

EX SITU CONSERVATION OF ENDANGERED *LIMONIUM* SPECIES IN THE BULGARIAN FLORA

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(Received February 16, 2012/Accepted May 5, 2012)

A B S T R A C T

Native populations of endemic, rare and threatened *Limonium* species (*L. meyeri*, *L. bulgaricum*, *L. latifolium*, *L. vulgare*, *L. asterotrichum* and *L. gmelinii*) in Bulgaria were monitored and found seriously declined. To preserve these wild genotypes, an approach involving *in vitro* propagation of explants isolated from immature inflorescence stems was applied at the Institute of Ornamental Plants, Sofia. The rooted plantlets produced were acclimated and grown outdoors under an optimized cultivation regime, which resulted in the establishment of an *ex situ* plantation. Plant performance *ex situ* (determined by leaf rosette diameter, plant height and the number of flower stems) was substantially improved and the variation in the biometric indices was found remarkably lower than in natural environment. The developmental stages of *ex situ* plants appeared with a delay in relation to their onset in the native environment, but occurred synchronously within each species. Analysis of germination of seeds harvested from *ex situ* and *in situ* grown plants showed species-specific behaviour, but in general, seed vitality remained relatively low in laboratory conditions, in the soil and *in vitro*. In order to assess the potential for protecting the native *Limonium* species from uncontrolled harvesting, the possibility for the production of cut flowers in *ex situ* conditions was studied. High yield of cut flowers from *ex situ* plants in comparison with the potential yield from the wild plants and extended vase life in comparison with commercially produced *Limonium sinuatum* were obtained. The results demonstrated that the applied micropropagation and agrotechnique for protected cultivation are reliable tools for *ex situ* conservation of the endangered *Limonium* genotypes in the Bulgarian flora. In addition to its advantage as a rescue measure, the developed system was shown to be suitable for obtaining cut flowers of competitive market quality.

Key words: *ex situ*, micropropagation, native, *Limonium*, endangered species, seeds, vase life, yield

INTRODUCTION

The species of the genus *Limonium* (Plumbaginaceae) are herbaceous perennial plants with thick spindle-shaped roots, dark green leaves, tiny purple or white flowers and inflorescences reaching a height of 30 to 100 cm. They form relatively scanty native populations in restricted locations, growing in poor, limy and dry soils with a slightly alkaline pH, and as halophytes on rocky and sandy terrains (Ančev, 1982). The *Limonium* species are popular for their distinct ornamental characteristics such as attractive flower colour, multiple flower stems and beautiful post-harvest appearance. These features make the plants excellent fresh or dry cut flowers, and a genetic resource for breeding. *Limonium* is also an appropriate ground cover plant for the urban landscape (Alarcón et al., 1999; Rizzotto, 1999; Mercuri et al., 2003; Rodríguez et al., 2003; Burchi et al., 2006). In Bulgaria, six wild *Limonium* species: *Limonium bulgaricum* Ančev, *Limonium gmelinii* (Villd.) O. Kuntze, *Limonium latifolium* (Sm.) O. Kuntze, *Limonium meyeri* (Boiss.) O. Kuntze, *Limonium asterotrichum* (Salmon) Salmon, and *Limonium vulgare* Mill. have been documented (Ančev, 1982); all of them are on the list of protected plants (Biological Diversity Act, 2005). *L. asterotrichum*, *L. meyeri* and *L. bulgaricum* are endemics and have been assessed as “critically endangered”; *L. gmelinii* has been assessed as “endangered” (Red Data Book of the Republic of Bulgaria, 2011); *L. latifolium* and *L. vulgare* are classified as “vulnerable” (Stoyanov et al., 1984).

The existence of wild *Limonium* populations is threatened by various environmental factors including climate changes (extreme temperatures, drought and flooding), landslides and erosion, soil and air pollution, and pathogen invasion. Human activities such as urbanisation of the areas of the native habitats and uncontrolled harvesting of cut flowers, and other anthropogenic factors also seriously alter the density and size of the wild populations and diminish their regeneration potential. On an international scale, the preservation of endemic flora and the possibilities to produce commercially available cut flowers from conserved (*ex situ* grown) *Limonium* plants have received a lot of attention (Fay, 1992; Hegazy, 1992; Francisco-Ortega et al., 2000; Heywood, 2004; Reyes-Betancort et al., 2008). Among the recommendations on how to conserve the diversity of native *Limonium* species are the creation of small-scale natural salt-marsh reserves within the developed agroecosystems, ecological research on *in situ* and *ex situ* survival, and development of propagation strategies (Hegazy, 1992). *In vitro* technology for the propagation of rare and endangered plants has been demonstrated as a potential strategy for conservation. In the Canary Islands and in some Mediterranean countries it has been implemented as a successful rescue measure for endangered *Limonium* species (Fay, 1992; Martin and Pérez, 1995; Amo-Marco and Ibañez, 1998; Mercuri et al., 1999; Francisco-Ortega et al., 2000; Savona et al., 2009). The advantage of tissue

