Dianthus superbus as a critically endangered species in Latvia: evaluation of its growth conditions and conservation possibilities

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Abstract

Dianthus superbus is one of the most endangered species in Latvia and is on the verge of local extinction. Therefore, the aim of this study was to inventory previously identified populations of D. superbus in Latvia and to develop activities to conserve this species in accordance with the results obtained in situ. Expeditions on 18 previously documented locations, according to the data of Nature Conservation Agency, revealed only three existing D. superbus localities in Latvia with a significant number of specimens located in the Latgale region near Silenieki. In 2020, for the first time, two more new D. superbus localities were found in the vicinity of these three approved locations. As it is not possible to create micro-reserves in the existing localities which are mainly located either on road sides or in a cemetery, additional activities are needed to preserve the D. superbus in Latvia. In vitro culture was initiated from shoot explants obtained in situ, from which, in turn, ex vitro plantings were done in the National Botanic Garden (NBG) and the Botanical Garden of the University of Latvia (BG UL). Their quality and compliance with plants in situ were analysed. Overall, it was concluded that the plants grown ex situ were qualitatively equivalent to those obtained in situ, as a very high percentage of seed viability and germination was found both for in situ and ex situ growing D. superbus. Based on the results obtained we could conclude that D. superbus is a species that is able to adapt to different soils with a wide range of nutrient levels, moisture conditions and pH, as long as there are three main preconditions—adequate lighting, low overgrowth of other plant species and non-destructive human activities.

Keywords: Conservation options; habitat characteristics in situ; seed viability and germination; soil agrochemical analysis.

Introduction

Dianthus superbus is widely but patchily distributed in Europe and northern Asia. Although not considered to fall within a threatened category in Europe as a whole, D. superbus is mentioned as a seriously threatened species in several European countries. It is considered to be possibly extinct in the Netherlands, critically endangered in the Czech Republic and Romania, vulnerable in France, Sweden and Poland, near threatened in Hungary and rare in Denmark (Mikulík and Vinter 2001–2002; Holobiuc et al. 2004–2005; Rosenthal 2010; Kostrakiewicz-Gierałt 2013; Hardion et al. 2018). Dianthus superbus is protected in the south of Finland, and a variant growing on the serpentines is critically endangered (Lehmuskallio 2014).
In the Baltic States D. superbus is included in the category of endangered species in Red Data Book of Lithuania (Rašomavičius 2007) and it is classified as vulnerable species in Estonia (https://elurikkus.ee/bie-hub/species/4309#redlist). In Latvia D. superbus as an endangered species on the edge of extinction is included in the Regulations of the Cabinet of Minister of Latvia No. 396 (Anonymous 2000) and the first category of threat in Red Data Book of Latvia (Kabucis 2003). Dianthus superbus is also included in the Red Data Book of the Baltic Region (Ingeló et al. 1993).

The decline of suitable habitats is considered to be a major threat for the species (Miranto et al. 2012; Allen et al. 2014). Due to changes in land use and agricultural practices, the natural or semi-natural habitats of D. superbus were intensively farmed or practically abandoned in the last century in Europe, resulting in highly fragmented or extinct habitats. Spatial isolation can lead to low genetic diversity, weakened fitness and declining population size, leading to a high risk of local extinction (Kostrakiewicz-Gieralt 2013, Hardion et al. 2018).

Given the progressive processes of extinction of many populations and the lack of data on variations in population characteristics, it should be emphasized that further demographic research is still needed. Demographic data can provide information not only on the status of population but also on its survival prospects in situ and serve as a basis for appropriate conservation programmes.

Seed germination is a significant phase in the reproductive cycle of species. An appropriate temperature, enough water and oxygen are very important factors for achieving a high germination rate. According to Mikulik and Vinter (2001–2003), the parameters of D. superbus germination were very high immediately after the maturation of capsules, exceeding 80%. At the same time, seeds of D. superbus are very susceptible to loss of germination ability. Thus, after 5 years of storage, germination energy and rate decreased twice (Kurek et al. 2019). Oxidative damage, deterioration of nucleic acids and fall of enzymatic activity are mentioned as potential drivers of the seed aging process (Kurek et al. 2019). In general, seed germination takes place over a certain temperature range, the most important of which is related to the phytogenetic adaptation of the species. An optimum temperature for D. superbus germination between 15 and 25 ºC was reported by Hiromasa and Noboru (2006). Studies in the Czech Republic also showed a high germination rate of D. superbus at 20 ºC (Šebánek 1992). Such a temperature from the germination point of view is very characteristic of many plant species also in the temperate and boreal zone.

One of the important factors for species growth and persistence is appropriate soil conditions. Only general soil characteristics for D. superbus are available. Dianthus superbus is considered a species favouring rich, well-drained sandy or loamy soil with neutral or slightly alkaline pH (Nature Gate 2021). Soils with a high content of organic matter in the upper horizons and a strong hydromorphic character have also been reported as appropriate for D. superbus (Gavrilova 1999; Hardion et al. 2018). Therefore, more detailed studies on soil properties and nutrient status are necessary not only to understand the ecology and the conservation potential of D. superbus in the wild, but also for preservation ex situ and reintroduction needs.

Although endangered plant species in Latvia are relatively well documented, information on D. superbus is mainly available from herbarium materials, literature and database of the Nature Conservation Agency. As mentioned by Priedits (2014) D. superbus is one of the most endangered plants in Latvia and is on the verge of extinction. There are very few data on changes in the characteristics of D. superbus population in recent decades and the current status of this species in Latvia (Jakobsone et al. 2020). So far, no research has been carried out on the possibilities of conservation of this species in Latvia. Therefore, the aim of this study was to inventory previously identified populations of D. superbus in Latvia in order to develop the activities for the conservation of this species in accordance with the results obtained in situ. To achieve this aim, a number of tasks were set: (i) to explore habitat characteristics of the D. superbus in situ, (ii) to determine soil agrochemical composition in situ and ex situ, (iii) to compare reproduction characteristics and potential in situ and ex situ, (iv) to reveal species conservation options.

