Turkish Journal of Botany

Volume 40 | Number 3 Article 4

1-1-2016

# The role of anthropogenic habitats as substitutes for natural habitats: a case study on Epipactis helleborine (L.) Crantz (Orchidaceae, Neottieae). Variations in size and nutrient composition of seeds

AGNIESZKA REWICZ

JEREMI KOLODZIEJEK

ANNA JAKUBSKA-BUSSE

TÜBİTAK

**Turkish Journal of Botany**

http://journals.tubitak.gov.tr/botany/

**Research Article**

Turk J Bot
(2016) 40: 258-268

doi:10.3906/bot-1404-69

# The role of anthropogenic habitats as substitutes for natural habitats: a case study on *Epipactis helleborine* (L.) Crantz (Orchidaceae, Neottieae). Variations in size and nutrient composition of seeds

**Agnieszka REWICZ[1,*], Jeremi KOŁODZIEJEK[1], Anna JAKUBSKA-BUSSE[2]**
[1]Department of Geobotany and Plant Ecology, University of Łódź, Łódź, Poland
[2]Department of Botany, Institute of Environmental Biology, Faculty of Biological Sciences, University of Wrocław, Wrocław, Poland

**Received:** 14.04.2014 • **Accepted/Published Online:** 08.01.2015 • **Final Version:** 08.04.2016

**Abstract:** Natural and anthropogenic habitats differed greatly in terms of soil nutrients. Soil at the anthropogenic sites contained significantly lower concentrations of nitrogen (N) than those at the natural sites. Seeds of *Epipactis helleborine* were sampled from these two contrasting growth habitats and their size and nutrient composition were recorded. The 8 populations of *E. helleborine* were different in seed and embryo volumes, but there were no significant differences in seed width. Habitat type significantly influenced seed area: in the anthropogenic areas, seed area was greater than in the natural areas. Two distinct groups of populations were evident with regard to seed length. On the whole, the seeds and embryos of *E. helleborine* plants from populations in the natural habitats were larger than those in the anthropogenic habitats. Seeds of *E. helleborine* are reticulated but their reticulation pattern is variable. Seeds varied in N content, with most of the variation being attributable to seed size. The reduced seed weight of *E. helleborine* in anthropogenic habitats may result, in part, from its inability to adapt to the lower nutrient availability in soils of these habitats.

**Key words:** Plastic responses, anthropogenic habitats, orchid seeds, seed mass and size, seed viability, adaptation

## 1. Introduction

Ecological comparisons between urban or industrial environments and natural areas emphasize their differences (Lundholm and Richardson, 2010). Human-modified ecosystems are considered to be ecologically novel in that climatic conditions, soils, toxins, hydrology, productivity, species composition, and interactions (Pickett et al., 2001) differ from conditions prevailing prior to human alterations (Lundholm and Richardson, 2010). The physical, chemical, and biological properties are generally less favorable than those in soil found on natural landscapes. For instance, urban soils are characterized by modified soil organism activity and modified soil temperature regimes, elevated soil reaction (pH), greater nutrient amounts and availability to plants, higher calcium levels, more rocks, greater decomposition rates, less accumulated organic matter, and compacted mixtures of anthropogenic materials compared with local remnants of the natural ecosystems (McDonnell et al., 1997).

Many orchid habitats are liable to be lost to development, since they are often postindustrial, and may even be contaminated. In contrast, anthropogenic analogues of natural habitats provide refuge for many rare or threatened and endangered species such as Orchidaceae (Pedersen et al., 2013). The most common colonizers of secondary habitats in temperate Europe are *Epipactis* and *Dactylorhiza*, species with short life cycles and broad ecological amplitudes. Substrates rich in $CaCO_3$, high light availability, and diminished competition resulting from disturbance of the upper soil level are common in habitats colonized by orchids (McCormick et al., 2004; Adamowski, 2006; Light and MacConaill, 2006; Adamowski et al., 2012).

Plants exhibit the capacity to modify their phenotypes, depending upon the environment in which they grow, so-called phenotypic plasticity (Liu et al., 2011). Genotypic plasticity is the ability of these plants to extend their range of tolerance or adaptation through changes in their genetic structure. Phenotypic and genotypic plasticity determine the ability of these plants to withstand disturbances and the rate at which recolonization occurs (Harper et al., 1970). The phenotypic plasticity of plant structure is an important characteristic in adaptation, and is one solution in adaptation to heterogeneous environments (e.g., Schlichting, 2002). The morphological traits of plants are affected both by abiotic factors (e.g., light and shade,

* Correspondence: stefa@biol.uni.lodz.pl

temperature, pH, and water and nutrient availability) and by biotic factors (e.g., pathogens, parasites, predators, pollinators, and competitors) (Schlichting, 2002). Morphological plasticity plays an important role in resource acquisition by plants. Knowledge of plasticity has the potential to provide useful biological information for conservation projects for rare and very rare native species, including endangered or protected species (Noel et al., 2007; Stachurska-Swakoń and Kuź, 2011).

Despite extensive research on plant plasticity, little is known about plant structures, e.g., the plasticity of seeds, in response to growth habitat. Differences in resource availability, which are reflected in microhabitat quality, may affect plant size and, consequently, the size of reproductive structures (Samson and Werk, 1986).

At present, little is known about seed and embryo size in *Epipactis helleborine* coming from anthropogenic habitats. It is therefore the purpose of this paper to present data on the morphologies of seeds and embryos of *E. helleborine* plants growing in different growth habitats. Our field investigation suggests that the adaptation of *E. helleborine* might by caused by its structural plasticity in response to growth habitats.

We quantified the morphological differences in seed morphological traits between and within different populations of *E. helleborine*, and correlated these variations with local environmental conditions. We tested the hypothesis that populations of *E. helleborine* growing in anthropogenic and natural habitats would differ in their plastic response to soil properties. The objectives of this study were (1) to quantify the degree of seed size variation in *E. helleborine*, among populations and plants within populations, and within a single plant; (2) to determine possible associations between seed size and soil conditions; and (3) to examine the relationship between seed mass and the nitrogen (N), phosphorus (P), and potassium (K) contents of the seeds. The investigated populations were chosen to represent a range of different growth habitats in which the plants grow.

## 2. Materials and methods

### 2.1. Study species

*Epipactis* Zinn is mostly a Eurasian genus with a south-central distribution. According to recent classification, the genus is divided into two monophyletic sister sections, distinguished based on the shape and color of the lip, the gynostemium structure, and the presence and shape of papillae and hairs; it presently contains 59 species clustered in 8 groups (Delforge, 2006).