culture technology is that a large amount of planting material from micropropagated plants can be used for re-introduction *in situ* and conservation *ex situ*. Following the description of native *Limonium* species and their habitats in the 1980s (Ančev, 1982), exploration of these protected plants in Bulgaria and the possibilities of preserving their genetic diversity through domestication have been poorly addressed.

The aim of this work was to establish a system for *ex situ* conservation of endangered *L. meyeri*, *L. bulgaricum*, *L. latifolium*, *L. vulgare* and *L. gmelinii* from the Bulgarian flora. Techniques for *in vitro* propagation and cultivation of these species have been developed; plant and seed performance have been evaluated; and the potential of *ex situ* grown plants for commercial production of cut flowers has been assessed.

MATERIAL AND METHODS

Plant material

The study was undertaken with whole plants and plant parts of wild and *ex situ* grown *L. vulgare*, *L. meyeri*, *L. latifolium*, *L. bulgaricum* and *L. gmelinii*. The wild populations were investigated in their natural habitats. *Ex situ* plants were studied in the experimental field of the Institute of Ornamental Plants, Sofia. *In vitro* experiments were performed with explants isolated from immature inflorescence stems of *ex situ* grown plants. Seeds were collected from both wild and *ex situ* grown plants and cut flowers were harvested from *ex situ* plants.

Monitoring of native populations

The condition of wild *Limonium* populations was monitored in the following habitats: *L. vulgare*, *L. meyeri*, *L. latifolium* and *L. gmelinii* – along the North and South Black Sea Coast, *L. asterotrichum* in South-East Bulgaria, and *L. bulgaricum* in the central part of North Bulgaria. The investigation was conducted according to the Instruction No. 2 of 18.12.2006 for the creation and functioning of the National System for Monitoring Biodiversity; Ministry of Environment and Waters, Bulgaria, and included identification of the species, number of plants in the populations and description of their developmental stages and health condition, determination of the size and structure of the populations, geographical coordinates of the habitats, type of terrain, accompanying species, and assessment of anthropogenic and environmental threats.

Measurements

Plants at the reproductive age (approximately 4 years old) from each species in their native locations were labelled and subjected to biometric measurements. Leaf rosette diameter (cm), plant height (cm) and the number of inflorescences per plant were measured. The presented data are means of three consecutive years (2005-2007) and were collected from at least 10-15 labelled native plants per a habitat. The same types of measurements were performed on 4-year-old *ex situ* grown plants. Data from *ex situ* plants were collected every year from at least 60 plants (20 plants per each of three

separate plots of 10 m²). The plots were isolated one from another and considered as replicates. Values are average from at least 180 plants in total for the three years period. each plot in three replicates

Phenological records

The time of the occurrence of the stages of initial (10% open buds) and full flowering (two-thirds of the flowers per branch opened) (VBN product specification, Limonium, 2004), and seed maturity were recorded for the plants growing both in native habitats and *ex situ*.

Generative propagation

In vivo

Mature seeds were collected from wild plants and, at the end of October, sown in the soil, in plastic-covered trays, in an unheated greenhouse. In April (*L. vulgare*, *L. meyeri*, *L. gmelinii* and *L. bulgaricum*) and in June (*L. latifolium*), the developed seedlings at the 3-4 leaf stage were transplanted outdoors in the experimental field of the Institute of Ornamental Plants in Sofia.

In vitro

The seeds harvested from wild plants were stratified by placing them in sealed plastic bags with a moistened filter paper at 4 °C for 45 days. For *in vitro* germination, seed coats were removed and the seeds were surface-sterilized by sequential immersion in 70 % ethanol for 1 min, followed by 1 min in 1% sodium hypochlorite solution, rinsing with 0.1% HgCl₂ and triple rinsing with sterile distilled water. Then the

seeds were laid out on MS basal salt media without hormones (6 g/l agar, 20 g/l sucrose, pH 5.7) to germinate. The seedlings produced from *in vitro* germinated seeds were acclimated and hardened as described in the protocol for the plants regenerated from inflorescence stems (see below).

