Adaptative Leaf Morphology of *Eurya japonica* Thunb. (Ternstroemiaceae) in Serpentine Areas

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Abstract

Serpentine soils are known to affect plant growth and plants in these soils have morphological and anatomical modifications. Comparative leaf morphology and anatomy studies of *Eurya japonica* Thunb. was conducted between serpentine and inland (control) areas. Our morphological analyses revealed that the individuals in the serpentine areas had significantly smaller and thicker leaves than those in the inland areas. Our anatomical analyses showed that the smaller leaves of serpentines had decreased numbers of cells, and their thicker leaves contributed to the increased height of epidermal cells, palisade tissue, and spongy tissue. Furthermore, the stomatal size of serpentines was significantly smaller than those from the inland areas. We concluded that *E. japonica* adapted to the serpentine areas by decreasing leaf size due to low levels of nutrients, by thickening the leaves to store water and reducing the stomatal size to minimize water loss via gas exchange.

Keywords: adaptation, anatomy, *Eurya japonica*, leaf, morphology, serpentine, stomata

1. Introduction

Soil conditions play dominant roles in terrestrial ecosystems, and nutrient mineralization in soil could affect biodiversity. Adaptation to edaphic factors such as soil physical, chemical, and biological characteristics have long been considered important components in plant distribution, diversification, and speciation (Kruckeberg, 1951; 1967). Serpentine soils occur in small patches along fault lines where igneous rocks appear and are characterized by abiotic factors such as low calcium-to-magnesium ratio, high heavy metal concentrations, nutrient deficiency, and low moisture retention, leading to sharp transitions in abiotic conditions at the boundaries of serpentine patches (Brady et al., 2005). Therefore, serpentine areas are a unique land type that hinder habitation by many plants, but they lead to the creation of new endemic species, including *Leontopodium hayachiinense* (Takeda) H. Hara et Kitam. (Asteraceae), *Primula yuparensis* Takeda (Primulaceae), *Hypericum tosaense* Makino (Hypericaceae), *Spiraea nipponica* Maxim. var. *ogawaee* (Nakai) Yamanaka (Rosaceae) (Kitamura, 1981; Yamazaki, 1981; Momiyama, 1982; Ohashi, 1989). Various unique plant flora have been reported in serpentine areas in Japan (Hara, 1937; Taniguchi, 1958; Yamanaka, 1973, 1974; Nakata, & Kojima, 1987; Kitamura, 1993), and it has been reported that even if the plant can invade and grow in serpentine areas, they often undergo morphological and anatomical modifications. For example, a comparative study using the serpentine ecotype and non-serpentine of *Adenophora triphylla* (Thunb.) A. DC. var. *japonica* (Regel) H. Hara (Campanulaceae) revealed that the former species had thinner stems and narrower leaves (Ohga et al., 2012). Moreover, it is interesting that this species adapted to the drought stress of serpentine conditions by reducing stomatal density (Ohga et al., 2012). Such stomatal changes have also been reported in the serpentine-endemic *Aster hispidus* Thunb. var. *leptocladus* (Makino) Okuyama (Asteraceae) (Hayakawa et al., 2012; Kumeekawa et al., 2013) and has been considered a plant adaptive mode in serpentine areas. Moreover, the fact that the reduced stomatal density was also found among different lineages suggested that this modification was likely an adaptation to serpentine areas. However, these studies were conducted using herbaceous species, and it remains unclear whether tree species growing in serpentine areas have acquired their own adaptations. Tree species can store water absorbed in various tissues (Goldstein et al., 1984; Goldstein et al., 1998; Borchert, 1994), meaning that trees in serpentine areas could have different stomatal adaptation strategies than herbaceous species. Despite the functional significance of leaf shape in environmental adaptation (Nicotra et al., 2011), few aspects of leaf...
shape have been linked directly to adaptive outcomes. 

_Eurya japonica_ Thunb. belongs to Ternstroemiaceae based on APG III (Haston et al., 2009) and is approximately 1–3.5 m tall and is a broad-leaved, evergreen, woody perennial that is widely distributed throughout Japan, from Honshu to Ryukyu, and Korea (Tsuyama, 1989). This species grows along the margins of evergreen forests, and Yamanaka (1959) reported that this species also grew in some serpentine areas of Shikoku, Japan. It is doubtful whether _E. japonica_ is accompanied by the above-mentioned modifications in the serpentine condition. As for the morphological and anatomical modification of this species to adapt to different environmental conditions, Shiba et al. (2021) recently indicated that the leaves of this species along rivers were significantly smaller to adapt to sudden flooding and strong river currents after heavy rain. Moreover, the stomatal density of riverside populations was significantly higher in response to soil moisture, which appears to correspond to riverside conditions (Shiba et al., 2021). This report explored adaptations of _E. japonica_ to other environmental conditions, with morphological and anatomical modifications. However, it is unclear whether _E. japonica_ undergoes morphological and anatomical modifications to adapt to serpentine conditions. Therefore, we characterized the variation in the leaves of _E. japonica_ using morphological and anatomical analyses.

