Physiological and anatomical differentiation of two sympatric weed populations

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Research article

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This publication is dedicated to Prof. Herbert Hurka’s 80th birthday
Abstract

In the vineyards of Rhineland-Palatinate (Germany), two different types of Shepherd’s Purse coexist: (1) the common type called ‘wild type’, and (2) the decandric type called *Capsella apetala* or ‘Spe’ with four stamens in place of petals. In this study, we compare the anatomical and physiological characters of rosette leaves of the respective types. Progeny of individual plants was cultivated in growth chambers under low- and high-light conditions. Under low-light conditions, the stomata densities of the adaxial and abaxial epidermis did not differ between the two types. When grown under high-light conditions, wild type and Spe, both exhibited increased stomata densities compared to low-light conditions, but Spe to a lesser extent than the wild type. The maximal photosynthetic capacity of Spe was lower in both, low-light and high-light conditions compared to wild-type plants. Even under ambient and sub-ambient CO$_2$ concentrations, Spe seemed to be less productive. The less effective CO$_2$ assimilation of the Spe mutant *C. apetala* was accompanied by later flowering. This fact prolonged the vegetative phase of Spe by about two weeks and was sufficient for the maintenance of both populations stably over years.

Introduction

In the vineyards of southwestern Germany (Rhineland-Palatinate) two morphologically distinct types of the common annual weed Shepherd’s Purse (*Capsella bursa-pastoris* (L.) Medik., Brassicaceae) occur side by side (Reichert 1998, Fig. 1).
The two populations can readily be differentiated by their characteristic flower structure. Wild type (wt) *C. bursa-pastoris* flowers exhibit the conserved body plan of Brassicaceae: it comprises four sepals in the first, four petals in the second, six stamens in the third, and two fused carpels in the fourth, the innermost whorl (Fig. 1).

In contrast, the flowers of the floral variant, also referred to as Spe (Hameister and Neuffer 2017, Nutt et al. 2006), can easily be identified by four additional stamens replacing the wild-type petals thus giving it its unique decandric phenotype (Fig. 1). This floral variant of *C. bursa-pastoris* has initially been described as *C. apetala* Opiz (Opiz 1821), a terminology which we follow here to differentiate between both types. Notably, the decandric flower shape does not affect floral symmetry, but the lack of petals might have an impact on pollination success (Hameister and Neuffer 2017). Recent field surveys in vineyards revealed that the wild type is the predominant taxon with tens of thousands of individuals, and the Spe occurs with a stable frequency of about 10% (Hameister et al. 2009). Intriguingly, potential pollinators, especially wild bees, and hoverflies, tend to prefer one over the other type, probably due to differing volatile emissions of the two flower types (Ziermann et al. 2009, Hameister and Neuffer 2017, Fig. 1). Recent investigations by Hameister and Neuffer (2017) demonstrated that flower induction is delayed in Spe when compared with wild type in both, field and controlled greenhouse conditions. Notably, the authors could show that in an overlapping period both types are fertile. However, both taxa exhibit a high “selfing” rate and a low crossing rate between the two flower types has been found (Hameister and Neuffer 2017, Fig. 1). Furthermore, pollen tubes of *C. apetala* self-pollen grew faster compared with *C. apetala* crossed with wild-type pollen (*spe x wt*, Neuffer and Paetsch...
2013). Field experiments in the Botanical Garden of the University of Osnabrueck revealed that the two types differ in the physiological investments into their respective offspring. Wild-type individuals produced more siliques per plant, whereas the Spe generated a higher number of seeds in the siliques (Hameister and Neuffer 2017). The total seed output per plant was balanced among the two flower types (Hameister and Neuffer 2017) indicating that the fitness-related factor seed production remains unaffected.