The study species

Superb Pink D. superbus (Caryophyllaceae) is a loosely tufted perennial, medium-sized (35–80 cm) (Gavrilova 1999), clonal forb with a strong primary root and an ascending or creeping rhizome. The vegetative stems are topped with oblong leaves, while generative shoots bear a solitary flower (Ø 3–5 cm) (Priedits 2014) or 3–15 flowers grouped in inflorescences (Kabucis 2003). Typical inflorescences have one primary flower, secondary flowers on each side of the central axis and tertiary flowers on each side of the lateral axis. The flowers are composed of five free, deeply cut fringed petals forming a functional tube enclosed by the tubular calyx, which is whitish in colour, have a long calyx tube, up to 2.5 cm long, and are strongly scented, especially during the night, indicating pollination by night-active flower visitors. However, the flowers do not exclude diurnal flower visitors (Jürgens et al. 2003).

Dianthus superbus is known to form subspecies, some of which have a limited geographical range. As in most species range, the subspecies found in Latvia is attributed to D. superbus subsp. superbus (https://www.latvijasdaba.lv/augi/dianthus-superbus-l/). To the east of Latvia, in Russia, and in Ukraine, another subspecies is common—D. superbus subsp. stenocalyx (Jalas and Suominen 1988). Recent studies have shown that the taxonomy of this species in Europe is still unresolved, calling into question the subsp. sylvestris, while supporting subs. alpestris (Hardion et al. 2020).

The ecotope for D. superbus is wet meadows and fens (Gavrilova 1999); individuals form small groups in fens, periodically wet meadows on peat or clay on carbonic deposits (Priedits 2014). In contrast to these findings, habitats for D. superbus in Finland are sandy and gravelly riverbanks, shore meadows, rocky embankments, dry commons and roadsides (Lehmuskallio 2014). In Greece, it is also found in grassy clearings (Laf几张inas and Sfkar 2009).

Methods

Inspection in situ

In 2017, numerous expeditions were carried out to verify the data on all previously known locations of D. superbus (Jakobsone et al. 2020). Unfortunately, in most of them D. superbus was no longer discovered. This study was continued and existing localities were re-surveyed in 2019 and 2020. During the expedition in 2020, D. superbus was additionally searched in Silenieki (Fig. 1), surveying a wider area near the only locality in Latvia with substantial population size. In 2020, D. superbus was found for the first time in a meadow between the River Pededze and a forest at the end of a sand road (MP) and on a hillock with clearing at the farmstead ‘Priežulejas’ (HC) (Fig. 1). A description of vegetation with an inventory of the accompanying species
was done for the localities where D. superbus specimens were found. All generative shoots and the number of flowers on them were counted. Seeds were collected for seed viability and germination tests, as well as for an ex situ collection.

**Preservation ex situ**

In order to survive, parallel conservation measures are needed for the endangered species: in situ and ex situ, including in vitro. Ex situ activities were performed in the National Botanic Garden (NBG) and in the Botanical Garden of the University of Latvia (BG UL). In vitro culture of D. superbus was developed in the Department of Eco-Physiology of NBG with cuttings collected in 2017 at the Silenieki findings. The propagation procedure is given below. In vitro obtained plantlets were used to establish plantations in both botanical gardens in the spring of 2018.

**Seed viability and germination**

To evaluate the existing in situ populations of D. superbus, tests were carried out on seed viability and germination. Seeds were collected in populations near Silenieki (GR, SR, MP) and in NBG and BG UL plantations, established under conditions close to natural. The embryo viability was determined using
1 % 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) test (modified Ramsey and Dixon 2003). One part of the seeds collected in situ in 2017 was analysed directly after collection, the other—in the following spring. For this part dry weight of 100 seeds was determined, the seeds were stored in a refrigerator at 4 °C during the winter and the viability of the seeds was tested in the spring of 2018. For TTC test, 50 seeds were analysed in three repetitions. For germination test, 30 seeds were put in Petri plates on double-layer filter paper, one on top, moistened with distilled water. The same experiments were repeated with seeds collected in autumn 2019 with seeds collected in situ and from in vitro obtained plants in both botanical gardens. Germination test was done in an artificial climate camera with a 16/8 h light/dark photoperiod and temperature regime 23 °C/15 °C. Seeds were counted every 3 days. The TTC test was repeated in 2020 after additional sites with D. superbus (HC, MP) were found.

**Soil analyses**

Soil samples were collected several times in the period from 2017 to 2020 from the populations near Silenieki (GR, SR), in 2020 from the newly discovered populations (MC, HC) and in 2019–20 from the ex situ plantations in NBG and BG UL. Samples were taken from the plant root zone at 0–20 cm depth, dried to air-dry condition and sieved through a 2-mm sieve. The plant-available concentrations of nutrients (N, P, K, Ca, Mg, S, Fe, Mn, Zn, Cu, Mo and B) in soil samples were extracted with 1 M HCl solution, in which the soil-extractant mixture volume ratio was 1:5 (Rinkis et al. 1987). The soil pH was detected in 1 M KCl solution (soil-extractant mixture 1:2.5), and electrical conductivity (EC) was measured by using distilled water extract (soil/distilled water ratio 1:5). The levels of K, Ca, Mg, Fe, Cu, Zn and Mn were estimated by microwave plasma atomic emission spectrometer (MP-AES) Agilent 4200 (Anonymous 2021), those of N, P, Mo and B were analysed by the colourimetry, S by turbidimetry (spectrophotometer JENWAY 6300). Soil pH was determined potentiometrically by pH-meter Sartorius PB-20, EC with the conductometer Hanna EC 215, soil organic matter content according to Tjurin method (Rinkis et al. 1987).