Assessment of laboratory seed germination

The laboratory germination of stratified seeds collected from wild and *ex situ* grown plants was assessed by the 'top of paper' method: seeds were placed on the surface of a moistened filter paper in closed Petri dishes and left to germinate in an incubator at 20 °C in the darkness. Seed viability was assessed in terms of germination energy (number of germinated seeds after one week) and germination rate (number of germinated seeds after one month). For each genotype, the experiments were carried out with 50 seeds per dish (the dishes in two replicates) and were repeated three times.

Establishment of *in vitro* culture

Segments (20-30 mm long) with closed buds were isolated from immature inflorescence stems of *ex situ* grown plants and sterilized by immersion in an aqueous 1 % sodium hypochlorite solution for 1 min, followed by triple rinsing with sterile distilled water. The sterile explants were then introduced onto solid (6 g/l agar) MS basal salt medium (Murashige and Skoog, 1962) with the addition of 20 g/l sucrose and pH adjusted to 5.7. Cultures were maintained under con-

trolled conditions: temperature 21-22 °C, 16/8 h day/night photoperiod and light intensity of 30 $\mu\text{mol}/\text{m}^2/\text{s}$ (cool-white fluorescent tubes, Philips).

Shoot multiplication, elongation and rooting

Single shoots with 2-3 leaves from 2-month-old explants were separated and transferred onto media for further development. To establish the composition of the media for multiplication, elongation and rooting, a range of concentrations (0.01-3.0 mg/l) and various combinations of plant growth regulators (BA, IBA and GA3) were tested. The tests for the determination of the type and concentration of the compounds for the media were conducted in three replicates, each replicate comprising 30 explants, and the experiments were repeated 4 times. The most efficient protocol (MS basal salt media supplemented with 100 mg/l myo-inositol, 0.1 mg/l IBA, 0.1 mg/l BA, 0.1 mg/l GA3 and 30 g/l sucrose; pH 5.7) was routinely used for producing plantlets for *ex situ* conservation. To obtain a sufficient number of explants, the shoots were subcultured at least five times on the same fresh medium every 30 days. Incubation conditions were as those mentioned in the subparagraph above.

Acclimatization of plantlets

Rooted plantlets produced *in vitro* from immature inflorescence stems were acclimated in plastic trays containing a 3:1 peat : perlite substrate, and kept under controlled glasshouse conditions with day/night

temperature of 25/18 °C, 70% relative humidity and regular ventilation. Under those conditions, the plantlets were grown for six weeks until the 3rd or 4th leaf had developed. For hardening before planting in a permanent location, the seedlings were transferred into trays filled with soil and placed outdoors for a week.

Growing conditions

Following the hardening, the plants were transplanted into moisturized alluvial-meadow soil (pH 6.8-7.2), with a humus content of 1.9-2.1%. The soil was fertilized with N:P:K = 1:2.5:1.5, ploughed deeply, and the soil clods were broken down into finer soil particles. Nitrogen nutrition was applied twice: before planting of the seedlings and before the initiation of budding. The seedlings were planted either at the beginning of October or in April according to the following design: *L. vulgare*, *L. bulgaricum* and *L. gmelinii* were planted in two-row beds – 70/90/60 cm (70 cm between rows, 90 cm between beds and 60 cm between plants in a row); *Limonium meyeri* in a two-row bed – 70/90/40 cm, and *Limonium latifolium* in a two-row bed – 70/90/80 cm. To avoid the risk of unwanted hybridization, the plots were secured by spatial isolation at a distance of 400 m between the species (according to Instruction No. 16 of 21.04.2004 for the production and trade of seeds and seedlings from ornamental plants; Ministry of Agriculture and Forestry, Bulgaria). Plants were watered once a week during rosette development, and from the flower bud initiation until

the end of the flowering period (July – August). To prevent the development of diseases, the watering was done carefully without wetting the upper parts of the plants or flower heads. Weed control consisted of removing weeds by hand or by hoeing in the spaces between the beds.

Yield of cut flowers

The yield of cut flowers from *ex situ* grown plants was determined by the number of harvested inflorescences with a length of at least 40 cm. The potential yield of cut flowers from native plants was calculated theoretically based on the number of flowering stems developed in the plants in native conditions. Data are presented as the number of cut flowers per 100 m².