Taken together, the previous investigations lead to the assumption that *C. bursapastoris* and *C. apetala* are separated by several smooth to strong isolation barriers. Thus, they form two morphologically distinct types that grow sympatrically in the vineyards of southwestern Germany (Reichert 1998). Further support for this hypothesis can be extracted from genetic work by Hameister et al. (2009). Here, the authors showed that wild type and Spe differed in their isozyme genotype. Furthermore, the genetic difference was confirmed by creating a mapping population between a wild-type individual and a Spe individual followed by comparative linkage analysis (Hameister et al. 2013).

In this study, we focus on the properties of the leaves of the two types in the vegetative stage when the plant accumulates assimilates which then are used for building up the inflorescence and production of seeds. Rosette leaves provide the basis for an individual to withstand harsh environmental conditions, e.g., cold temperatures, high irradiation, and drought stress. When the apical meristem switches from the vegetative to the reproductive state, no further rosette leaves are generated. The question was: what is the contribution of leaf differentiation to the ecotypic variation
between sympatrically growing individuals with normal (wild-type) flowers in contrast to individuals with decandric (Spe) flowers? Here, we studied the ecotypic differentiation of the rosette leaf after growth under stress (high-light) and no stress (low-light) conditions using anatomical and physiological methods.

Material and Methods

Plant material and cultivation of plants

Seeds of individual plants of Capsella bursa-pastoris (wild type) and Capsella apetala Opiz (Spe) have been collected in the vineyards located near to Gau-Odernheim (Rhineland-Palatinate, 25 km southwest of Mainz, 49.7847 N, 8.1942 E, elevation 150 m, Germany) and subsequently stored at minus 20°C in the seed gene bank of the Botanical Garden of Osnabrueck. The material refers to the gene bank numbers 1951/19 and 1960/25 for the wild type and 1956/5 and 1961/4 for the decandric type. Note that plant material subjected to photophysiological and anatomic characterization in this publication is derived material from seeds of two independent population subgroups of each flower morphological type. Plants were initially cultivated under controlled conditions programmed with a 12-h photoperiod per day, resulting in four subgroups with six individuals each. The temperature was adjusted to 15°C during day time and 5°C during the night phase (initial growth conditions for seedlings). After six weeks, the plants were divided into two experimental groups. Three individuals of each subgroup were transferred into two different light conditions in an 8-h light period at 20°C: (i) high-light setting (600 µmol quanta m⁻²s⁻¹); (ii) low-light setting
(100 µmol quanta m\(^{-2}\)s\(^{-1}\)) (Fig. 2). Later on sister individuals have been planted in the experimental field of the Botanical garden.

**Morphology and anatomy**

Leaf characterization was performed on three-month-old leaves. Epidermal leaf tissue was prepared for microscopic examination (Fig. 3) by a customized technique applying nail polish. After solidification of the nail polish, the epidermal cell layer was removed by transparent adhesive tape (Tesafilm). The nail polish imprint, including adhesive tape, was observed with a microscope. Subsequently, stomata were quantified in a representative area of 400 x 400 µm\(^2\) on one rosette leaf of every individual plant (Fig. 4).

**Photosynthetic characterization**

All measurements were performed using one fully expanded rosette leaf from plants two months after sowing when they did not yet flower. The CO\(_2\) gas exchange was measured using the LI-6400/XT Portable Photosynthesis System (LI-COR Environmental, Lincoln, NE, U.S.A.). All measurements were conducted under saturating light intensities of 800 µmol quanta m\(^{-2}\)s\(^{-1}\) at 20°C. The relative humidity was adjusted to approximately 50%. The rate of CO\(_2\) assimilation (A) was measured as a function of sub-stomatal CO\(_2\) concentration (C\(_i\)). C\(_i\) values were applied in the following order: 400 ppm; 200 ppm; 100 ppm; 50 ppm; 0 ppm; 400 ppm; 600 ppm; 1000 ppm; 1750 ppm. Here, the initial decrease in C\(_i\) concentration was chosen to ensure a sufficient leaf conductance. The leaf conductance is widely considered as an indicator
of effective gas exchange of the leaf with the surrounding environment and is
predominantly determined by the extent to which the stomata are opened. By applying
decreasing CO\textsubscript{2} concentrations in the first half of a photosynthesis measurement, a
conductance of at least 0.2 was maintained throughout the entire measurement
(Fig. S1). In this way, a possible limitation of CO\textsubscript{2} assimilation due to inefficient gas
exchange was avoided. Data points were logged within 2 min after the start of each C\textsubscript{i}
concentration when infrared gas analyzer (IRGA) parameters reached a steady-state
value (raw data for 22 individuals Suppl. Tab. S2).