*Epipactis helleborine* (L.) Crantz, Broad-leaved Helleborine, the object of our study, is the most common and widely distributed species in the genus, occurring from Mediterranean to boreal zones up to 2000 m a.s.l. (Ehlers et al., 2002; Delforge, 2006). *E. helleborine* is a clonal taxon, growing in broadleaved forests and coniferous (also secondary) forests, on forest edges, and also in anthropogenic habitats such as rural and urban road verges, railway embankments, postmining sites, tracks, quarries, poplar plantations, parks, sandy beaches, and lawns (Dickson, 1990; McKendrick and Dickson, 1994; Hollingsworth and Dickson, 1997; Pedersen and Ehlers, 2000; Stefaniak et al., 2011). This species is rather indifferent as to habitat and behaves as a pioneer (Delforge, 2006). It grows on humus soils, moderately wet, acidic to neutral, and sometimes on substrates rich in calcium carbonate (Hollingsworth and Dickson, 1997; Pedersen and Ehlers, 2000). The Broad-leaved Helleborine flowers from June to September, depending on latitude and altitude and demonstrates a wide range of phenotypic plasticity, which hinders taxonomic diagnosis (Ehlers et al., 2002).

### 2.2. Study sites

The study was carried out in Poland during fruiting months (from the end of June to the beginning of September) from 2010 to 2011. The study sites were located in three different geographic regions along a latitudinal gradient of c. 1000 km, from the Białowieża Primeval Forest, NE Poland, via Central Poland, to the province Lower Silesia, SW Poland (see Figure 1). Eight *E. helleborine* populations each in natural and anthropogenic habitat types (habitat type is subsequently referred to as habitat), representative of the major occurrences of *E. helleborine*, were sampled in Poland, as summarized in Table 1. Within regions, populations of *E. helleborine* were separated from each other by 1.0 to 20.0 km (mean = 17.4 km). The selected populations each contained more than fifty individual plants, and often many hundreds of plants. *E. helleborine* can produce clonal offshoots, but we attempted to sample fruits from only one ramet per genet. Because it was difficult to distinguish between ramets and genets, we considered all apparently attached shoots to be part of a single clump (hereafter referred to as a plant), but we counted and measured seeds of individual shoots. Within populations, the sampled individuals were separated from each other by at least 5 m to avoid sampling from closely related individuals. We sampled 3 capsules per individual ramet from 30 individuals per population.

### 2.3. Soil properties

To document edaphic differences among the sites, soil chemistry was analyzed. A soil sample was collected from the surface layer (0–10 cm) in the immediate vicinity of each individual or group of individuals sampled, and sent to the Regional Agrochemical Station in Łódź for analysis. The pH and quantities of potassium (K), phosphorus (P), magnesium (Mg), calcium (Ca), chlorine (Cl), iron (Fe), and nitrate nitrogen ($NO_3N$) in the soil samples were determined. Soil pH was measured in a 1:2.5 soil–water

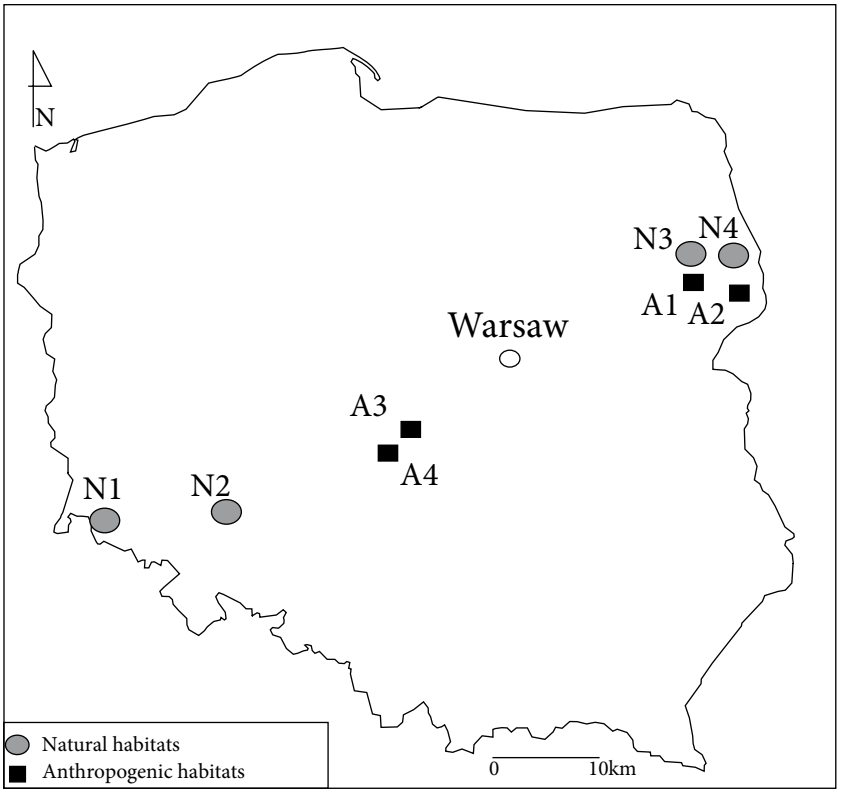


**Figure 1.** The distribution of the studied populations of *Epipactis helleborine*.

**Table 1.** List of study populations used in the study. The region is indicated in the population code (A1, A2 – Białowieża Primeval Forest, N3, N4, – NE Poland; A3, A4 – Central Poland; N1, N2 – Lower Silesia). Population size is given as number of flowering plants.

| Region | Code | Characterization of locality | Population size | Geographical coordinates |
|---|---|---|---|---|
| anthropogenic sites - roadside | | | | |
| NE Poland | A1 | Guszczewina | 127 | N 52.83160, E 23.794836 |
| | A2 | Hajnówka | 102 | N 52.734217, E 23.603314 |
| Central Poland | A3 | Sulejów | 80 | N 51.353793, E 19.883155 |
| | A4 | Sulejów | 152 | N 51.349757, E 19.882484 |
| natural sites – mixed forest | | | | |
| Lower Silesia | N1 | Kotowice | 300 | N 50.963255, E 15.963255 |
| | N2 | Góry Kaczawskie | 150 | N 51.041241, E 17.176701 |
| NE Poland | N3 | Białowieża Primeval Forest | 34 | N 52.828706, E 23.797095 |
| | N4 | | 41 | N 52.832427, E 23.763069 |

suspension and in 1 N KCl. Exchangeable Ca and Mg were determined in soil extracts of 1 N KCl; exchangeable K and available P and Fe were determined in Mehlich's extract of a diacid mixture of 0.05 N HCl and 0.025 N $H_2SO_4$. The cations in soil extracts were determined by atomic absorption spectrophotometry and P by colorimetry (Allen, 1989). Total N in soil was determined by Kjeldahl digestion and distillation. Soil organic matter was determined by the method of Walkley and Black (Allen, 1989).