Vase-life test

Vase-life tests were performed by following the methods of Doi and Reid (1995) and Shimamura et al. (1997) with slight modifications. Flowering stems from *ex situ* grown 4-year-old plants were harvested at the stage of commercial maturity in accordance with the minimum ripeness requirement for *Limonium* (VBN Product Specification, Limonium, 2004), trimmed to a 40 cm in length and the cut ends were immersed in tap water in a controlled environment: temperature 20 °C ±1 °C, 12/12 h day/night photoperiod, light intensity 15 μmol/m²·s and 70% relative humidity. The vase life of *ex situ* harvested *Limonium* flowers was compared with that of commercially-grown *L. sinuatum* ‘Mid-

night Blue’ and the scoring was terminated when 80% of the open florets had lost turgor. The experiments were conducted in five replicates, each with eight flower stems per vase. Data represent an average from three consecutive years.

Data analysis

Data for the diameter of the leaf rosette, plant height, yield and vase life of cut flowers are compared using SEM_(n-1). The variation in the indices is shown by the coefficient of variation (CV%). Statistical significance of the differences was evaluated by one-way Anova (Duncan’s multiple range test, confidence level $p \leq 0.05$).

RESULTS AND DISCUSSION

Condition of wild populations

The exploration of wild *Limonium* species revealed a serious shrinkage of the habitats and decline in the populations. The populations were of a small size, with a mosaic pattern of distribution (limited number of plants growing in scattered patches). *L. asterotrichum* in South-East Bulgaria was found nearly extinct. Because of insufficient availability of plants, the data collected for this species were inconsistent and are not presented. It was found that, in addition to climate changes, extremely harmful effects on the native *Limonium* plants in Bulgaria had been exerted by the intensive housing construction along the Black Sea Coast and elsewhere, changes of land ownership, farming, trampling, fire and overgrazing. It was also observed

that the wild *Limonium* species are subjected to intensive harvesting for cut flowers. In summary, the monitoring confirmed the necessity of urgent conservation measures.

Programmes for the preservation of biodiversity of endemic, rare and threatened species from the genus *Limonium* along the Mediterranean coasts of Europe and Africa, around the coasts of the British Isles and in Asia have already been developed (Boormann, 1968; Fay, 1992; Hegazy, 1992; Francisco-Ortega et al., 2000; Heywood, 2004). Here we report on the first research-based approach to *ex situ* conservation of endangered *Limonium* species in the Bulgarian flora.

Establishment of starting initial *ex situ* plantation

From the native seeds that were germinated in soil, only 5-9 % produced healthy seedlings. The amount of sprouting seeds *in vitro* was also low – not exceeding 10% (data not shown). Nevertheless, the seedlings produced from native seeds *in vitro* and *in vivo* were transplanted outdoors and grown at the experimental field of the Institute of Ornamental Plants, at an altitude of 560 m above sea level, with standard weed control, regular watering and nutrient supply. In that initial plantation, only 8-10 plants from each species survived and reached the phase of full flowering. This stage of the investigation was considered a starting point in the *ex situ* preservation of *L. vulgare*, *L. meyeri*, *L. latifolium*, *L. gmelinii* and *L. bulgaricum* in Bulgaria. For *ex situ* conservation on

a larger scale, explants from immature inflorescence stems from *ex situ* grown plants were introduced into culture and regenerated *in vitro* to produce planting material.

Micropropagation

In vitro cloned healthy and genetically identical plants are a good source for obtaining a desired quantity of planting material for conservation purposes (Amo-Marco and Ibañez, 1998; Savona et al., 2009). Combinations of plant growth regulators stimulated the growth of new shoots, shoot elongation and rooting. In the presented medium, after 30 days of subculture an average of 7-10 vegetative shoots from a single explant could be separated (Tab. 1). In that time span the shoots reached an average length of 17.8 mm and at least 80% of the explants developed into healthy *in vitro* rooted plantlets (Tab. 1). The obtained results indicated that immature inflorescence stems are a suitable starting material for the establishment of *in vitro* cultures of the studied *Limonium* species, which supports the findings of Amo-Marco and Ibañez (1998). Using inflorescence pieces as initial explants, these authors achieved successful micropropagation of *L. cavanillesii* Erben, a threatened and endemic static species in Valencia Community in Spain.