Determination of flowering time under field conditions
Seventy days after sowing sister plants were planted into the experimental field of the
Botanical Garden Osnabrueck and flowering time was determined by observation of the
emerging inflorescence bud.

Results
Stomata density on both sides of rosette leaves
After growth for three months in the climate chambers, epidermal peel from upper
and lower leaf surfaces was prepared and inspected by light microscopy (Fig. 3). The
number of stomata was determined for wild type and Spe after growth in low light and
high light. As expected, the stomata density of the lower epidermis was found to be
higher compared to the upper surface for both types in all light conditions (Fig. 4). When
grown under low-light conditions, no obvious differences could be detected between the
two flower types. In contrast, both types after growth under high-light conditions
exhibited a significantly increased stomata density on both leaf surfaces (Fig. 3 and 4, Suppl. Table S1). Interestingly, the number of stomata on the adaxial (upper) surface of the leaves of Spe was significantly less increased when compared with wild-type individuals after growth in high-light conditions (Fig. 4). Notably, this trend could not be observed for the abaxial (lower) epidermis under the same conditions.

**Photosynthetic characteristics of rosette leaves**

To further elucidate the impact of the differentially increased stomata density observed in plants grown under high-light conditions, the photosynthetic efficiency was determined under saturating light using gas-exchange measurements. The rate of carbon assimilation (A) in response to various externally applied CO$_2$ concentrations given as internal CO$_2$ (C$_i$) was monitored for wild type and Spe plants. The resulting A/C$_i$ plots show the typical saturation behavior for all plants grown under both light conditions (Fig. 5).

Photosynthesis was limited by the availability of CO$_2$ until a calculated internal CO$_2$ concentration (C$_i$) of approximately 350 ppm was reached. The initial linear increase of CO$_2$ assimilation reflects the maximal carboxylation rate of RubisCO (Fig. 5, C and D). Above 350 ppm, the curves enter saturation as net photosynthesis becomes limited by other factors such as the electron transport and, at higher CO$_2$ concentrations, the utilization of triose phosphate. At saturating CO$_2$ concentrations, maximum net-photosynthesis rates of Spe were always lower than of the wild type when cultivated under both, low- and high-light conditions (Fig. 5, A and B). However, the initial carboxylation rate was rather similar for the two types. At ambient CO$_2$ concentrations of
approximately 400 ppm which more accurately reflect the photosynthetic efficiency of the plants the rates of CO$_2$ assimilation are higher when the plants have been grown under high light, and significantly lower when grown under low-light conditions (Fig. 5, C and D). No premature stomatal closure had limited gas exchange as shown by plotting the stomatal conductance (gs) against the C$_i$ values for each measurement (Suppl. Fig. S1).

Flowering time

In order to identify traits of Spe, which explain the observed stable co-existence in the field even with a lower photosynthetic performance, the flowering time was analysed. After 70 days sister plants (wt 72 individuals, Spe 63 individuals) were transferred from the initial growth conditions for seedlings to the experimental field in the Botanical Garden of Osnabrueck. Under these field conditions, wild-type individuals in average flowered about two weeks earlier (x = 77.64 +/- 3.562 days after sowing) than Spe individuals (x = 91.75 +/-12.618 days after sowing (Fig. 6).

