures of *E. sp. B* and *C. brevicomi* were analyzed to determine growth rates at a range of temperatures. Most isolates exhibited growth at each temperature tested, although at the highest temperature, 34°C, four isolates of *E. sp. B* failed to grow (two from SB and two from SierraII). Temperature significantly influenced daily growth rate for both fungi (Tables 1 and 2) and there were significant differences in growth rates of the two species within all sites (Table 1, Table 2, Fig 2). Importantly, there was a significant interaction between fungal species and temperature (Table 1). In particular, at all four sites (SB, SierraII, LA, SU), *C. brevicomi* exhibited highest mean growth at 22°C, while at three of four sites (SierraII, LA, SU), *E. sp. B* showed highest mean growth at 28°C (Table 2). Generally, *C. brevicomi* exhibited growth over a broader range of temperatures than did *E. sp. B* (Fig 2) and showed pronounced, and often statistically significant, differences in growth from *E. sp. B* at the two lowest temperatures, and highest temperature tested in our experiment (Table 2). We also observed differences in growth rates among isolates of the same fungal species among sites, as seen by the statistically significant Site*Temperature interaction (Table 3, Fig. 2). However, only a few statistically

significant pairwise comparisons were observed when comparing isolates from different sites, and these always occurred in either the lowest, or highest temperature tested (Table 4).

Generally, *E. sp. B* isolates showed more variable growth rates than did *C. brevicomi* isolates (Fig. 2). No clear clustering of population level differences in growth rate characteristics was evident in either fungal species (table 4).

Discussion

Differences in temperature ranges and optima among poikilothermic partners in symbioses can have profound effects on their interactions and stability. This has been well demonstrated in scleractinian corals whose *Symbiodinium* partners are prone to loss and substitution, sometimes resulting in massive die-offs of the host (bleaching events) when temperatures exceed the tolerances of the symbionts (Brown 1997, Fisher et al. 2012). While most symbioses on Earth involve poikilotherms, how differential temperature tolerances among partners may circumscribe these relationships is seldom investigated. Hence, in few cases can we predict how temperature variability, including that resulting from anthropogenic climate change, influences their dynamics (Six 2009).

This remains the case for most bark beetle-fungus symbioses. Many of the world's most serious tree-killing bark beetle species depend upon fungal symbionts for nutrient supplementation. However, only one system, the *D. ponderosae*-fungus symbiosis, has been investigated in-depth as to how temperature influences the relative prevalence of the symbionts and their persistence through time with the host (Six and Bentz 2007, Addison et al. 2013).

With this study, we assessed how temperature may affect another important bark beetle-fungus symbiosis, that which occurs among *D. brevicomis* and its two mycangial fungi, *C.*

brevicomi and *E. sp. B*. We found that, like in the *D. ponderosae* system, the two symbiotic fungi associated with *D. brevicomis* differ in their responses to temperature. Such differential responses can have strong effects on beetle fitness through effects on the rate of resource capture by each symbiont within the tree, and thus, how much each contributes to host beetle nutrition. Evidence suggests that *E. sp. B* is superior to *C. brevicomi* in conferring benefits to the host (Bracewell and Six 2014). Thus, the relative prevalence of each fungus should translate to differential fitness effects on the host (Bracewell and Six 2014). Rates of resource capture also influence fungal fitness by affecting the number of new beetle hosts that each fungus encounters and thus their potential for dispersal to the next tree and the next generation of beetles.

Our results are in contrast to Davis et al. (2010), where differences in growth rate in response to temperature between the fungal species from a single site did not alter. We were likely able to identify differences in growth rate between species due to increased sampling, greater replication, and by using temperatures above 30°C. The use of area measurements rather than radial measurements may have also reduced error, especially for *E. sp. B*, which exhibits highly irregular growth margins in culture.

The relatively similar responses we observed for the two fungi at intermediate and high temperatures may have important implications in how stability is maintained in this three-way symbiosis and how it will respond to a changing climate. In the *D. ponderosae* symbiosis, differential responses of its fungi to temperature appear to reduce competition between its fungal symbionts by allowing each to dominate at different times as temperatures vary seasonally, annually, and as beetles migrate between different locations (Addison et al. 2013). These differences are also likely to determine the order of in which symbionts are lost with increasing warming.

For *D. brevicomis*, instead of having one fungus grow best at cooler temperatures and the other best at warmer temperatures, one fungus, *C. brevicomi*, grows best at cooler and higher temperatures but mostly similar to *E. sp. B* at intermediate temperatures (although some *E. sp. B* isolates grew at a faster rate). This implies that differential growth in response to temperature is likely to relieve competition between these two fungi only when temperatures are at the coolest or warmest bounds of growth for the two fungi. Because of its ability to grow at higher temperatures, *C. brevicomi*, the less beneficial symbiont, may be at an advantage as warming increases.