2. Method

We collected samples from five localities in Kochi Prefecture, Japan. These localities were named as follows: two serpentine areas (Ikou and Kagami) and three inland areas (Okina, Shigeto, and Noichi) (Figure 1; Table 1). These localities are indicated in Figure 1 and Table 1. _E. japonica_ was analyzed in a total of 60 individuals from the serpentine areas (30 from Ikou and 30 from Kagami) and 93 individuals from the inland areas (30 from Okina, 33 from Shigeto, and 30 from Noichi) (Figure 2).

Shiba et al. (2021) reported methods of morphological and anatomical analyses in _E. japonica_. Therefore, we followed these methods in this study.

![Figure 1. Sampling localities in this study](image)

White and black circles indicate serpentine and inland (control) populations, respectively. Number in circles corresponds to that given in Table 1.

<table>
<thead>
<tr>
<th>Type</th>
<th>Locality name &amp; number*</th>
<th>Locality</th>
<th>Latitude and longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpentine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ikou</td>
<td>1</td>
<td>Ikou, Yusuhara-cho, Takaoka-gun, Kochi Pref.</td>
<td>33°27’N  132°50’E</td>
</tr>
<tr>
<td>Kagami</td>
<td>2</td>
<td>Kagami-ohri, Kochi City, Kochi Pref.</td>
<td>33°35’N  133°29’E</td>
</tr>
<tr>
<td>Inland (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Okina</td>
<td>3</td>
<td>Okina, Hidaka-mura, Takaoka-gun, Kochi Pref.</td>
<td>33°30’N  133°20’E</td>
</tr>
<tr>
<td>Shigeto</td>
<td>4</td>
<td>Kashinotani, Tosayama-cho, Kami City, Kochi Pref.</td>
<td>33°41’N  133°39’E</td>
</tr>
<tr>
<td>Noichi</td>
<td>5</td>
<td>Ohtani, Noichi-cho, Konan City, Kochi Pref.</td>
<td>33°34’N  133°42’E</td>
</tr>
</tbody>
</table>

*: locality no. corresponds to that given in Figure 1.
3. Results

We analyzed the leaf morphology of *Eurya japonica* using morphological and anatomical approaches. A summary of the morphological measurements is presented in Table 2. We calculated the mean leaf length, width, thickness, and angle of the leaf base for each individual. Leaf length and width in the serpentine populations tended to be smaller than those of the inland (control) populations, and leaf area in the former was significantly smaller than that in the latter. To detect morphological differentiation in serpentine areas, the leaf index values were calculated as the ratio of leaf length to leaf width, based on Tsukaya (2002). If the results of comparing these leaf index values between serpentine and inland populations showed a significant difference, it indicates that the population with the large value has relatively narrow leaves (Tsukaya, 2002). No significant difference between the serpentine and inland populations was detected in the leaf index. Furthermore, there was no significant difference in the angle of the leaf base between the populations, indicating that they have similar leaf silhouettes. However, our results indicated that leaves in the serpentines had significantly thicker leaves than those in the inland areas.

![Figure 2. Eurya japonica Thunb. A: serpentine (Kagami), B: inland (Noichi)](image)