Discussion

Finely scaled ecotypic differentiation for Shepherd’s Purse is well known and quite substantial regarding germination, the onset of flowering, and growth forms (Linde et al. 2001, Neuffer and Linde 1999, reviewed in Neuffer et al. 2011). Already at the beginning of the 20$^{th}$ century, Almquist (1907) determined several elementary species based on fruit and rosette-leaf morphology. This variable leaf morphology attracted the interest of the geneticist Shull (1909, 1911). Aksoy et al. (1999) included the system of
Shull in their attempt towards a simplified taxonomy of *C. bursa-pastoris* and Ianetta *et al.* (2007) widened the system of Shull.

Combining anatomical and physiological traits provides the first insight into the value of leaf differentiation other than the morphology of leaf types after Shull (Neuffer *et al.* 2018). The ecotypic differentiation of the leaf in various habitats and conditions at geographically and climatically different places of origin of many populations is an obvious character that somehow serves adaptation and persistence in new growth conditions. The occurrence of a specific flower morphology of *C. bursa-pastoris* individuals in the vineyards south of Mainz in southwestern Germany is known for quite some time (Reichert 1998). At first sight, this natural mutant is predominantly important for studies of plant geneticists (Nutt *et al.* 2006, Ziermann *et al.* 2009, Hintz *et al.* 2006, Hameister *et al.* 2013). With a more detailed analysis, several traits became apparent, and population studies led to the insight that the wild type of *C. bursa-pastoris* and its variant *C. apetala* even form two different ecotypes, sympatrically occurring at one place over many years (Hameister and Neuffer 2017, Hameister *et al.* 2009). This differentiation is also evident from genetic differences (Isozyme Genotypes, AFLPs, see Hameister *et al.* 2009).

As a typical leaf characteristic, stomata density was determined in the experimental populations (Fig. 4). In general, all leaves had developed more stomata under high-light growth conditions than in low-light as was already observed for *C. bursa-pastoris* provenances from Morocco and Norway, but not from Russia (Neuffer *et al.* 2018). Furthermore, stomatal density on the abaxial epidermis was about twice as high as in the adaxial leaf surface both in wild type and *Spe* after growth under high
light. However, as an exception, Spe had not increased its stomata density on the upper leaf surface to the same extent as did the wild type. It had developed about 35% fewer stomata per area unit on the upper surface of its rosette leaves as compared to the wild type (Fig. 4). A possible explanation for this phenomenon might lie in the fact that the incident light primarily impacts the adaxial surface of the rosette leaves and is exposed to adverse conditions more than the abaxial surface.

In our study, wild type individuals compared to Spe exhibited a higher maximal photosynthetic capacity both when grown under low light as well as high light. At saturating CO$_2$ and light, the rates of CO$_2$ assimilation were always higher for the wild type compared to C. apetala (Fig. 5, A and B). This points to a limitation at the level of electron transport and would cause a disadvantage for Spe. However, as can be taken from the initial slopes of the A/C$_i$ curves, under ambient conditions with limiting CO$_2$ concentrations up to 400 ppm, only slight differences were apparent when both types were compared (Fig. 5, C and D).

One way to explain the coexistence of wild type and Spe in the same habitat is based on the fact that the flowering phenology of both types differs. Wild-type plants flower earlier by several days after sowing under greenhouse conditions (Hameister et al. 2009, 2013) as well as under field conditions at the Botanical Garden of Osnabrueck (Hameister and Neuffer 2017). The appearance of the inflorescences was monitored also for sister plants after transfer to field conditions in the Botanical Garden in Osnabrück (Fig. 6). On average flowering started two weeks later in Spe individuals as compared to wild type. This might be explained by the disadvantage of Spe over the total growth period due to somewhat lower photosynthetic capacity and consequently a
lower growth rate resulting in a delay of biomass production in the vegetative stage. Either due to the difference in the nutritional status or due to environmental signals, flowering induction occurs significantly later in Spe thus leading to a temporal niche for successful pollination and seed set. A small but stable population of *C. apetala* can apparently coexist in the presence of the large wild-type population. Both types might be adapted to slight differences in their growth properties and the environment by growing in the rosette stage for a longer or shorter time resulting in a difference in flowering time of about two weeks. Taken together, the selection pressure for both types is apparently similar leading to stable populations growing side by side.