### 2.4. Seed shape, size, and mass

For each population a systematic sampling approach was used. The sampling was designed to cover as much morphological variation as possible. Seeds were taken from live plants from 3 dehisced centers of capsules from the same population. To determine both seed and embryo morphometry, 90 seeds were measured from 1 ramet. The identified habitat types were separated into two categories: populations found in human disturbance habitats, such as roads (A1, A2, A3, A4), and populations found in natural habitats (N1, N2, N3, N4), such as peat, conifer forest, or deciduous forest. Patterns of between-population differentiation in seed size were analyzed in three separate canonical variate analyses (CVAs), based on the 4 populations of natural habitats, the 4 populations of human disturbance habitats, and the pooled data (8 populations) from both habitats.

The observations and documentation of both seeds and embryos size, and seed surfaces were performed with the use of a Nikon SMZ-800 optical stereomicroscope (Nikon Instruments, Europe B.V.) connected to a Coolview camera (Nikon). Scanning electron microscopy (SEM Vega 5135 MM Tescan) was used to analyze the number of cells along the longitudinal axis, the morphology of the total seed, and the apical, medial, and chalazal cells.

The seed terminology and the methods for measuring morphological characters were taken from previous studies, such as Arditti et al. (1979, 1980). Eight seeds and embryos traits were quantified: i) seed length (SL), ii) seed width (SW), iii) seed circuit (SC), iv) seed area (SA), v) embryo length (EL), vi) embryo width (EW), vii) embryo circuit (EC), and viii) embryo area (EA). We also calculated the volume of seeds using the formula 2 (W/2)2(1/2L) (1.047)], where W = width, L = seed length, and 1.047 – π/3. The volume of the embryo was calculated by using the formula 4/3 $\pi ab^2$, where a = 1/2 its length and b = 1/2 its width. Percent free air space in the testa was calculated as: (seed volume - embryo volume)/seed volume) × 100. Seed mass was defined as the weight of the embryo plus the seed coat. To establish correlations between the weight and number of seeds from each population, three individuals were randomly selected. One capsule was collected from each individual, from which all the seeds were counted and weighed (N = 24).

### 2.5. Seed nutrient analyses

Eight capsule samples (middle of the shoot) from eight plants per site were harvested from each nutrient treatment. Total seed N concentration was determined using a micro-Kjeldahl digestion unit and an auto-analyzer. Phosphorus and K concentrations were determined by emission spectrometry in inductively coupled plasma. Nitrogen, P, and K contents are all expressed on a dry mass basis.

### 2.6. Data analysis and seed plasticity index

To determine whether the magnitude of phenotypic plasticity varied among traits, we calculated the coefficient of variation (CV) for each combination of population and trait. Furthermore, we calculated separate regressions for trait and soil properties. The distribution of the pooled data was generally not normal, and therefore the nonparametric Mann–Whitney U-test was used. Statistical analysis was based on the Kruskal–Wallis test (for $P \leq 0.05$), which is a nonparametric alternative to ANOVA. Correlation between pairs of morphological characters was evaluated using Pearson's correlation coefficient. Ordinations of the locations and populations were analyzed using principal component analysis (PCA). The index of leaf plasticity sensu Valladares et al. (2000) was calculated as the difference between the maximum and minimum mean value divided by the maximum mean value (per trait). Leaf plasticity index scales ranged from 0 to 1. The software package STATISTICA PL. ver. 10 was used for all mentioned numerical analyses (Statsoft Inc., 2011).

## 3. Results

### 3.1. Soil properties

Comparison of soil properties between sites revealed that concentrations of Ca were significantly lower in the natural sites ($P < 0.05$). The soil at all population sites had a high content of Ca, from 695 to >5000 mg/L. The soil samples collected from the natural sites also had a higher content of $N\text{-}NO_3$ (from 46.5 to 87.0 mg/L) than samples collected at the anthropogenic sites (<10.0 and 19.8 mg/L, respectively). The anthropogenic sites were poor in organic matter and NaCl; the soil was particularly deficient in NaCl at the A4 population site. None of the other soil attributes differed between sites (Table 2). The regression analysis carried out among all the morphological traits and the soil data showed significant differences between the characteristics of EW, EA, EC, AS, SL, and $N\text{-}NO_3$. The high regression coefficient ($R^2 = 0.99$) confirms the dependence of the studied trait's dependent value ($N\text{-}NO_3$).

### 3.2. Seed and embryo shape and size

The seeds of all examined populations of *E. helleborine* were elongate fusiform and the coat was brown. Generally, the medial cells were longer than the apical and basal cells. The apical pole mainly consisted of polygonal cells. The seeds varied in length from 0.53 mm to 0.82 mm, and in width from 0.56 to 0.76 mm. The number of cells at the longest axis of the testa varied from 18 to 20 (natural habitats) and from 28 to 33 (anthropogenic habitats). The average length of testa cells from seeds from the natural habitats was greater than the testa cells in seeds from the anthropogenic habitats (0.48 µm and 0.72 µm, respectively) (Figure 2; Table 3).

**Table 2.** Means (±1 SE) of pH measured in KCl, organic matter, total nitrogen (N), available phosphorus (P), available potassium (K), and calcium (Ca) in samples of soil (n = 8; 10 cm depth) at the seed provenance sites (natural and anthropogenic habitats).

| | A1 | A2 | A3 | A4 | N1 | N2 | N3 | N4 |
|---|---|---|---|---|---|---|---|---|
| NaCl/1 (mg) | 0.30 | 0.25 | 0.16 | 0.28 | 0.63 | 0.52 | 0.2 | 0.36 |
| pH in $H_2O$ | 7.2 | 7.5 | 7.6 | 7.9 | 7.4 | 5.8 | 7.6 | 7.6 |
| $N\text{-}NO_3$ (mg $L^{-1}$) | 19.3 | <10.0 | <10.0 | 19.8 | 87.0 | 69.7 | 46.5 | 60.9 |
| P (mg $L^{-1}$) | 34.4 | 23.8 | <15.0 | <15.0 | 21.0 | <15.0 | 16.1 | 16.8 |
| K (mg $L^{-1}$) | 64.0 | 76.0 | <20.0 | 52.0 | 130.0 | 60.0 | 24.0 | 20.0 |
| Ca (mg $L^{-1}$) | 1520.0 | 2000.0 | 695.0 | 4105.0 | >5000.0 | 850.0 | 2695.0 | 4645.0 |
| Mg (mg $L^{-1}$) | 220.0 | 228.0 | <10.0 | 90.0 | 294.0 | 110.0 | 142.0 | 212.0 |
| Cl (mg $L^{-1}$) | 11.7 | <10.0 | <10.0 | <10.0 | 28.9 | <10.0 | <10.0 | <10.0 |
| Fe (mg $L^{-1}$) | 113.4 | 63.8 | 37.3 | 25.0 | 14.8 | 397.5 | 43.7 | 40.4 |
| Organic matter (%) | 6.1 | 7.8 | <1.0 | 1.5 | 14.3 | 7.3 | 2.3 | 5.1 |