Table 2. Leaf morphology (average ± standard error) of *Eurya japonica*

<table>
<thead>
<tr>
<th></th>
<th>Ikou</th>
<th>Kagami</th>
<th>Okina</th>
<th>Shigeto</th>
<th>Noichi</th>
</tr>
</thead>
<tbody>
<tr>
<td>length (mm)</td>
<td>45.52±1.13 c</td>
<td>50.22±0.95 b</td>
<td>53.58±1.25 ab</td>
<td>55.00±1.13 a</td>
<td>54.01±1.13 ab</td>
</tr>
<tr>
<td>width (mm)</td>
<td>19.86±0.45</td>
<td>17.76±0.49 d</td>
<td>21.77±0.36 ab</td>
<td>20.84±0.33 bc</td>
<td>22.92±0.48 a</td>
</tr>
<tr>
<td>size (mm²)</td>
<td>911.83±37.09 b</td>
<td>900.81±36.43 b</td>
<td>1,177.75±41.92 a</td>
<td>1,149.85±27.62 a</td>
<td>1,252.92±47.81 a</td>
</tr>
<tr>
<td>leaf index*</td>
<td>2.31±0.06  c</td>
<td>2.88±0.07 a</td>
<td>2.47±0.05 bc</td>
<td>2.66±0.05 ab</td>
<td>2.37±0.04 c</td>
</tr>
<tr>
<td>angle of leaf base (°)</td>
<td>62.71±1.47 a</td>
<td>52.75±1.47 b</td>
<td>53.58±1.18 b</td>
<td>55.10±1.02 b</td>
<td>62.89±1.66 a</td>
</tr>
<tr>
<td>thickness (μm)</td>
<td>283.83±8.27 a</td>
<td>269.13±5.57 a</td>
<td>183.85±6.34 b</td>
<td>191.43±8.90 b</td>
<td>173.00±7.90 b</td>
</tr>
</tbody>
</table>

Columns marked by different letters differ significantly according to the Tukey’s HSD test (p < 0.05).

*: Tsukaya (2002)

We calculated the mean epidermal cell size using the length and width of a cell on both adaxial and abaxial sides of the leaf measured from SUMP samples of this species (Table 3; Figure 3). The results showed that the cell length and width and the cell size on both adaxial and abaxial sides of the leaf were not significantly different between serpentine and inland populations, suggesting that the difference in leaf size was caused not by the difference in the number of cells on sides of the leaf. In addition, the results of measuring the height of epidermal
cells, palisade tissue, and spongy tissue that contributes to the thickness of the leaves using the longitudinal section of the leaf are shown in Table 4 and Figure 4. The results showed that the heights of epidermal cells, palisade tissue, and spongy tissue were significantly different between serpentine and inland populations.

We measured and calculated the mean stomatal size (guard cell area, based on guard cell pair length and width) and density using SUMP samples of serpentine and inland areas of *E. japonica* from all localities we examined (Table 5 and Figure 5). Although the stomatal size of serpentines was significantly smaller than that of the inland populations, stomatal density did not differ significantly.

Table 3. Anatomical measurements (average ± standard error) of epidermal cell in *Eurya japonica*

<table>
<thead>
<tr>
<th></th>
<th>Serpentine</th>
<th>Inland (control)</th>
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<tbody>
<tr>
<td></td>
<td>Ikou</td>
<td>Kagami</td>
<td>Okina</td>
<td>Shigeto</td>
<td>Noichi</td>
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<tr>
<td><strong>adaxial surface</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>length (μm)</td>
<td>20.98±0.24</td>
<td>20.13±0.19</td>
<td>ab</td>
<td>20.14±0.24</td>
<td>ab</td>
<td>20.13±0.27</td>
<td>ab</td>
</tr>
<tr>
<td>width (μm)</td>
<td>21.05±0.26</td>
<td>20.21±0.17</td>
<td>ab</td>
<td>20.39±0.29</td>
<td>ab</td>
<td>19.91±0.20</td>
<td>b</td>
</tr>
<tr>
<td>size (μm²)</td>
<td>444.35±10.67</td>
<td>409.77±7.19</td>
<td>ab</td>
<td>411.94±9.59</td>
<td>ab</td>
<td>401.69±7.76</td>
<td>b</td>
</tr>
<tr>
<td><strong>abaxial surface</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>length (μm)</td>
<td>20.51±0.16</td>
<td>18.97±0.24</td>
<td>b</td>
<td>19.32±0.18</td>
<td>ab</td>
<td>19.70±0.66</td>
<td>ab</td>
</tr>
<tr>
<td>width (μm)</td>
<td>18.42±0.14</td>
<td>17.08±0.19</td>
<td>b</td>
<td>18.01±0.22</td>
<td>a</td>
<td>17.99±0.22</td>
<td>a</td>
</tr>
<tr>
<td>size (μm²)</td>
<td>378.77±4.74</td>
<td>328.16±7.81</td>
<td>b</td>
<td>328.79±3.77</td>
<td>a</td>
<td>354.63±12.45</td>
<td>ab</td>
</tr>
</tbody>
</table>

Columns marked by different letters differ significantly according to the Tukey’s HSD test (*p* < 0.05).