**In vitro**

In vitro culture initiation was done with cuttings from plants obtained near the Silenieki at the edge of a gravel road. Sterilization procedure: cuttings with two internodes were soaked in weak K permanganate solution overnight, then rinsed in distilled water and immersed in 1/4 AE (commercial solution with active Cl) for 15 min. After the sterilization procedure, the cuttings were rinsed four times in sterile distilled water and placed in culture tubes. For the initiation of in vitro culture, 16 × 100 mm Pyrex culture tubes (SIA MyLab, Latvia, Riga) were used with 14 mL medium and closed with foil. Hereafter the cultivation was made in 24 × 200 mm Duran culture tubes (‘Antonidis Electronics Educational Ltd’, Cyprus) with 25 mL medium, closed with foil; in each tube—three explants. The cultivation media based on Murashige and Skoog (1962) inorganic substances. The list of organic substances was modified in the Department of Eco-Physiology, NBG: the culture initiation medium contains 0.12 mg L⁻¹ 6-benzylaminopurine, 0.03 mg L⁻¹ α-naphthalene acetic acid and β-iodole acetic acid, whereas the cultivation medium was used without phytohormones. The reagents for in vitro cultivation were obtained from Duchefa Biochemie B.V., Haarlem, the Netherlands. The pH of the culture media was adjusted to 5.8 using either HCl or NaOH (both reagents from Sigma-Aldrich®, St Louis, MO, USA) before gelling with agar (Duchefa Biochemie B.V.) prior to autoclaving for 15 min at 121 °C and 1.05 kg cm⁻². There were 15.25 × 15 cm culture tubes per treatment closed with polypropylene cups (Sigma-Aldrich®).

Culture tubes with explants were placed in a cultivation chamber under master TL-D Super 80 58W/840 white luminescent lamps (Philips, Pila, Poland), with a light intensity of 27.6 μmol m⁻² s⁻¹ and a 16-h photoperiod for 2 × 2 months, i.e. transplanting in vitro was done every 2 months.

For slow growth conditions, the plantlets were periodically held in a cultivation chamber under master TL-D Super 80 58W/840 white luminescent lamps (Philips), with a light intensity of 27.6 μmol m⁻² s⁻¹ and a 12-h photoperiod and 4–6 months.

Transplanting in vitro was done every 2 months.

**Ex vitro in outdoor conditions**

The cuttings from in vitro obtained plantlets were replanted in substrate in ex vitro conditions using plant boxes with neutral fertilized peat (pH 6–7) on March 2018. The plant boxes were placed under white luminescent lamps for 2 months. The rooted plants were then transplanted outdoors in the territory of NBG and BG UL. In NBG, the plants were planted in two places: in a perennial plant bed on 5 m² plot and on the southern slope of the pond, in semi-natural conditions on four plots (1.2 × 1.2 m). In BG UL, in vitro derived plants were transplanted (i) in ornamental, (ii) in semi-natural conditions on the shore of a pond with Taxus baccata and Tussilago farfara (2 × 2 m) and (iii) in semi-natural conditions on the shore of a pond with Matteuccia struthiopteris (2 × 2 m). The first observations were made in 2019 during flowering; they were repeated in 2020. From the beginning of flowering, plants were evaluated by counting the generative shoots and the number of flowers per shoot.

Statistical analysis of the results was done using MS Excel 2016. Standard errors (SEs) were calculated to reflect the mean results of seed viability and germination rate, as well as the number of flowers per generative shoot. Heterogeneity of soil nutrient concentrations was characterized by the coefficient of variation (CV). The Student’s t-test “Two-Sample Assuming Unequal Variances” (P < 0.05) was used to check the significance of the differences between D. superbus sites in situ and ex situ in the number of flowers per generative shoot and seed quality parameters.

**Results**

**In situ**

Examination of 18 previously dated localities revealed that D. superbus had survived only in four: one in Kurzeme region—in the micro-reserve Dzirnieki at Lake Vigilje (DZ) and in Latgale region—locality near Lake Lubins: in the Silenieki-cemetery (SC), at a gravel road edge near the village Silenieki (GR), on a sand road and on the side of the road in a pine forest near the village Silenieki (SR) (Fig. 1). Overgrowth with other tall herbaceous species or shrubs was found in areas where carnations were no longer observed. Cattle grazing and trampling were also found at some previously dated D. superbus sites. In the only micro-reserve for D. superbus in Latvia (DZ), which is located in a private territory, the meadow was mowed early and low. In 2017, only one generative shoot with one flower and two buds was found in August (Fig. 2A). During the expedition in 2020, D. superbus was first found in two new locations near Silenieki: in a meadow between the Pededeze River and the forest at the end of the
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sand road (MP), as well as on the hillock with a clearing at the farmstead ‘Priežulejas’ (HC) (Fig. 1).