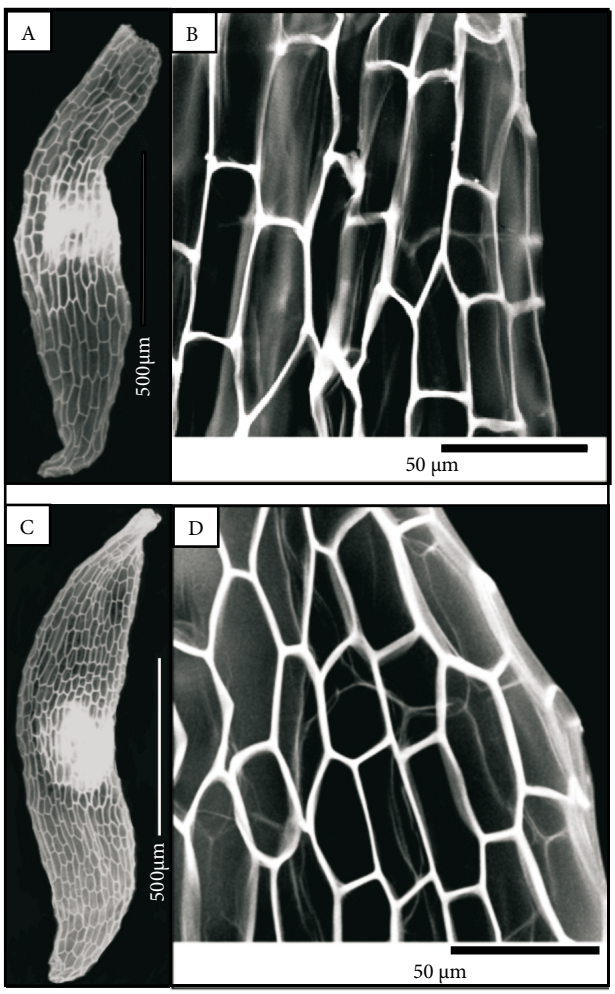


**Figure 2.** Seed micromorphology (SEM) of *Epipactis helleborine*. A, B – natural habitat, C, D – anthropogenic habitat.

Among the eight populations of *E. helleborine* there was considerable variability in seed and embryo volumes. There was no significant difference in either seed air space or weight between habitats (H = 547.03, P > 0.05 Kruskal–Wallis test). When the results were pooled for all populations, it could be seen that the anthropogenic populations had significantly higher seed and embryo volumes than the natural populations (H = 2825.78, P < 0.05 Kruskal–Wallis test). Both seed area and circuit from the anthropogenic plant populations were significantly higher than those of the natural plant populations (H = 1946.04, H = 3712.65, respectively; P < 0.05 Kruskal–Wallis test). Plants from the anthropogenic populations generally had seeds with a larger surface area than corresponding plants from the natural populations (H = 1946.04, P < 0.05 Kruskal–Wallis test). Similarity in seed length was also significantly higher in the anthropogenic populations than in the natural plant populations (H = 1556.53, P < 0.05 Kruskal–Wallis test).

The analysis of correlation revealed significant associations between traits of *E. helleborine* (Table 4). The strongest correlation (P < 0.05) was found between the volume and circuit of seeds. Seed area showed a significant correlation (P < 0.05) with seed volume. Similarity, there was a significant correlation (P < 0.05) between embryo width and volume. In general, populations from the anthropogenic habitats showed more moderate to strong correlations between traits than populations from the natural habitats. No significant correlation was found between the traits EL – AS, EC – AS, EA – AS, or AS – VE. The calculated variability coefficient values (CVs) showed that the examined seeds coming from the natural habitats were less variable (V = 45.17% to 50.26%) than seeds

**Table 3.** Biometric traits of *E. helleborine* in the studied populations.

| | A1 | A2 | A3 | A4 | $(\overline{X})$ | IP | N1 | N2 | N3 | N4 | $(\overline{X})$ | IP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Seed | | | | | | | | | | | | |
| SW (mm) | 0.35 | 0.34 | 0.33 | 0.29 | 0.33 | 0.13 | 0.29 | 0.36 | 0.22 | 0.29 | 0.29 | 0.24 |
| SL (mm) | 0.82 | 0.64 | 0.70 | 0.73 | 0.73± | 0.22 | 0.53 | 0.62 | 0.58 | 0.71 | 0.61 | 0.20 |
| SC (mm) | 3.26 | 2.62 | 3.07 | 3.01 | 3.00± | 0.20 | 2.36 | 2.91 | 2.57 | 3.02 | 2.71 | 0.19 |
| SA (µm) | 0.15 | 0.14 | 0.15 | 0.14 | 0.15± | 0.37 | 0.10 | 0.14 | 0.12 | 0.13 | 0.12 | 0.28 |
| Weight (µg) | $163 \times 10^{-6}$ | | | | | | $214 \times 10^{-6}$ | | | | | |
| SV ($mm^3$) | 0.58 | 0.39 | 0.50 | 0.46 | 0.48 | 0.37 | 0.30 | 0.44 | 0.34 | 0.46 | 0.39 | 0.36 |
| AS ($mm^3$) | 98.55 | 96.97 | 98.97 | 98.94 | 98.48 | 0.76 | 98.47 | 98.89 | 97.72 | 98.85 | 98.36 | 0.33 |
| Embryo | | | | | | | | | | | | |
| EW ( mm) | 0.19 | 0.18 | 0.18 | 0.18 | 0.18 | 0.05 | 0.18 | 0.19 | 0.19 | 0.18 | 0.19 | 0.05 |
| EL (mm) | 0.21 | 0.18 | 0.17 | 0.18 | 0.19 | 0.19 | 0.17 | 0.17 | 0.18 | 0.18 | 0.18 | 0.10 |
| EC (mm) | 1.12 | 0.96 | 1.13 | 1.12 | 1.08 | 0.15 | 1.03 | 1.10 | 1.06 | 1.08 | 1.07 | 0.06 |
| EA (mm) | 0.02 | 0.01 | 0.01 | 0.02 | 0.02 | 0.07 | 0.02 | 0.01 | 0.02 | 0.03 | 0.02 | 0.14 |
| EV ($mm^3$) | 0.0079 | 0.0045 | 0.0042 | 0.0040 | 0.0051 | 0.09 | 0.0039 | 0.0044 | 0.0070 | 0.0051 | 0.0052 | 0.03 |

Explanation: (SL) seed length, (SW) seed width, (SC) seed circuit, (SA) seed area, (EL) embryo length, (EW) embryo width, (EC) embryo circuit, (EA) embryo area, (VS) volume of seeds, (VE) volume of embryo, (AS) air space

**Table 4.** The correlation coefficients between parameters for seeds and embryos of *E. helleborine* in A) anthropogenic and B) natural habitats. Distinguished by an absolute correlation r > 0.50. All values are significant at $P < 0.05^*$. The numbers correspond to correlations significant at P < 0.05.