Data obtained for the number of shoots, shoot length and rooting percentage of the different species were not significantly different, which indicated that the selected media composition was equally effective for micro-

Table 1. Characterisation of *in vitro* propagated *Limonium* species

Species	No. of shoots per explant	Shoot length [mm]	% Rooting
	M±SE	M±SE	
<i>L. vulgare</i>	7.3±0.6 a	19.3±1.9 a	84 a
<i>L. meyeri</i>	6.4±0.8 a	15.8±3.1 a	79 a
<i>L. bulgaricum</i>	10.6±0.8 a	16.4±2.7 a	89 a
<i>L. gmelinii</i>	9.8±0.4 a	17.1±2.3 a	81 a
<i>L. latifolium</i>	8.1±0.7 a	20.2±2.9 a	86 a

Medium composition: MS basal salt media supplemented with 100 mg/l myo-inositol, 0.1 mg/l IBA, 0.1 mg/l BA, 0.1 mg/l GA3 and 30 g/l sucrose; pH 5.7. Experiments were conducted in three replicates, each replicate comprising 30 explants. The trials were repeated 4 times. Data were recorded after 30 days of culture. In each column means followed by the same letter are not significantly different (Duncan's multiple range test, $p \leq 0.05$). M = mean; SE = SEM_(n-1) (standard error of the means)

propagation of the five *Limonium* species under study. Depending on the type of explants used (immature flower stems, immature floral scape tip or young leaf explants), *in vitro* culturing of *L. cordatum*, *L. gmelinii* and *L. sinuatum* has been achieved by supplementing the media with various combinations of cytokinins and auxins such as BA, 2iP, IAA (Gabryszewska and Podwyszynska, 1992; Gabryszewska et al., 2000; Rufoni et al., 2000; Mercuri et al., 2003; Savona et al., 2009). For several threatened *Limonium* species it has been found that a high BA concentration and very low concentrations of 2iP suppress shoot development *in vitro* (Harazy et al., 1985; Martin and Pérez, 1995; Lledó and Amo-Marco, 1993). Kinetin (2-5 mg/l) and 5 mg/l 2iP have been recommended for the multiplication and elongation of *L. cavaniillessi* Erben, whereas no significant

difference in the effect of different concentrations of IBA and IAA on *in vitro* rooting of the same species has been detected (Amo-Marco and Ibañez, 1998).

Plant performance *ex situ* and in native environment

Approximately 90% of *in vitro* produced acclimated plantlets developed into adult synchronously flowering plants. The results showed that the applied combination of alluvial-meadow soil, the optimized regime of phosphorus and potassium fertilization, double nitrogen feeding, the scheduled watering and weed control stimulated the growth and development of *ex situ* *Limonium* plants. Under cultivation, leaf rosette diameter, plant height and the number of flowering stems per plant were significantly greater in comparison with wild plants (Tab. 2). Moreover,

Table 2. Genotype x origin of plants interaction for biometric characteristics of *Limonium* species (average for a period of three years (2005-2007))

Species	Leaf rosette diameter [cm]		Plant height [cm]		Number of inflorescences	
	M ± SE	CV	M ± SE	CV	M ± SE	CV
<i>L. vulgare</i> 1	21.33±3.53 c	16.55	53.00±4.80 c	9.05	19.00±3.06 d	16.11
<i>L. vulgare</i> 2	34.30±2.33 e	6.79	83.67±2.96 e	3.54	35.67±2.96 e	8.30
<i>L. meyeri</i> 1	7.67±2.33 a	30.38	21.67±3.28 a	15.14	4.00±1.15 a	28.75
<i>L. meyeri</i> 2	11.33±1.76 b	15.33	42.00±2.64 b	6.29	8.33±0.88 b	10.56
<i>L. bulgaricum</i> 1	16.33±1.97 c	12.06	33.67±6.98 b	20.73	8.00±1.63 b	20.37
<i>L. bulgaricum</i> 2	26.66±1.76 d	7.44	49.00±2.08 c	4.24	18.33±1.57 d	8.57
<i>L. gmelinii</i> 1	18.67±2.04 c	10.92	38.12±6.75 b	17.71	9.16±1.92 b	20.96
<i>L. gmelinii</i> 2	30.02±2.13 e	7.09	51.68±2.36 c	4.57	15.82±2.45 c	15.49
<i>L. latifolium</i> 1	35.42±3.11 e	8.78	68.05±5.24 d	7.70	6.52±1.45 a	22.24
<i>L. latifolium</i> 2	59.62±3.78 f	6.34	94.15±3.11 f	3.30	12.41±2.11 c	17.00

1 native plants; 2 *ex situ* plants; CV = Coefficient of variation (%); M = mean; SE = SEM_(n-1) (standard error of the means). In each column the mean values marked with different letters are significantly different (Duncan's multiple range test, $p \leq 0.05$)

in *ex situ* grown plants the high variability in biometric indices found in the native populations was reduced 2-3-fold (Tab. 2).