Dianthus superbus was growing in cemetery, located on a sandy pine forest hill (SC), on gravel road edge in clearing (GR); near a pine forest with some other tree species on sandy, dry roadsides (SR); in a meadow between the shore of the Pededze River and the edge of a forest at the end of a sand road (MP); and on the hillock with ~2 years old clearing at the farmstead ‘Priežulejas’ (HC). The study showed that the accompanying plant species in these localities form a low and sparse ground cover (Fig. 2B–E). Detailed characteristics of the D. superbus habitats in situ are given in Table 1, characteristics of the environment of D. superbus in semi-natural conditions and as ornamental plant ex situ—in Table 2. Significant differences in soil reaction were found for five sites where D. superbus was currently distributed in situ: pHKCl from 3.75 to 6.96. The substrate reaction from acidic to neutral was also found for D. superbus ex vitro in NBG and BG UL (3.66–7.14) (Table 2).

Soil analyses

In general, wide ranges of nutrient concentrations, soil reaction and organic matter content were found at various D. superbus sites in Latvia (Table 3). Of the macronutrients, the highest CV was determined for Ca (216 %) and Mg (259 %), the lowest for S (44 %). For micronutrients, the highest concentration variability was determined for Fe (81 %) and Cu (68 %), the lowest for Mo (28 %). Since the EC characterize the level of soluble ions in the soil, the CV for EC (59 %) coincided with the average of N, K, S and Na.

According to soil analyses, D. superbus localities in Latvia were mainly found on mineral soils with an organic matter content of 2.85–8.55 %. In general, soil pH and EC, as well as nutrient (N, P, K, Ca, S, Fe, Mn and Mo) concentrations for ex situ cultivation of D. superbus in NBG and BG UL were within the range found in the wild (Tables 3 and 4). The maximum concentrations of Ca, Zn, Cu, B and Na found in soils of botanic gardens were higher. While in semi-natural growing sites ex situ the soil organic matter content ranged from 4.0 to 7.5 %, there was also one site developed on peat soil (perennial plant bed NBG) with an organic matter content of 39 %.

Due to the large dispersion of pH values (3.75–6.96) in wild growth sites of D. superbus, the concentrations of Ca and Mg also differed significantly—52 and 106 times, respectively, comparing the minimum and maximum values. Nevertheless, the Ca:Mg ratio ranged only from 5.0 (meadow at the Pededze River) to 8.3 (hillock with a clearing at the farmstead ‘Priežulejas’) indicating the presence of similar soil bedrocks.
| Site                | Accompanying species                                                                 | pH/KCl |
|---------------------|---------------------------------------------------------------------------------------|--------|
| DZ Micro-reserve    | **EU grassland**, **Pimpinella saxifraga**, **Deschampsia flexuosa**, **Galium boreale**, **Achillea millefolium** | 4.38   |
| Lake Vilg variant   | **Anthoxanthum odoratum**, **Anthriscus sylvestris**, **Angelica sylvestris**, **Lychnis flos-cuculi**, **Campanula patula**, **Centaurea sylvestris**; inclusions of **G. verum**, **Festuca ovina s.str.**, **Dianthus deltoides** | 6.27   |
| Cemetery            | **Hieracium pilosella s.l.**, **E. canadensis**, **Erigeron acris**, **H. umbellatum**, **Jasione montana**, **Festuca rubra s.l.**, **campestris**, **Leontodon autumnalis**, **Scleranthus**, **Rumex acetosa**, **Potentilla argentea**, **R. acetosella**, **annuus**, **Sieglingia decumbens**, **Trifolium repens**, **Equisetum arvense**, **Vicia cracca**, **Pinus sylvestris**, **C. rotundifolia**, **C. oleraceum**, **Campanula patula** | 6.96   |
| Road edge           | **Pinus sylvestris**, **Juniperus communis**, **Thymus serphyllum**, **Vaccinium myrtillus**, **V. vitis-idaea**, **Agrostis stolonifera** | 6.75   |
| Semi-natural        | **Antennaria dioica**, **Agrostis stolonifera**, **Artemisia campestris**, **Dianthus barbatus** | 4.52   |
| Cemetery            | **Antennaria dioica**, **Agrostis stolonifera**, **Artemisia campestris**, **Dianthus barbatus** | 4.47   |
| Cemetery            | **Antennaria dioica**, **Agrostis stolonifera**, **Artemisia campestris**, **Dianthus barbatus** | 4.47   |
| Cemetery            | **Antennaria dioica**, **Agrostis stolonifera**, **Artemisia campestris**, **Dianthus barbatus** | 4.47   |
| Cemetery            | **Antennaria dioica**, **Agrostis stolonifera**, **Artemisia campestris**, **Dianthus barbatus** | 4.47   |
| Cemetery            | **Antennaria dioica**, **Agrostis stolonifera**, **Artemisia campestris**, **Dianthus barbatus** | 4.47   |

Table 1. Characteristics of the examined species habitats in situ.
in D. superbus growth sites in situ. Only one lower Ca:Mg ratio (2.9) was found for soil from the edge of the gravel road near the village Silenieki, which indicates the presence of dolomite impurities.

**Seed viability and germination**

Generative capacity—the total number of generative shoots and flowers of D. superbus for in situ localities—was registered. It was found that none of the localities had <130 generative shoots and 650 flowers. The greatest number of generative shoots and the most abundant flowering was found on the hillock with clearing at the farmstead ‘Priežulejas’ (HC)—in summary 699 generative shoots with 7340 flowers. Seed viability and germination tests performed in our study showed a high percentage of viable seeds of D. superbus from all sites in situ and ex situ (Fig. 3; see Supporting Information—Table S1.1). The highest viability (98 %) was found for seeds sampled in BG LU, where the lowest number of flowers per generative shoot was recorded. It is notable that seeds collected from the D. superbus grown in natural conditions of site HC, with significantly higher number of flowers per shoot, exhibited the lowest viability—70.7 %.