| A) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | SW | SL | SC | SA | EW | EL | EC | EA | VS | VE | AS |
| SW | 1.00 | −0.42 | 0.44 | 0.43 | 0.42 | −0.23 | 0.14 | 0.00 | 0.52* | 0.12 | 0.04 |
| SL | | 1.00 | 0.47 | 0.43 | −0.26 | 0.39 | 0.07 | 0.00 | 0.49 | −0.02 | 0.06 |
| SC | | | 1.00 | 0.72* | 0.13 | 0.13 | 0.20 | 0.00 | 0.72* | 0.08 | 0.09 |
| SA | | | | 1.00 | 0.13 | 0.11 | 0.19 | 0.00 | 0.70* | 0.05 | 0.08 |
| EW | | | | | 1.00 | 0.40 | 0.73* | 0.06 | 0.16 | 0.76* | −0.25 |
| EL | | | | | | 1.00 | 0.69* | 0.06 | 0.15 | 0.56* | −0.18 |
| EC | | | | | | | 1.00 | 0.06 | 0.18 | 0.64* | −0.15 |
| EA | | | | | | | | 1.00 | 0.00 | 0.08 | −0.03 |
| VS | | | | | | | | | 1.00 | 0.11 | 0.06 |
| VE | | | | | | | | | | 1.00 | −0.38 |
| AS | | | | | | | | | | | 1.00 |
| B) | | | | | | | | | | | |
| N | SW | SL | SC | SA | EW | EL | EC | EA | VS | VE | AS |
| SW | 1.00 | −0.41 | 0.50 | 0.37 | 0.36 | −0.22 | 0.12 | 0.01 | 0.48* | 0.11 | −0.01 |
| SL | | 1.00 | 0.41 | 0.40 | −0.25 | 0.38 | 0.05 | −0.01 | 0.52* | −0.01 | 0.17 |
| SC | | | 1.00 | 0.63 | 0.09 | 0.09 | 0.14 | 0.01 | 0.70* | 0.08 | 0.11 |
| SA | | | | 1.00 | 0.07 | 0.14 | 0.18 | 0.00 | 0.62* | 0.06 | 0.10 |
| EW | | | | | 1.00 | 0.41 | 0.76* | −0.02 | 0.10 | 0.75* | −0.78 |
| EL | | | | | | 1.00 | 0.73* | −0.02 | 0.17 | 0.54* | −0.48 |
| EC | | | | | | | 1.00 | −0.02 | 0.15 | 0.66* | −0.64 |
| EA | | | | | | | | 1.00 | 0.00 | 0.02 | −0.02 |
| VS | | | | | | | | | 1.00 | 0.12 | 0.12 |
| VE | | | | | | | | | | 1.00 | −0.82 |
| AS | | | | | | | | | | | 1.00 |

Explanation: (SL) seed length, (SW) seed width, (SC) seed circuit, (SA) seed area, (EL) embryo length, (EW) embryo width, (EC) embryo circuit, (EA) embryo area, (VS) volume of seeds, (VE) volume of embryo, (AS) air space

coming from plants growing in the anthropogenic habitats. The highest coefficient of variation was observed for the width of the populations (A1, A2, A3, N3, N4), and ranged from 41.45 to 46.97. The highest coefficient of variation for length (47.62) was observed in population. The lowest coefficient of variation in all analyzed populations was observed for the circuit (Figure 3). The highest coefficient of variation for embryo was observed for the area of the embryo in all populations studied, ranging from 71.71 to 1333.21. The lowest value for the coefficient of variation was observed for the length of the embryo (39.90) in population A2.

PCA revealed that the first two principal components explained 98.75% of the total variance. The first component accounted for 97.08% of the total variance and the second component 1.67%. Similarly, PCA of populations based on embryo morphological traits also showed a clear separation between populations. According to PCA of seeds, a scatter diagram/plot for the eight populations showed the populations from the anthropogenic habitats concentrating closer to the center, with a small separation from the populations of the natural habitats (Figures 4 and 5).

### 3.3. Plasticity index, seed nutrient concentration, and source of seed-mass variance

The median of seed traits was higher for seeds from the anthropogenic habitats than for seeds traits from the natural habitats (PI = 0.24 and PI = 0.18, respectively; $P < 0.05$ in both cases) (Table 3), and among them, PI = 0.84 and PI = 0.71, respectively; $P < 0.05$ in both cases.

Both the average concentration and total N, P, and K in *E. helleborine* seeds from plants in the anthropogenic habitats were lower than those in seeds from plants in the natural habitats ($P < 0.05$, Kruskal–Wallis test). Heavier seeds from plants in the natural habitats contained more N, P, and K than lighter seeds did. Site differences in seed nutrient content did not perfectly reflect the nutrient content of the soil. The two sites with the greatest amounts of P and K had plants that produced seeds with the lowest P and K content (Table 5).

The total seed number per capsule was determined in a subsample of 12 fruits per habitat. The mean number of seeds in the capsule was significantly higher in the anthropogenic populations than in the natural plant populations ($P < 0.05$, Kruskal–Wallis test). When all the data from the populations were pooled, simple linear regression showed that the mean number of seeds in the capsule was negatively correlated with seed weight in the natural habitats.

There was no evident relationship between a fruit's total seed number and the mean size of its seeds ($r = -0.015$ in natural habitats, $r = 0.107$ in anthropogenic habitats; $P = 0.005$). The correlation between the seed size and mass was slightly positive for the natural habitats ($r = 0.75$; $P = 0.005$).