Table 4. Anatomical measurements (average ± standard error) of mesophyll tissues using longitudinal section in *Eurya japonica*

<table>
<thead>
<tr>
<th></th>
<th>Serpentine</th>
<th>Inland (control)</th>
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<tbody>
<tr>
<td></td>
<td>Ikou</td>
<td>Kagami</td>
<td>Okina</td>
<td>Shigeto</td>
<td>Noichi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>epidermal cell at adaxial side (μm)</td>
<td>28.15±0.50</td>
<td>23.03±0.64</td>
<td>b</td>
<td>17.65±0.63</td>
<td>c</td>
<td>13.28±0.70</td>
<td>d</td>
</tr>
<tr>
<td>palisade tissue (μm)</td>
<td>102.60±3.96</td>
<td>87.80±2.94</td>
<td>b</td>
<td>61.58±3.45</td>
<td>c</td>
<td>63.20±3.90</td>
<td>c</td>
</tr>
<tr>
<td>spongy tissue (μm)</td>
<td>130.30±4.04</td>
<td>138.13±2.70</td>
<td>a</td>
<td>90.43±3.82</td>
<td>b</td>
<td>105.38±6.33</td>
<td>b</td>
</tr>
<tr>
<td>epidermal cell at abaxial side (μm)</td>
<td>22.78±0.57</td>
<td>20.18±0.34</td>
<td>b</td>
<td>14.20±0.63</td>
<td>c</td>
<td>9.58±0.49</td>
<td>d</td>
</tr>
</tbody>
</table>

Columns marked by different letters differ significantly according to the Tukey’s HSD test (*p* < 0.05).

Table 5. Anatomical measurements (average ± standard error) of stomata in *Eurya japonica*

<table>
<thead>
<tr>
<th></th>
<th>Serpentine</th>
<th>Inland (control)</th>
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<tr>
<td></td>
<td>Ikou</td>
<td>Kagami</td>
<td>Okina</td>
<td>Shigeto</td>
<td>Noichi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>length (μm)</td>
<td>18.74±0.26</td>
<td>17.68±0.23</td>
<td>c</td>
<td>19.58±0.21</td>
<td>ab</td>
<td>20.10±0.18</td>
<td>a</td>
</tr>
<tr>
<td>width (μm)</td>
<td>4.89±0.02</td>
<td>4.92±0.03</td>
<td>b</td>
<td>5.12±0.06</td>
<td>a</td>
<td>5.22±0.05</td>
<td>a</td>
</tr>
<tr>
<td>size (μm²)</td>
<td>91.59±1.53</td>
<td>87.03±1.36</td>
<td>c</td>
<td>100.17±1.64</td>
<td>b</td>
<td>104.75±1.34</td>
<td>a</td>
</tr>
<tr>
<td>density (N/μm²)</td>
<td>213.39±5.13</td>
<td>251.64±4.85</td>
<td>ab</td>
<td>259.19±4.90</td>
<td>a</td>
<td>243.33±6.49</td>
<td>ab</td>
</tr>
</tbody>
</table>

Columns marked by different letters differ significantly according to the Tukey’s HSD test (*p* < 0.05).

Figure 3. Epidermal cell on adaxial surface

A: serpentine (Kagami), B: inland (Noichi). Bar = 30 μm
4. Discussion

The morphological characteristics of an edaphic ecotype may be the result of a physiological adaptation to the environment and serpentine soils, which have an excess of magnesium, chromium, and nickel and a deficiency of plant nutrients such as potassium, phosphorus, and calcium, that adversely affect plant growth (Kruckeberg, 1954). In particular, high magnesium content inhibits calcium uptake in plants, due to antagonistic uptake between the two cations (Gardner & Macnair, 2000; Brady et al., 2005). Therefore, plants that grow in serpentine soils must adapt to their environment by developing plant bodies capable of detoxifying heavy metals and tolerating low nutrient levels (Kruckeberg, 1954; Brady et al., 2005). The characteristic vegetation in serpentine outcrops is associated with the presence of edaphic endemic species, and the morphological characteristics may differ from those of ancestral species in nonserpentine areas (Kruckeberg, 1954). In fact, previous studies have indicated that serpentine-adapted plants have narrow leaves (Hayakawa et al., 2012; Ohga et al., 2012; Kumekawa et al., 2013, 2016), indicating that these reductions could be a response to an edaphic environment that exhibits nutrient deficiencies in serpentine soils (Kruckeberg, 1954). Our results indicated that *E. japonica* in the serpentine populations had thicker leaves and smaller stomata than those of the inland populations (Table 2), suggesting that *E. japonica* had been morphologically and anatomically modified to adapt to serpentine soils. Moreover, it is interesting that *E. japonica* did not develop narrow leaves in serpentine areas although previous studies of serpentine ecotypes have reported decreased leaf size due to low nutrient levels in serpentine areas (Ohga et al., 2012; Kumekawa et al., 2013). Therefore, our results revealed that the leaf size of *E. japonica* was not affected by low levels of nutrients. However, the leaves were significantly thicker in the serpentine area, which is discussed below.