To carry out the seed viability test, seeds were soaked in distilled water for 2 days. Within this 2-day period, 41.3 % (HC, 20) to 84 % (BG LU) of the soaked seeds had already germinated (Table 5).

| Table 2. Characteristics of the environment of Dianthus superbus in semi-natural conditions and as ornamental plant ex situ in National Botanic Garden (NBG) and in the Botanic Garden of the University of Latvia (BG UL). |
| Botanic garden | Planting place | Substrate characteristic | pH\text{KCl} |
| NBG | Pond south slope, semi-natural conditions | Dry sandy soil | 6.25–6.51 |
| NBG | Perennial-bed | Peat | 3.66 |
| BG UL | Bed for threatened plants | Dry cultivated soil | 7.14 |
| BG UL | Pond slope | Dry sandy soil | 6.81–6.9 |
| BG UL | Pond slope | Dry sandy soil | 6.88–6.9 |

| Table 3. Nutrient concentration (mg L\(^{-1}\), 1 M HCl extraction), soil pH and EC and organic matter content in air-dry soils from Dianthus superbus in situ sites in Latvia, 2017–20. *Coefficient of variation. |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Micro-reserve | Gavel road edge near the village Silenieki | Gavel road edge near the village Silenieki | Sand road and at the side of the road in pine forest near the village Silenieki | Meadow between Pededze shore and forest edge | Hillock with clearing | Range | CV*, % |
| Dzirnieki at the Lake Vilgāle (2017) | Gravel road edge near the village Silenieki/1 (2017–20) | Gravel road edge near the village Silenieki/2 (2017) | (2017–20) | (2020) | (2020) | | |
| N | 113 | 8–33 | 25 | 28–55 | 23 | 10 | 8–113 | 83.5 |
| P | 256 | 150–213 | 213 | 119–610 | 278 | 116 | 116–610 | 61.5 |
| K | 165 | 77–180 | 175 | 65–327 | 80 | 63 | 63–327 | 61.5 |
| Ca | 770 | 375–409 | 14 250 | 275–690 | 1043 | 778 | 275–14 250 | 216.2 |
| Mg | 120 | 50–57 | 5000 | 47–80 | 208 | 94 | 47–5000 | 258.4 |
| S | 13 | 4–7 | 16 | 6–11 | 7 | 6 | 4–16 | 43.7 |
| Fe | 1840 | 221–270 | 630 | 524–995 | 399 | 262 | 221–1840 | 81.4 |
| Mn | 190 | 65–65 | 100 | 95–120 | 91 | 51 | 51–190 | 41.8 |
| Zn | 7.5 | 3.5–3.6 | 6.0 | 3.5–8.0 | 6.0 | 10.5 | 3.5–10.5 | 41.9 |
| Cu | 2.50 | 0.50–0.50 | 1.85 | 0.70–0.75 | 0.90 | 0.65 | 0.50–2.50 | 68.3 |
| Mo | 0.03 | 0.03–0.04 | 0.03 | 0.03–0.05 | 0.04 | 0.02 | 0.02–0.05 | 27.6 |
| B | 0.2 | 0.2–0.3 | 0.2 | 0.1–0.6 | 0.3 | 0.3 | 0.1–0.6 | 53.0 |
| Na | 9.0 | 4.9–12.0 | 18.5 | 6.0–21.0 | 10.5 | 8.9 | 4.9–21.0 | 49.0 |
| pH\text{KCl} | 4.38 | 4.22–4.59 | 6.96 | 4.13–4.56 | 5.27 | 3.75 | 3.75–6.96 | 19.9 |
| EC\text{1\,M HCl}, mS/cm | 0.24 | 0.12–0.19 | 0.56 | 0.12–0.2 | 0.25 | 0.22 | 0.12–0.56 | 59.2 |
| Organic matter, % | 4.15 | 3.40–4.00 | 5.70 | 2.85–7.50 | 4.15 | 8.55 | 2.85–8.55 | 38.2 |
| Bulk density, g cm\(^{-3}\) | 1.09 | 1.03–1.11 | 1.07 | 0.93–1.19 | 1.07 | 0.90 | 0.9–1.19 | 9.3 |
Table 4. Nutrient concentration (mg L\(^{-1}\), 1 M HCl extraction), soil pH and EC and organic matter content in air-dry soils from *Dianthus superbus* ex situ sites in National Botanic Garden (NBG) and Botanic Garden of the University of Latvia (BG UL), 2019–20. *Coefficient of variation.