The comparison of the time of initial flowering, full flowering and seed maturation between *ex situ* and native *Limonium* plants showed that in *ex situ* conditions plant development was retarded (Tab. 3). Under cultivation, flowering was delayed by 8-10 days for *L. vulgare*, *L. meyeri*, *L. gmelinii* and *L. latifolium*, and by approximately two weeks for *L. bulgaricum*. Seed maturation of all *Limonium* species was delayed by

10-15 days in comparison with the wild plants. Basically, the later occurrence of the generative phase could be due to the phenomenon of juvenility (delay of the generative stage and intensification of vegetative growth in plants propagated *in vitro*). However, the records were taken from 4-year-old plants, which make this cause unlikely. We suggest that it is rather a response to the geographic location of *ex situ* collections and the associated intensity of solar radiation, air humidity and temperature fluctuations that differ from those of the native environment.

Table 3. Phenological records of *ex situ* grown and wild *Limonium* species. The presented time of developmental events is average for a period of three years (2005-2007)

Species	<i>Ex situ</i> plants			Native plants		
	initial flowering	full flowering	seed maturation	initial flowering	full flowering	seed maturation
<i>L. vulgare</i>	29 June	02 July	25 Oct.	15 June	25 June	15 Oct.
<i>L. meyeri</i>	15 June	25 June	05 Oct.	05 June	15 June	20 Sept.
<i>L. bulgaricum</i>	30 June	19 July	17 Oct.	20 June	05 July	15 Oct.
<i>L. gmelinii</i>	26 June	10 July	28 Oct.	18 June	01 July	15 Oct.
<i>L. latifolium</i>	30 Sept.	15 Oct.	30 Nov.	20 Sept.	05 Oct.	21 Nov.

Despite the delay in timing, the developmental phases occurred synchronously within each species and an overall improvement in plant appearance was soundly observed.

Characterization of seed performance

To evaluate seed performance, germination of stratified seeds was tested in the laboratory. Germination energy and germination rate significantly varied among the species. The lowest vitality was determined for *ex situ* collected seeds of *L. latifolium* and for the seeds collected from the wild *L. gmelinii* (Tab. 4). By contrast, the native seeds from *L. latifolium*, followed by *L. meyeri* showed the highest germination energy and rate in comparison with the wild seeds of the other genotypes tested. Interestingly, the vitality of the wild seeds from *L. latifolium* and *L. meyeri* appeared also better in comparison with *ex situ* harvested seeds of the same species, whereas the vitality of *ex situ* seeds from *L. bulgaricum*, *L. vulgare* and *L. gmelinii* was substantially improved in comparison with the native seeds.

Seed quality was also examined under a microscope with a “cut-test” for the presence of the embryo. The percentage of embryo-containing seeds corresponded to the percentage of sprouting seeds (data not shown). In general, the tests showed that the germination capacity in laboratory conditions (Tab. 4), in soil and *in vitro* (data not shown) of seeds produced by *ex situ*- and wild-grown plants, was relatively low. These results indicate that the *ex situ* conditions did not equally alter the seed viability of the studied species and suggest species-specific seed behaviour in nature and *ex situ*, thus substantiating the suggestion that the variation in the germination of native *Limonium* seeds is partly genetically and partly environmentally determined (Boormann, 1968; Reyes-Betancort et al., 2008).

Yield and quality of cut flowers

The largest quantity of cut flowers (more than 7,000 per 100 m²) was obtained from *ex situ* grown *L. vulgare*, followed by *L. bulgaricum*, *L. gmelinii*, *L. meyeri* and *L. latifolium* (Fig. 1).

Table 4. Laboratory germination of seeds collected from *ex situ* grown *Limonium* plants

Species	Native seeds		<i>Ex situ</i> seeds	
	germination energy [%]	germination rate [%]	germination energy [%]	germination rate [%]
<i>L. vulgare</i>	5.40 a*	18.33 b	18.33 b	23.18 c
<i>L. bulgaricum</i>	8.10 b	16.38 b	21.40 c	26.70 d
<i>L. meyeri</i>	16.42 c	28.10 c	15.08 b	17.44 b
<i>L. gmelinii</i>	4.73 a	4.33 a	24.15 d	28.60 d
<i>L. latifolium</i>	20.15 d	38.32 d	10.85 a	12.17 a

*In each column the mean values marked with different letters are significantly different (Duncan's multiple range test, $p \leq 0.05$)