Little variability in growth rate at the various temperatures tested occurred among isolates of *C. brevicomi* either within or among sites. In contrast, *E. sp. B* exhibited highly variable responses to mid-range temperatures among sites and within some sites, and, unlike *C. brevicomi*, grew not at all or only very poorly at the highest and lowest temperatures tested (Fig. 2). Why *E. sp. B* is more variable in its response to temperature is unknown. However, this variability may be related to innate differences between the fungi given that one is an ascomycete and the other a basidiomycete and each use wood substrates in very different ways. It could also be related to the amount of genetic variation and degree of sexual outcrossing in each species. *Ceratocystiopsis brevicomi* is sexually outcrossing while *E. sp. B* is thought to be primarily clonal.

Because *C. brevicomi* can grow at cooler and warmer temperatures than *E. sp. B*, it may, at least partly, maintain its presence with the host by capturing resources at times when *E. sp. B* is unable to grow. The high variability in response to some temperatures among isolates of *E. sp. B* in some populations (e.g., SierraII and SB) indicates that the ability to capture spatial and nutritional resources can vary greatly within this species which is likely to have considerable

impacts on the outcome of both inter- and intra-specific competition among the fungi within trees and the short- and long-term dynamics of the fungi with the host beetle. This also has important implications in the long term stability of the symbiosis as climatic warming continues and where the interplay between genotypic and phenotypic variability will influence adaptability to new conditions.

Future work in this system should include investigations on the effects of temperature on the outcomes of inter- and intra-specific competition including trials incorporating isolates that exhibit substantial variability in their responses. Future work tracking the growth of the fungi in their natural substratum (phloem and bark), while difficult, can help us scale the growth of the fungi in controlled lab assays to that which happens in nature. With scaled growth rates, models, such as those developed in Addison et al. (2013) for *D. ponderosae* and its symbiotic fungi, could be applied to investigate how variability in fungal responses affect the dynamics of this system over a variety of temporal scales and thermal conditions and help predict if and when the symbiosis will decouple in response to increasing warming. Because poikilothermic partners involved in symbioses respond idiosyncratically to changes in environmental conditions, future studies investigating responses of symbionts to thermal conditions will need to be conducted on a symbiosis-by-symbiosis basis. For bark beetles and their fungi, which occur in cryptic habitats, controlled lab experiments coupled with careful intensive sampling from trees in the field (along with temperature data from phloem) will be needed to accurately estimate how the symbionts respond to temperature and to changing thermal regimes. This dual approach would also allow for different initial lag periods of growth that can be expected to occur for saprotrophic (such as *E. sp. B*) and biotrophic fungi (such as most bark beetle-associated Ophiostomatales) when they are first introduced into a still living and defensive host tree.

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Figure legends:

Figure 1. Collection sites of *Entomocorticiium* sp. B and *Ceratocystiopsis brevicomi* used to determine the effect of temperature on growth rate. The westernmost portion of the range of *Pinus ponderosa*, the host tree for western pine beetle and their symbiotic fungi, is shown in dark grey.

Figure 2. Growth response to temperature of *Entomocorticiium* sp. B and *Ceratocystiopsis brevicomi* isolates from four different geographic locations.

Table 1. Linear mixed model results testing for interspecific differences in the growth of *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi* isolated from *Dendroctonus brevicomis* from four geographic locations (SB, SierraII, LA, SU).

Site		F statistic	df, residual df	P value
SB	Species	130.99	1, 110	<0.0001
	Temperature	79.29	4, 110	<0.0001
	Species*Temperature	18.62	4, 110	<0.0001
SierraII	Species	31.88	1, 110	<0.0001
	Temperature	57.04	4, 110	<0.0001
	Species*Temperature	12.6	4, 110	<.0001
LA	Species	125.392	1, 110	<0.0001
	Temperature	59.18	4, 110	<0.0001
	Species*Temperature	9.231	4, 110	<0.0001
SU	Species	4.071	1, 110	0.04605
	Temperature	34.314	4, 110	<0.0001
	Species*Temperature	4.948	4, 110	<0.0001

Table 2. Mean growth rates (SE) of *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi* isolated from *Dendroctonus brevicomis* from different geographic locations (SB, SierraII, LA, SU) grown at a range of temperatures. Values within a column from the same location with the same letter and values within rows followed by the same number are not statistically different (Tukey HSD test).