|          | NBG Semi-natural meadow (2019–20) | NBG Ornamental plant bed (2019) | NBG Bed for threatened plants (2019–20) | NBG Shore of central pond (2019–20) | NBG Shore of fern pond (2019–20) | Range | CV*, % |
|----------|----------------------------------|--------------------------------|------------------------------------------|------------------------------------|----------------------------------|-------|-------|
| N        | 68–88                            | 40                            | 28–93                                    | 40–89                              | 33–65                            | 28–93 | 42.8  |
| P        | 192–234                          | 73                            | 534–556                                  | 338–360                            | 256–256                          | 73–556 | 50.3  |
| K        | 122–183                          | 124                           | 125–139                                  | 141–196                            | 115–142                          | 115–196 | 19.7  |
| Ca       | 2412–2700                        | 1045                          | 11 513–11 700                            | 4400–4898                          | 4450–4513                        | 1045–11 700 | 71.7 |
| Mg       | 373–550                          | 325                           | 6067–6500                                | 1753–1800                          | 1538–1600                        | 325–6500 | 103.1 |
| S        | 14–15                            | 4                             | 18–24                                    | 16–18                              | 15–16                            | 4–24   | 33.7  |
| Fe       | 895–1099                         | 245                           | 1326–1740                                | 849–900                            | 475–495                          | 245–1740 | 51.7  |
| Mn       | 145–145                          | 16.5                          | 116–125                                  | 63–65                              | 88–110                           | 16.5–145 | 44.1  |
| Zn       | 1.80–3.4                         | 4.8                           | 53–60                                    | 17.5–18                            | 16.5–19.5                        | 1.8–60 | 97.2  |
| Cu       | 2.00–2.25                        | 0.70                          | 9.45–10.5                                | 4.65–5.1                           | 4.50–7.0                         | 0.7–10.5 | 67.1  |
| Mo       | 0.04–0.04                        | 0.025                         | 0.05–0.05                                | 0.04–0.07                          | 0.04–0.04                        | 0.025–0.07 | 27.8 |
| B        | 0.5–0.5                          | 0.3                           | 0.2–0.5                                  | 0.7–0.9                            | 0.5–0.8                          | 0.2–0.9 | 41.2  |
| Na       | 10.9–22.0                        | 25.0                          | 43.0                                     | 37.5                               | 20.0                             | 10.9–43.0 | 44.9 |
| pH\(_{KCl}\) | 6.25–6.51                      | 3.66                          | 7.14–7.14                                | 6.81–6.90                          | 6.88–6.90                        | 3.66–7.14 | 16.9 |
| EC\(_{KCl}\) mS/cm | 0.38–0.44                    | 0.21                          | 0.27–0.33                                | 0.40–0.41                          | 0.39–0.41                        | 0.21–0.44 | 21.0 |
| Organic matter, % | 4.00–5.70                | 38.68                         | 48.0–5.50                                | 5.20–7.50                          | 6.20–6.70                        | 4.00–38.68 | 117.9 |
| Bulk density, g cm\(^{-3}\) | 1.16–1.20                  | 0.39                          | 1.26–1.30                                | 1.03–1.15                          | 1.10–1.15                        | 0.39–1.30 | 25.1  |
In 2018, D. superbus was successfully introduced in vitro and started to be maintained in the in vitro collection of NBG. Plant tissue culture left for preservation in the in vitro collection was alternately kept at a low temperature and in a warm cultivation chamber in line with the methodology. If necessary, it can also serve as a basis for the renewal of outdoor plantings.

Ex vitro

Dianthus superbus in semi-natural conditions in NBG (Fig. 4A) was characterized by the number of flowers per generative shoot and the percentage of germination and viable seeds (Figs 3 and 5; see Supporting Information—Table S1.1 and S1.2). The results showed high and comparable seed germination and viability, while the number of flowers per generative shoot was lower than under natural conditions of D. superbus. The observations made during flowering in 2019 revealed that D. superbus in the ornamental bed for perennials in NBG demonstrated high decorativeness when planted closely (Fig. 4B). For D. superbus plantings in BG UL (Fig. 4C and D), the light conditions on the shore of the ponds could be described as partial shading, but in the decorative plant bed—as full lighting. In the first year after planting, the largest flowering was found in the site of the ornamental bed, but in the second year, the flowers no longer developed. A more stable situation was found on the shores of ponds, with shoots of D. superbus developing in the first year and flowers in the second year. In general, plants grown ex situ were qualitatively equivalent to those obtained in situ.

Discussion

Habitat characteristics

Dianthus superbus which is native to most European countries is also an endangered species throughout Europe. Nowadays it is very rarely found as a wild plant in Latvia. Expeditions to update information on 18 previously registered locations revealed only three existing D. superbus localities in Latvia with a significant population size (<400 generative shoots) near Lake Lubāns. However, during repeated expeditions, two more sites with flowering plants were successfully found near the above-mentioned locations, increasing the number of deposits to five.

In 2016–20, national monitoring of habitats, plant species and other values of nature called ‘a Nature Census’ was carried out, including nationwide mapping, to gather detailed and complete...
information on Latvia’s natural capital. Although this project did not include studies on specific plant species, both many rare species and new species were identified. Unfortunately, no new localities of *D. superbus* were found during this survey. This indirectly confirms that *D. superbus* is indeed very rare in Latvia.

A number of literature sources indicated on fens and moderately moist meadows as *D. superbus* habitats (Fleischer and Lindeman 1839; Gavrilova 1999; Kabucis 2003; Kostrakiewicz-Gieralt 2013; Hardion *et al.* 2018). The only place that met this characteristic was micro-reserve Dzirnieki (DZ), where *D. superbus* was growing in a meadow with a dense accompanying species cover. In addition, the meadow was regularly mowed low. This generally led to near-disappearance of *D. superbus* in this deposit, as only one specimen was found. This species, which was observed in the lower reaches of the Pededze River even in 2015, was no longer found in 2017 (Jakobsone *et al.* 2020). Here, too, the vegetation cover was probably too high and dense, but *D. superbus* needs enough sunlight, and the growth and development of this species may require a less disruptive root system of other plants. The competition for light and resources in the distribution areas was reported as the main factor determining the growth and development of *Dianthus* species. Decreasing light entering the fen meadows at ground level can inhibit plant growth or even cause mortality (Onete *et al.* 2010; Kostrakiewicz-Gieralt 2014). Studies in Poland conducted in unmanaged *Molinietum caeruleae* meadows situated along the successional gradient have shown that the number of generative shoots, flowers, fruits and seeds of *D. superbus* gradually decreased from places overgrown by shrubs and trees to places with low meadow species. However, significant production of generative structures may not be sufficient to ensure the sustainability of populations in meadows in progressive stages of succession (Kostrakiewicz-Gieralt 2013; Kostrakiewicz-Gieralt 2014). Thus, there is a high risk of extinction of *D. superbus* in wet and humid meadows.