**Conclusions**

In this study, we were able to analyse two independent sympatrically occurring populations of Shepherd’s Purse. These two populations (wild type and Spe) occur intermingled with each other in stable frequencies of 9:1 over the years. This situation is rather specific and the question arose: What are the characters enabling these populations to coexist as they actually do? Both populations are isolated by strong (selfing) and smooth (flowering, reduced pollen growth activity between taxa) barriers. We have been able here to quantify anatomical and physiological traits which, with all probability, stay under selection pressure. Of course, many more characters are included in the ecotypic differentiation and already have been studied elsewhere (e.g. seed production). Here, in Spe, photosynthetic capacity appears to be lower, while growing for a longer time in the vegetative stage and later flowering induction. As both populations occur in stable frequencies, the selection pressure on single traits might be
different, however, when combined, they seem to be neutralized. The specific
combination of characters in each taxon is linked due to predominantly selfing.

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Figure 1

Flower morphology

**Figure 1** Typical wild-type flower (A arrow points to the petal) and its typical pollinator, a wild bee (D). Typical decandric flower (C) and its typical pollinator, a hoverfly (E). Proof for a rare hybrid between both flower types with a developing petal and pollen sacs below (B). In the field, both types are growing in mixed populations (F). Fotos: Gitta Schüttler, Birgit von Höveling.
Figure 2

Habitus of individual plants cultivated under different light conditions

Habitus of individual plants of the same age (8\textsuperscript{th} to 9\textsuperscript{th} week after sowing) that had been cultivated under different light conditions. The inflorescence shoot was distorted since the light source was mounted 20 cm above the rosettes.
Figure 3

Stomata density of plants grown under high-light conditions.

Microscopical views of stomata distribution of plants grown under high-light conditions. Nail polish tracks of upper and lower leaf surfaces were taken. Black scale bar to avoid different font sizes.

Wild type

Adaxial epidermis

Specimen A

Abaxial epidermis

Specimen C

Spe

Adaxial epidermis

Specimen B

Abaxial epidermis

Specimen D
Figure 4

Stomata density

Stomata density of wild-type *C. bursa-pastoris* versus *C. apetala* (= Spe) grown under different light conditions. For significant differences between experimental groups see Suppl. Table 1.
Figure 5

$\text{CO}_2$-concentration dependence of the rate of $\text{CO}_2$ assimilation

**Figure 5** $\text{CO}_2$-concentration dependence of the rate of $\text{CO}_2$ assimilation of wild type (WT, five individuals low light, seven individuals high light) and *C. apetala* (Spe, three individuals low-light, seven individuals high light) grown under different light conditions. The plants had been grown either under low-light (A and C) or under high-light conditions (B and D), respectively. Determination of the rates of $\text{CO}_2$ assimilation was performed under saturating light using one leaf for each individual as described in Material and Methods. **A** and **B**: After application of descending $\text{CO}_2$ concentrations, the internal $\text{CO}_2$ concentration ($C_i$) was calculated in each case, and similar $C_i$ values were clustered (horizontal error bars). Stomata aperture was recorded for each measurement and is shown in **Suppl. Fig. 1**. **B** and **D**: Single measurements in the linear range up to 350 ppm were plotted against $C_i$. 
Manuscript to be reviewed
Figure 6

Onset of flowering in days after sowing.

Sister individuals of the plants that had been used for the physiological and anatomical analyses have been planted into the experimental field of the Botanical Garden Osnabrück on day 70 after sowing.