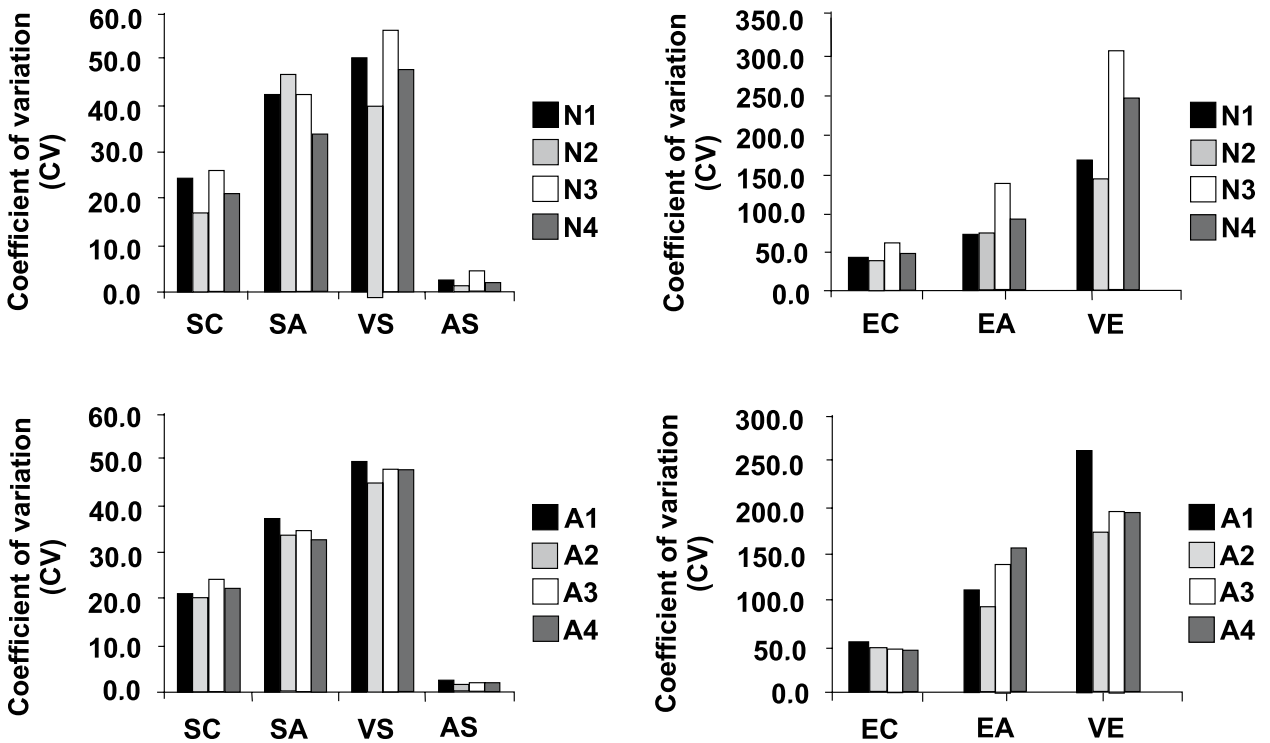


**Figure 3.** The coefficient of variation of seeds and embryos (±1 SE) for seven traits.

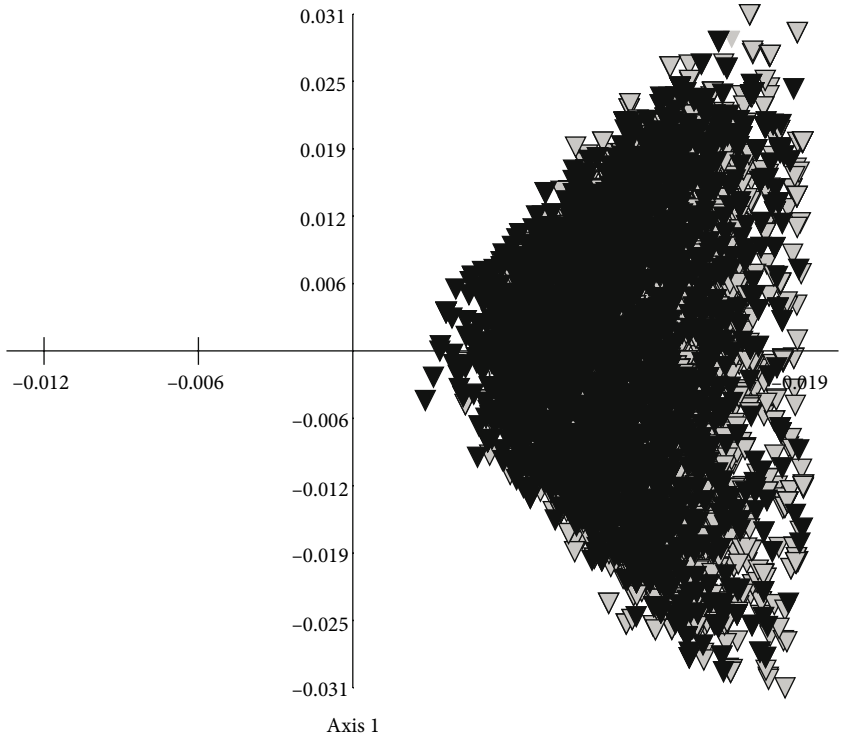


**Figure 4.** Box plots for comparison of 8 populations of *E. helleborine* embryos. Gray dots – anthropogenic habitats, black dots – natural habitats.

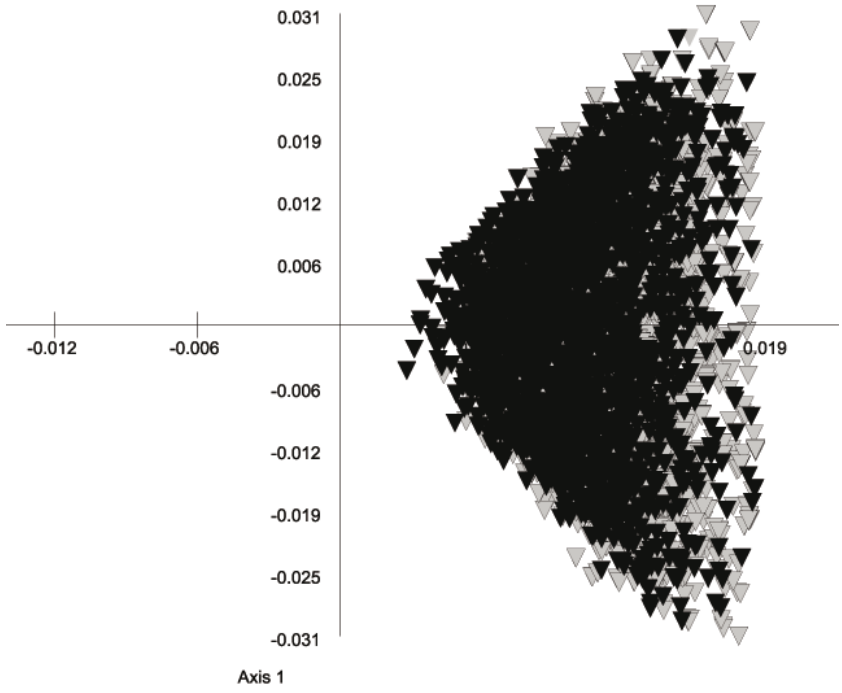


**Figure 5.** Box plots for comparison of 8 populations of *E. helleborine* seeds. Gray dots – anthropogenic habitats, black dots – natural habitats.

**Table 5.** Seed's content of nitrogen (N), phosphorus (P), and potassium (K).

| | Habitats | |
|---|---|---|
| | Anthropogenic | Natural |
| $N\text{-}NO_3$ | 14.8 | 66.0 |
| K | 53.0 | 58.5 |
| P | 22.1 | 17.2 |

## 4. Discussion

As in other studies (e.g., Lombardo et al., 2011), our results showed phenotypic variability in seed size within a species, population, individual, or even fruit, whereas no evidence for regional differences has been found. Seed flow among local populations within regions is likely greater than seed flow among regions. Occasional long distance dispersal may have a considerable effect on genetic structure, retarding the development of regional differences. A similar result was obtained by Pedersen and Ehlers (2000). They studied three species of *Epipactis* and found significant among-population differentiation, but found no differences between the regions.