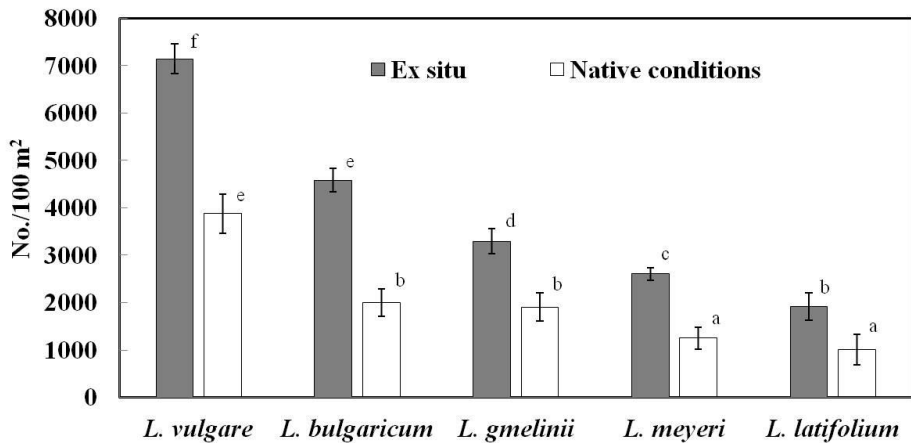


Figure 1. Yield of cut flowers from *Limonium* species

Flowers were harvested from 4-year-old plants. Vase-life was tested in tap water. Control: *L. sinuatum* 'Midnight Blue'. Error bars indicate \pm SEM(n-1). Mean values marked with different letters are significantly different (Duncan's multiple range test, $p \leq 0.05$)

In comparison with the potential yield of cut flowers in the native environment, a twice as high productivity of cut flowers from *ex situ* grown plants was obtained. Although the number of cut flowers harvested

from *L. meyeri*, *L. gmelinii* and *L. latifolium* was relatively lower than from *L. vulgare*, *L. bulgaricum*, the post-harvest longevity of the inflorescences from all the species was impressively high.

Vase life of *ex situ* harvested *Limonium* flowers was compared with the vase life of the classical *L. sinuatum* ‘Midnight Blue’ (grown also at the experimental field of the Institute of Ornamental Plants in Sofia, under the cultivation regime applied to the *ex situ Limonium*). Whereas the vase-life of cv. ‘Midnight Blue’ lasted only 6.3 days, remarkably longer keepability was recorded for *L. latifolium* (22 days) and *L. vulgare* (20 days) followed by *L. bulgaricum*, *L. meyeri* and *L. gmelinii* (16.3, 14.6 and 14.3 respectively) (Fig. 2). The softening of flower stems observed during post-harvest handling of cv. ‘Midnight Blue’ occurred later and to a lesser extent in the naturally harder stems of *ex situ* grown *Limonium* species. It has been reported that, regardless of their harvest maturity, the longevity of cut inflorescences of cv. ‘Fantasia’ (an interspecific hybrid between *L. bellidifolia* Gouan and *L. latifolium* Kuntzn), held in deionised water, averaged 5 days. Addition of sucrose and α -aminoisobutyric acid to the vase solution has promoted bud opening and retarded petal senescence (Doi and Reid, 1995; Shimamura et al., 1997). Our results show that the fresh appearance of *ex situ* harvested cut *Limonium* flowers can be maintained for an extended period in tap water without additional post-harvest treatment.

Taken together, the improved yield and longevity of cut flowers

from *ex situ* plants demonstrate that in addition to the preservation of genetic diversity of native *Limonium* genotypes in Bulgaria, the protected cultivation also seems promising for commercial production of cut flowers of highly competitive quality.

In summary, a system for *ex situ* conservation of endangered wild *Limonium* species in the Bulgarian flora has been developed. The system combines *in vitro* regeneration from immature inflorescence stems and optimization of the cultivation regime. Plant performance in *ex situ* conditions was substantially improved in comparison with plant performance in the native environment. In *ex situ* plants, the occurrence of phenological phases was delayed, but plant development proceeded synchronously within each species. The yield and quality of cut flowers harvested from *ex situ* grown plants were of high commercial quality. Further investigations for developing a strategy for re-introduction *in situ* are envisaged.

Acknowledgements: This work was partially supported by project: “Breeding, introduction, agrotechnology, enrichment and preservation of the genetic resources of ornamental plants” financed by Agricultural Academy, Sofia, Bulgaria. The authors are thankful to Dr. Jeremy Burdon, Plant & Food Research Ltd, Auckland, New Zealand, for his helpful opinion on the English language of the manuscript.