Thickening of the leaves plays an important role in maintaining water potential when the water supply is low.
(Coneva & Chitwood, 2018). Ogburn and Edwards (2010) indicated that a variety of morphological and anatomical features that could vary with the aridity of the habitat underlie this adaptive feature. In fact, some studies have reported that taxa with thick leaves were found in low water supply areas, such as coastal areas (Hayakawa et al., 2012; Ohga et al., 2012). Serpentine soils are also poor in soil organic matter accumulation, which decreases water-holding capacity, and these soils can cause drought stress in plants (Kruckeberg, 1954; Gardner & Macnair, 2000; Hughes et al., 2001). Therefore, our finding that *E. japonica* developed thicker leaves in the serpentine area indicated that this species might morphologically adapt to the dryness of serpentine soils. On the cellular level, leaf thickness has been broadly associated with increased cell size (Watson, 1942), which promotes water storage (Becker, 2007), and some studies have reported that the differentiation of leaf thickness is directly related to the height of palisade cells (Garnier & Laurent, 1994; Roderick et al., 1999; Sack & Froel, 2006; John et al., 2013). For example, Coneva et al. (2017) reported that palisade cells were linked to increased leaf thickness in a desert-adapted wild species.

Furthermore, our anatomical results showed that the differentiation of *E. japonica* leaf thickness in serpentine areas was attributed to an increase in the height of epidermal, palisade, and spongy cells. Although our anatomical results were considered to be a morphological adaptation to the dry environment in serpentine areas, it has been shown that the differentiation in leaf cell height affects other functions. Oguchi, Hikosaka and Hirose (2005) and Terashima et al., (2011) reported that the increased height of palisade cells led to improved uptake of carbon dioxide and improved photosynthesis in thick leaves. Moreover, the shape of palisades has also been functionally linked to a more efficient distribution of light throughout the leaf (Brodersen et al., 2008; Brodersen & Vogelmann, 2010). From these studies, thicker leaves were correlated with rapid growth and drought conditions (Edwards et al., 2014). However, our anatomical results indicated that the stomatal size of *E. japonica* in serpentine areas was smaller than that in the control localities. Stomata are small pores formed by a pair of guard cells on the epidermis of leaves, which control the water loss in transpiration and the carbon dioxide uptake for photosynthesis (Hetherington & Woodward, 2003; Franks & Beerring, 2009). Smaller stomata sizes reduce water loss, but there is also a decrease in the absorption efficiency of carbon dioxide. Therefore, our anatomical results suggested that *E. japonica* in the serpentine areas adapted through not only thicker leaves for storing water but also by reduced stomatal size, at the expense of photosynthesis, to minimize water loss. From these results, we concluded that *E. japonica* experienced significant stress in the serpentine areas and it could not grow without these anatomical differentiations. In addition, future comparative studies of morphological and anatomical modifications of other organs, such as stem and root, between inland areas and serpentine areas will boost up our understanding of the adaptation of *E. japonica* into serpentine areas.

Shiba et al. (2021) indicated that six independent riverside populations of *E. japonica* experienced differentiation, including smaller leaves and higher stomata density. Moreover, populations of *E. japonica* are located in a small mosaic area of Kochi Prefecture in accordance with serpentine outcrops. The riverside or serpentine ecotypes of this species are found in Kochi Prefecture and its neighboring areas. Although these ecotypes of *E. japonica* might have been derived from Kochi Prefecture and its neighboring areas, it is doubtful whether allopatric ecotypes have expanded their distribution through a single origin or in parallel origin at each location. In fact, some studies indicated that the origin of the ecotype involves an assessment of its being an ecotype derived from the result of parallel evolution (Nicholls & McNeilly, 1982; Wolf, Howe, & Hamrick, 2000; Nyberg Berglund, Dahlgren, & Westerbergh, 2003; Yokoo et al., 2009). Further molecular phylogenetic analyses of this species will provide a practical scenario to verify the origin of these *E. japonica* ecotypes.

**Acknowledgments**

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