Almost all *D. superbus* localities in Latvia, found in the Lubana wetlands near Silenieki, were on dry sand (forest road) and sand-gravel substrate (road edge, clearing) with accompanying species such as *D. arenarius* and *Thymus serpyllum* etc. Therefore, *D. superbus* habitats in Latvia are similar to Finland, where sandy

Figure 4. *Dianthus superbus* ex vitro in 2019: (A) pond south slope, semi-natural conditions in NBG (National Botanic Garden); (B) perennial-bed in NBG (photo: Gunta Jakobsone); (C and D) in collection in BG UL (Botanical Garden of University of Latvia) (photo: Ieva Akmane).
and gravelly river banks, shore meadows, rocky embankments, dry calcareous grasslands, roadsides are reported as habitat of this species (Lehmuskallio 2014). Obviously, D. superbus has a rather wide range of growth conditions.

Soil agrochemical characteristics

*Dianthus superbus* is generally considered to be a species that prefers fertile, moist but well-drained soil with high organic matter content and a neutral to alkaline soil reaction in the pH range of 6–8 (Dansereau et al. 2007; https://pfaf.org 2021). However, some sources suggest that, although preferring alkaline soils, *Dianthus* tolerates low acidity (https://plants.ces.ncsu.edu/plants/dianthus). In Latvia *D. superbus* localities were found mainly on acid soils with pH KCl 3.75–5.27. In contrast, in botanic gardens, vital growth of *D. superbus* was also observed in soils with near-neutral pH level and significantly higher Ca and Mg concentrations (Tables 3 and 4). Therefore, soil reaction for sustainable growth for *D. superbus* could not be considered as a significant limiting factor.

Adequate levels of macronutrients in the soil are an important aspect of ensuring a favourable plant growth condition. From the point of view of plant mineral nutrition, the optimal Ca:Mg ratio in soils using 1 M HCl extraction is 5–8:1 (Rinkis and Nollendorf 1982; Cekstere and Osvalde 2013). Our results revealed that despite the exact soil content of Ca and Mg, their ratio was optimal for the accumulation of these nutrients in the plant. While the levels of P and K in *D. superbus* soils in Latvia were generally comparable to those recommended for crop plants in 1 M HCl extraction, concentrations of N and S can be assessed as very low (Osvalde 2011). However, the relatively high content of soil organic matter (between 3 and 6 %), during mineralization, can provide sufficient amounts of N and S for successful plant growth.

Although relatively wide concentration ranges of mobile forms of micronutrients Fe, Mn, Zn, Cu, Mo and B in soil were found for *D. superbus* in situ and ex situ in Latvia, the higher concentrations of Zn, Cu and B were found in botanical gardens. However, considering the low pH of soils from *D. superbus* localities, which promotes the uptake of trace elements, the low status of Cu and B in wild soils may be sufficient to meet plant needs.

Thus, soil agrochemical analyses showed a high probability of successful growth of *D. superbus* in soils with a wide nutrient and pH range. In addition, several studies have shown that *D. superbus* is also salt-tolerant (Ma et al. 2017) and resistant to heavy metal contamination (Yang et al. 2017). Thus, soil chemical content could not be considered as limiting factors for the growth and development of this species. However, optimal nutrient supply would improve the vitality of *D. superbus* in both ex situ plantings and wild populations in situ.

Species conservation options

The most effective approach for conservation of endangered plant species can be achieved by combining in situ and ex situ conservation methods (Miranto et al. 2012; Cristea et al. 2013). Although *D. superbus* is included in the list of specially protected vascular plants for which micro-reserves shall be established (Regulations of the Cabinet of Ministers of the Republic of Latvia No. 940), the prospect of in situ conservation to ensure the protection of this species in Latvia by establishing micro-reserves is limited, as the existing deposits are mainly located in a cemetery and on roadsides. Therefore, ex situ conservation of *D. superbus* outside the natural habitats was started by introducing and maintaining in vitro collections and field collections in the Latvian Botanic Gardens.

Conservation of *D. superbus* using in vitro methods ensures a high rate of propagation and the possibility of obtaining healthy plant material. However, there are potential weaknesses of in vitro conservation in relation to vegetative means. For instance, there is the need to develop a propagation protocol for the specific

Figure 5. Germination of *Dianthus superbus* seeds: collected in 2017, germinated in spring 2018; collected in 2019, germinated in spring 2020. NBG—National Botanic Garden; GR—gravel road edge; SR—sand road and the side of the road in pine forest.
species, the need for a relatively high level of technology and high maintenance costs (Thor mann et al. 2006). In addition, the most important issue could be the microplant acclimatization ex vitro (Muszyńska and Hanus-Fajerska 2017). The simultaneous exposure of plants to many stressors under natural conditions, such as broad-spectrum sunlight, variable temperatures, antagonistic soil microbial communities, can lead to high mortality. On the other hand, the break-up of potential symbiotic interactions could also be a disadvantage of the in vitro propagated plants.

Micropropagation and medium-term preservation protocols were developed for D. superbus using explants of flower buds, single node stem fragments and sterile sowing of seeds in Murashide and Skoog (1962) medium with various modifications and supplantations (Mikulík 1999; Holobiuc et al. 2004–2005). Mikulík (1999) reported the best results in medium without growth regulators, when 86.6 % of rooted shoots and 4.4 axillary shoots per plantlet were obtained. Our study shows that D. superbus could be successfully induced and maintained in vitro tissue cultures using shoot explants obtained in situ. The obtained results revealed that in vitro propagation of this species is very simple and fast, and no problems were found for further adaptation of seedlings to ex situ conditions. Cuttings from in vitro derived seedlings were well rooted in a commercial fertilized sphagnum peat substrate with low microbiological activity. Furthermore, high survival rates were recorded after transplantation under soil conditions. Thus, in vitro propagated D. superbus was hardly sensitive to soil microbiological composition.