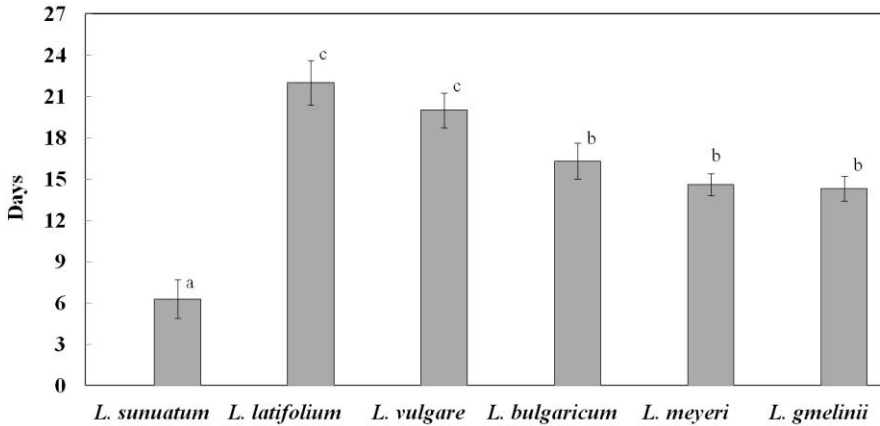


Figure 2. Vase-life of cut flowers from *ex situ* grown *Limonium* species

Flowers were harvested from 4-year-old *ex situ* grown plants. The potential yield from the wild plants of the same age was calculated theoretically based on the number of flowering stems developed in the plants in native conditions. Error bars indicate \pm SEM(n-1). Mean values marked with different letters are significantly different (Duncan's multiple range test, $p \leq 0.05$)

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OCHRONA *EX SITU* ZAGROŻONYCH GATUNKÓW *LIMONIUM* FLORY BUŁGARSKIEJ

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S T R E S Z C Z E N I E

Monitorowano rodzime populacje endemicznych, rzadkich i zagrożonych gatunków *Limonium* (*L. meyeri*, *L. bulgaricum*, *L. latifolium*, *L. vulgare*, *L. asterotrichum* i *L. gmelinii*) w Bułgarii i stwierdzono, że znacznie się one zmniejszyły. Aby zachować te dziko rosnące genotypy, zastosowano rozmnażanie *in vitro* eksplantów wyizolowanych z niedojrzałych pędów kwiatostanowych w Instytucie Roślin Ozdobnych w Sofii. Wyprodukowane ukorzenie roślinki zaaklimatyzowano i hodowano na zewnątrz w zoptymalizowanych warunkach uprawy, co zaowocowało utworzeniem plantacji *ex situ*. Wzrost roślin *ex situ* (określany średnicą rozety liściowej, wysokością rośliny i liczbą pędów kwiatowych) znacznie się poprawił. Stwierdzono, że różnicowanie wskaźników biometrycznych było znacząco niższe niż w środowisku naturalnym. Fazy rozwojowe roślin *ex situ* pojawiały się z opóźnieniem w stosunku do ich występowania w środowisku naturalnym, ale występowały one synchronicznie w obrębie poszczególnych gatunków. Analiza kiełkowania nasion zebranych z roślin rosnących dziko i *ex-situ* wykazała charakterystyczne dla każdego gatunku zachowanie, ale na ogół żywotność nasion była stosunkowo niska w warunkach laboratoryjnych, w glebie i *in vitro*. Aby ocenić możliwości ochrony rodzimych gatunków *Limonium* przed niekontrolowanym zbieraniem, badano możliwość produkcji kwiatów ciętych w warunkach *ex situ*. Otrzymano wysoki plon kwiatów ciętych z roślin *ex situ* w stosunku do potencjalnego uzysku z roślin dzikich i dłuższą ich trwałość w wazonie w porównaniu z produkowanymi komercyjnie roślinami *Limonium sinuatum*. Wyniki pokazały, że mikrorozmnażanie i agrotechnika zastosowana w uprawie ochronnej są niezawodnymi narzędziami dla ochrony *ex situ* zagrożonych genotypów *Limonium* flory bułgarskiej. Oprócz korzyści jako środek ratunkowy, opracowany system przyczynił się do uzyskiwania kwiatów ciętych o konkurencyjnej jakości rynkowej.

Słowa kluczowe: *ex situ*, mikrorozmnażanie, gatunki rodzime, *Limonium*, gatunki zagrożone, nasiona, trwałość w wazonie, plon