Each of the habitats we examined showed a characteristic quantity or pattern of plasticity for some traits. This supports the theory of Schlichting and Levin (1984) that ecologically similar individuals should have similar plasticities. A similar result was found in *Ophrys* (Aybeke, 2007). Overall, the results seem to support the theoretical prediction that phenotypic plasticity should be of greater magnitude in more heterogeneous environments (Alpert and Simms, 2002).

We found that the *E. helleborine* plants from anthropogenic populations produced more seeds per fruit than those from natural populations (the average figure for anthropogenic populations was 2265 and for natural populations was 1700). However, recent studies have suggested that fruit production in one season may incur a cost to reproduction such that reproductive output and/or vegetative growth is lower in future seasons (reviewed in Neiland and Wilcock, 1998). Hence, these preliminary results of *E. helleborine* seeds should be further confirmed.

There was an observable trend that plants were taller in the anthropogenic area than in the natural area (Rewicz, data unpublished). Both the number of seeds and their size are probably dependent in part on plant size. Hence, large plants growing in the natural areas produced more seeds than small plants growing in the anthropogenic areas.

Here, both seed and embryo volumes and the ratios between them in *E. helleborine* were variable. This is similar to measures given by Arditti et al. (1967, 1980) in populations of plants in North America. Variations in seed and embryo volume also exist in other orchid species, for example in *Corralorhiza striata* (Arditti et al., 1980). According to Arditti et al. (1979), the seed volume in orchids is a reflection of the size of the seeds. Therefore, studies to understand the seed volume are very important. In the present study, there were no significant differences in either seed or embryo width. Similar results were obtained by Arditti et al. (1980) and Augustine et al. (2001).

Anthropogenic habitats, e.g., poplar plantations, may not replace the original conditions of a forest, but is possible that these and other orchid species can survive and reproduce in poplar plantations that provide appropriate microclimate conditions for the plants, including pollinators.

In *E. helleborine*, higher seed volume was found to be the result of a greater width rather than length of the testa. This finding is in agreement with those previously published by Swamy et al. (2004).

Several studies have reported that an increase in the nutrient concentration of the growing environment often leads to the production of heavier seeds (e.g., Aarssen and Burton, 1990) and greater quantities and concentrations of nutrients (e.g., Austin, 1966). Studies of closely related species also have found that species from fertile soil had larger seeds with more seed nutrients than species from infertile sites (e.g., Vaughton and Ramsey, 2001). Because reduced soil fertility in anthropogenic habitats decreased the seed weight of *E. helleborine*, we propose that the gradient in soil fertility between the two growth habitats helps to explain the heavier seeds of *E. helleborine* in more fertile natural habitats.

In our study, seed weight was negatively correlated with the numbers of seeds that a flower produced. A similar result was found in *Ranunculus acris* (Totland and Birks, 1963) and *Bromus sacalinus* (Skrajna et al., 2012).

Besides the nutrient solutions, light conditions (shade vs. sun) in different habitats can also be expected to affect seed size variation (Wyllie-Echeverria et al., 2003). Two major studies of seed weight in relation to the environment have shown shade and drought to be the most important correlates of seed size in the native flora of Britain and California, respectively (Salisbury, 1942). Moreover, Agren (1989) found that differences in light conditions resulted in differences in seed weight among populations of *Rubus chameomorus*. Our data support this trend to some degree. In the present study, eight populations with contrasting light availability were chosen. Four populations were located on urban road verges and had relatively high light levels with no overhead canopy but a dense herbaceous cover composed primarily of grasses. Four populations were in forest interiors that had patchy canopy shade, resulting in less available light, and a lower

density of herbaceous plants than the open sites. Seeds in the shaded habitats were heavier than seeds from habitats that had relatively high light levels. *E. helleborine* seems to prefer shady areas, and hence seeds from the population in the forest are larger and heavier. Besides the effects of light, water and nutrient solutions and air temperature in the different habitats can also be expected to affect seed size variation.

Seed weight may also affect seedling survival. Decreased seed weight can be disadvantageous, since small seeds are often associated with a lower germination percentage and smaller seedlings that would decrease the chances of seedling establishment and survival to reproduction (Agren, 1989; Wyllie-Echeverria et al., 2003). Salisbury (1942) discussed the fact that in some species larger (heavier) seeds can produce 'larger more vigorous seedlings' and may be better suited to endure 'unfavourable climatic conditions' to produce a seedling.

It is not possible to dissect the genetic and environmental components of variation in seed mineral content in wild species. *E. helleborine* shows considerable genetic variability as a result of outcrossing; however, a major portion of the morphological variation is the product of phenotypic plasticity. So far, chromosome numbers 2n = 38, 40 (Hollingsworth and Dickson, 1997) and 2n = 36, 38, 40, 44, 52, 60 (Delforge, 2006) have been determined. Plasticity probably contributed to this species' successful colonization of a wide variety of temperate habitats, and, as a result, cytotype and the habitat occupied are not apparently correlated.

This study of seed trait differences may contribute to our understanding of optimum habitat conditions and the ecophysiological adaptations of plants. We recommend that further research be performed to investigate the effects of other environmental factors on morphological traits. Within its broad distribution, there is considerable morphological variation between *E. helleborine* individuals, which may correlate with local environmental conditions. These should integrate with detailed studies on flowering time, morphology, and habitat and correlations between them. As pointed out by Krannitz (1972), studies are needed in wild plant species to determine the relative importance of seed size vs. seed mineral content on seedling establishment and final adult size.

## References


Aarssen LW, Burton SM (1990). Maternal effects at four levels in *Senecio vulgaris* (Asteraceae) grown on a soil nutrient gradient. Am J Bot 77: 1231–1240.

Adamowski W (2006). Expansion of native orchids in anthropogenous habitats. Pol Bot Stud 22: 35–44.

Adamowski W, Stefaniak A, Święczkowska E (2012). Giant *Epipactis helleborine* (L.) Crantz on the outskirts of the Białowieża Forest. Acta Bot Siles 8: 129–135.

Agren J (1989). Seed size and number in *Rubus chamaemorus*: between habitat variation, and effects of defoliation and supplemental pollination. J Ecol 77: 1080–1092.

Allen SE (1989). Chemical Analysis of Ecological Materials. 2nd ed. Oxford, UK: Blackwell Scientific Publications.

Alpert P, Simms EL (2002). The relative advantages of plasticity and fixity in different environments: when is it good for a plant to adjust? Evol Ecol 16: 285–297.

Arditti J, Michaud JD, Healey PL (1979). Morphometry of orchid seeds. I. *Paphiopedilum* and native California and related species of *Cypripedium*. Am J Bot 66: 1128–1137.