The preservation of biodiversity is closely connected with the task of growing endangered species in botanical gardens for possible reintroduction of these in situ. Therefore, after acclimatization, in vitro obtained D. superbus seedlings were transplanted outdoors in the cultivation sites in the territory of NBG and BG UL. Overall good adaptation and growth success were found in both botanic gardens—the plants bloomed and matured seeds. In general, seed viability and germination are decisive indicators for assessing the reproductive prospects of in vitro derived plants. Our results revealed high potential of these living D. superbus collections as raw material for reintroduction as a very high percentage of seed viability and germination was found both for in situ and ex situ growing plants.

New approaches have been proposed in recent decades, integrating ex situ and in situ conservation for endangered plant species. The in situ approach includes the establishment of species by reintroduction to locations outside the current range— not only in the priority areas within the species past range, but also including locations with some degree of environmental degradation. Such assisted colonization of threatened species also demands agricultural management, invasive species control and plant protection measures (Volis 2017). Restoration of endangered species, combined with the simultaneous restoration of degraded habitat, could be promising nowadays.

Given our data on the width of the species niche in terms of soil conditions (wide nutrient and pH range), D. superbus would be suitable for such inter situs conservation approach. The first attempts to preserve D. superbus inter situs have already taken place in Latvia, as in vitro propagated seedlings were planted in Ščuvara Dendrological Park, where they began to successfully grow, bloom and produce seeds. Due to the ornamental nature of the species and the pleasant aroma of flowers, some places in the urban environment could also serve as alternative habitats for inter situs conservation. In this respect, Tallinn, the capital of Estonia, is known to have habitats where protected plant species, including D. superbus, thrive (Vacht et al. 2018).

In the conditions of sufficient funding for reintroduction projects and national-level support, the integration of in situ, ex situ and inter situs conservation strategies for D. superbus in Latvia could be the most optimal. As D. superbus is a species that is able to adapt to different soil conditions and is decorative, reintroduction inter situs in different environments, including the urban environment, can be considered as a very promising conservation measure.

Reproduction characteristics and potential

Comparing the generative indicators, a negative correlation was found between the number of flowers per shoot and seed viability (Fig. 3). Thus, the highest number of flowers per generative shoot and the lowest seed viability was found for the site hillock with clearing (HC), while the opposite was characteristic for D. superbus growing in the botanical garden (BG UL). This could be explained by a lack of resources, as the leaves and roots of the plant were less able to supply seeds with nutrients to shoots that produced 10.5 flowers per shoot than to shoots that produced only 2.9 flowers.

One of the conditions for the spread of the species is the presence of pollinators. The flowers of D. superbus are strongly scented, especially during the night, indicating pollination by night-active flower visitors. According to Erhardt’s research in the Swiss Alps (1991) D. superbus is adapted to crepuscular and nocturnal hawk-moth and is clearly not pollinated by butterflies, not even diurnal moths. Insect pollinators have not been studied in our work, but a significant number of generative shoots and flowers found both in situ and ex situ may enhance the chances for nocturnal pollinator visits. The increase in seed production could contribute to the continued survival of the D. superbus until overgrowth with other species increases or destructive human activity prevails.

Conclusions

Expeditions on 18 previously documented locations revealed only three existing D. superbus localities in Latvia with a significant number of specimens located in the Latgale region near Silenieki. In 2020, for the first time, two more new D. superbus deposits were found in the vicinity of these three approved locations. In situ conservation to ensure the protection of D. superbus in Latvia by establishing micro-reserves is limited, as the existing deposits are mainly located in a cemetery and on roadsides. Therefore, ex situ conservation of D. superbus outside natural habitats was carried out by introducing and maintaining in vitro collections and field plantings in the National Botanic Garden (NBG) and the Botanical Garden of University of Latvia (BG UL). Our study revealed equivalent quality and high potential of these living D. superbus collections as raw material for reintroduction since a very high percentage of seed viability and germination was found both for in situ and ex situ growing plants.

The conservation of this species can also be implemented inter situs—in urban areas and dendrological parks by planting in vitro propagated plants in perennial ornamental plant beds with the recommendation to use concentrated plantings. The plantings will be ornamental not only for ~1.5 months until a large part of the shoots have stopped flowering, but also after pruning, because, young small shoots with greenish silver leaves are also decorative. The agrochemical results showed that D. superbus is a species that is able to adapt to a wide range of soil conditions in terms of nutrient concentrations, soil reaction and organic matter contents. Thus, it can be concluded that there are three

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main preconditions for the successful growth of D. superbus in Latvia—adequate lighting, low overgrowth of other plant species and non-destructive activity of humans.

Supporting Information
The following additional information is available in the online version of this article—
Table S1.1. Data on mean seed viability and number of flowers per generative shoot of Dianthus superbus in Latvia, 2020.
Table S 1.2. Data on mean germination rate of Dianthus superbus seeds (%): germinated in spring 2018 (collected in 2017); germinated in spring 2020 (collected in 2019). NBG – National Botanic Garden; GR - gravel road edge; SR - sand road and the roadside in pine forest.

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Contributions by the Authors
A.O. performed soil chemical analyses. G.J. supervised the study, introduced D. superbus in vitro and transplanted ex vitro, performed seed tests. I.A. carried out an inventory of accompanying species and characterized habitats. A.S and I.D. participated in fieldworks. G. J. and A.O. wrote the manuscript with the help of I.A.

Conflict of Interest
None declared.

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Data Availability
The data are provided as Supporting Information.

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