Site	Temperature (°C)	<i>C. brevicomi</i> mean (SE) daily growth rate	<i>E. sp. B</i> mean (SE) daily growth rate
SB	10	31.79 (3.20)a1	4.22 (0.62)a2
	15	79.28 (4.85)b1	13.81 (2.74)a2
	22	158.80 (0.12)c1	148.97 (24.87)b1
	28	106.93 (4.10)d1	121.83 (11.84)b1
	34	39.21 (2.22)a1	5.31 (1.94)a2
SierraII	10	43.80 (2.59)a1	7.95 (2.32)a2
	15	95.46 (4.10)b1	23.65 (3.48)a2
	22	123.31 (9.72)c1	224.40 (39.44)b1
	28	99.97 (7.86)bc1	229.04 (36.26)b1
	34	25.32 (4.83)a1	5.74 (1.90)a2
LA	10	20.25 (1.95)a1	1.60 (0.30)a2
	15	60.21 (3.98)b1	9.43 (2.02)a2
	22	138.15 (13.40)c1	73.42 (11.78)b1
	28	100.05 (5.38)d1	78.74 (8.09)b1
	34	55.37 (6.15)b1	14.40 (3.17)a2
SU	10	26.38 (0.97)a1	18.95 (4.23)a1
	15	67.15 (2.50)b1	40.89 (7.96)a2
	22	149.26 (8.66)c1	208.05 (34.24)b1
	28	108.19 (7.02)d1	273.52 (47.17)b1
	34	33.06 (3.81)a1	30.92 (2.28)a1

Table 3. Results of linear mixed model testing for intraspecific differences in growth of *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi* isolated from *Dendroctonus brevicomis* at four sites.

Species		F statistic	df, residual df	P value
<i>C. brevicomi</i>	Site	1.43	3, 220	0.235
	Temperature	90.992	4, 220	<0.0001
	Site*Temperature	5.121	12, 220	<0.0001
<i>E. sp. B</i>	Site	16.924	3, 220	<0.0001
	Temperature	127.312	4, 220	<0.0001
	Site*Temperature	4.244	12, 220	<0.0001

Table 4. Results of *post hoc* pairwise comparisons (Tukey's HSD) of growth rates of the *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi* from different sites. Statistically significant comparisons are shown in bold.

Temperature	<i>Ceratocystiopsis brevicomi</i> population		<i>Entomocorticium</i> sp. B population	
	comparison	P value	comparison	P value
10	SB vs SierraII	0.948	SB vs SierraII	1.000
	SB vs LA	0.882	SB vs LA	0.978
	SB vs SU	1.000	SB vs SU	0.769
	SierraII vs LA	0.018	SierraII vs LA	0.725
	SierraII vs SU	0.752	SierraII vs SU	0.985
	LA vs SU	0.987	LA vs SU	0.015
15	SB vs SierraII	1.000	SB vs SierraII	1.000
	SB vs LA	0.998	SB vs LA	0.999
	SB vs SU	1.000	SB vs SU	0.991
	SierraII vs LA	0.675	SierraII vs LA	0.676
	SierraII vs SU	0.983	SierraII vs SU	1.000
	LA vs SU	1.000	LA vs SU	0.326
22	SB vs SierraII	0.999	SB vs SierraII	1.000
	SB vs LA	0.982	SB vs LA	0.978
	SB vs SU	1.000	SB vs SU	1.000
	SierraII vs LA	1.000	SierraII vs LA	0.314
	SierraII vs SU	1.000	SierraII vs SU	1.000
	LA vs SU	0.999	LA vs SU	0.461
28	SB vs SierraII	1.000	SB vs SierraII	1.000
	SB vs LA	1.000	SB vs LA	1.000
	SB vs SU	1.000	SB vs SU	0.999
	SierraII vs LA	1.000	SierraII vs LA	0.754
	SierraII vs SU	1.000	SierraII vs SU	1.000
	LA vs SU	1.000	LA vs SU	0.670
34	SB vs SierraII	0.001	SB vs SierraII	1.000
	SB vs LA	0.999	SB vs LA	0.020
	SB vs SU	0.998	SB vs SU	0.000
	SierraII vs LA	0.000	SierraII vs LA	0.088
	SierraII vs SU	0.098	SierraII vs SU	0.000
	LA vs SU	0.472	LA vs SU	0.384

Summerland, BC (SU)

Latitude: 49° 41' N

Elevation: 655 m

La Grande, OR (LA)

Latitude: 45° 19' N

Elevation: 1,079 m

Avery, CA (Sierrall)

Latitude: 38° 13' N

Elevation: 1,075 m

San Bernardino, CA (SB)

Latitude: 34° 10' N

Elevation: 1,908 m

Figure 2