Arditti J, Michaud JD, Healey PL (1980). Morphometry of orchid seeds. II native California and related species of *Calypso*, *Cephalanthera*, *Corallorhiza* and *Epipactis*. Am J Bot 67: 347–360.

Augustine J, Yogendrakumar K, Sharma J (2001). Orchids of India II. Biodiversity and status of *Bulbophyllum* Thou. New Delhi, India: Daya Publishing House.

Austin RB (1966). The growth of watercress (*Rorippa nasturtium aquaticum* (L) Hayek) from seed as affected by the phosphorus nutrition of the parent plant. Plant Soil 24: 113–120.

Aybeke M (2007). Pollen and seed morphology of some *Ophrys* L. (Orchidaceae) taxa. J Plant Biol 50: 387–395.

Delforge P (2006). Orchids of Europe, North Africa and the Middle East. London, UK: AC Black Publishers.

Dickson JH (1990). *Epipactis helleborine* in gardens and other urban habitats: an example for apophytism. In: Sukopp H, Hejny S, editors. Urban Ecology: Plants and Plant Communities in Urban Environments. The Hague, the Netherlands: SPB Academic Publ, pp. 245–250.

Ehlers BK, Olesen JM, Gren JA (2002). Floral morphology and reproductive success in the orchid *Epipactis helleborine:* regional and local across-habitat variation. Plant Syst Evol 236: 19–32.

Harper JL, Lovell PH, Moore KG (1970). The shapes and sizes of seeds. Annu Rev Ecol Syst 1: 327–356.

Hollingsworth PM, Dickson JH (1997). Genetic variation in rural and urban populations of *Epipactis helleborine* (Orchidaceae) in Britain. Bot J Linn Soc 123: 321–331.

Krannitz PG (1997). Variation in magnesium and nitrogen content in seeds of antelope bitterbrush (*Purshia tridentata*, Rosaceae). Am J Bot 84: 1738–1742.

Light MHS, MacConaill M (2006). Appearance and disappearance of a weedy orchid, *Epipactis helleborine*. Folia Geobot 41: 77–93.

Liu W, Deng RF, Liu WP, Wang ZM, Ye WH, Lan YW, Hong LC, Hao S (2011). Phenotypic differentiation is associated with gender plasticity and its responsive delay to environmental changes in alternanthera philoxeroides – phenotypic differentiation in alligator weed. PLoS One 6.

Lombardo TV, Cafasso D, Cristaudo A, Cozzolino S (2011). Phylogeographic patterns, genetic affinities and morphological differentiation between *Epipactis helleborine* and related lineages in a Mediterranean glacial refugium. Ann Bot 107: 427–436.

Lundholm JT, Richardson PJ (2010). Habitat analogues for reconciliation ecology in urban and industrial environments. J Appl Ecol 47: 966–975.

McCormick MK, Whigham DF, O'Neill J (2004). Mycorrhizal diversity in photosynthetic terrestrial orchids. New Phytol 163: 425–438.

McDonnell MJ, Pickett STA, Groffman P, Bohlen P, Pouyat RV, Zipperer WC, Parmelee RW, Carreiro MM, Medley K (1997). Ecosystem processes along an urban-to-rural gradient. Urban Ecosyst 1: 21–36.

McKendrick SL, Dickson JH (1994). The autecology of *Epipactis helleborine* in the Glasgow area. Proceedings of the 14th World Orchid Conference 1: 161–169.

Neiland MRM, Wilcock CHC (1998). Fruit set, nectar reward, and rarity in the Orchidaceae. Am J Bot 85: 1657–1671.

Noel F, Machon N, Porcher E (2007). No genetic diversity at molecular markers and strong phenotypic plasticity in populations of *Ranunculus nodiflorus*, an endangered plant species in France. Ann Bot 99: 1203–1212.

Pedersen H, Ehlers BK (2000). Local evolution of obligate autogamy in *Epipactis helleborine* subsp. *neerlandica* (Orchidaceae): a recent response to water stress? Plant Syst Evol 223: 173–183.

Pedersen HÆ, Watthana S, Srimuang K (2013). Orchids in the torrent: on the circumscription, conservation and rheophytic habit of *Epipactis flava*. Bot J Linn Soc 172: 358–370.

Pickett STA, Cadenasso ML, Grove JM, Nilon CH, Pouyat RV, Zipperer WC, Costanza R (2001). Urban ecological systems: linking terrestrial ecological, physical, and socioeconomic components of metropolitan areas. Ann Rev Ecol Evol Syst 32: 127–157.

Salisbury EJ (1942). The Reproductive Capacity of Plants: Studies in Quantitative Biology. London, UK: G. Bell and Sons Ltd.

Samson DA, Werk KS (1986). Size dependent effects in the analysis of reproductive effort in plants. Am Nat 127: 667–680.

Schlichting CD (2002). Phenotypic plasticity in plants. Plant Species Biol 17: 85–88.

Schlichting CD, Levin DA (1984). Phenotypic plasticity of annual Phlox. Tests of some hypothesis. Am J Bot 71: 252–260.

Skrajna T, Kubicka H, Rzymowska Z (2012). Phenotypic variation in relation to seed storage protein polymorphism in *Bromus secalinus* L. (Gramineae) populations from north-eastern Poland. Pol J Ecol 60: 41–55.

Stachurska-Swakoń A, Kuź K (2011). Phenotypic response of *Doronicum australicum* Jacq (Asteraceae) to diverse mountain and lowland condition. Pol J Ecol 59: 249–262.

Stefaniak A, Adamowski W, Święczkowska E, Jakubska-Busse A (2011). Changes in the abundance and population density of *Epipactis helleborine* (L.) Crantz (Orchidaceae) in anthropogenic habitats in northeastern Poland. Acta Bot Siles 7: 189–196.

Swamy KK, Kumar HNK, Ramakrishna TM, Ramaswamy SN (2004). Studies on seed morphometry of epiphytic orchids from western Ghats of Karnataka. Taiwania 2: 124–140.

Totland Ø, Birks HJB (1996). Factors influencing inter population variation in *Ranunculus acris* seed production in an alpine area of southwestern Norway. Ecography 19: 269–278.

Valladares F, Martinez-Ferri E, Balaguer L, Perez-Corona E, Manrique E (2000). Low leaf-level response to light and nutrients in Mediterranean evergreen oaks: a conservative resource-use strategy? New Phytol 148: 79–91.

Vaughton G, Ramsey M (2001). Relationships between seed mass, seed nutrient, and seedling growth in *Banksia cunninghami* (Proteaceae). Int J Plant Sci 162: 599–606.

Wyllie-Echeverria S, Cox PA, Churchill AC, Brotherson JD, Wyllie-Echeverria T (2003). Seed size variation within *Zostera marina* L. (Zosteraceae). Bot J Linn Soc 142: 281–288.