In vitro cultivation of plant species from sandy dunes along the Bulgarian Black Sea Coast

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Abstract. The human impact on the seashore was intensive over the last years which resulted in severe damage to the sandy dunes along the Bulgarian Black Sea Coast. *In vitro* cultivation was recognised as one of the important tools in the *ex situ* conservation and sustainable use of biodiversity. *In vitro* cultures from fourteen sandy dune plants (*Pancratium maritimum*, *Scabiosa argentea*, *Cionura erecta*, *Jurinea albicaulis* subsp. *kilaea*, *Peucedanum arenarium*, *Linum tauricum* subsp. *bulgaricum*, *Aurinia uechtritziana*, *Silene thymifolia*, *Glaucium flavum*, *Stachys maritima*, *Astrodaucus littoralis*, *Otanthus maritimus*, *Plantago arenaria*, *Verbascum purpureum*) were initiated. The first observations presented a promising propagation of several rare, endangered and medicinal plant species.

Key words: conservation, *in vitro*, sandy dunes, seeds

Introduction

The human impact on the seashore resulted in severe destruction of sandy habitats along the Bulgarian Black Sea Coast over the last years (extensive building of tourist infrastructure, sand-pits and pollution). Conservation of the Western Pontic grey and white dunes requires special areas to be designated for conservation (Habitat Directive 92/43/EEC). However, some of the vulnerable localities stay out of the protected areas and the measures taken for their conservation are not effective enough. The sandy dunes are naturally inhabited by psammophytes, including *Scabiosa argentea* L., *Cionura erecta* (L.) Griseb., *Jurinea albicaulis* subsp. *kilaea* (Azn.) Kožuharov, *Peucedanum arenarium* Waldst. & Kit., *Silene thymifolia* Sm., and some rare and endangered plant species [*Pancratium maritimum* L., *Aurinia uechtritziana* (Bornm.) Cullen & T.R. Dudley, *Verbascum purpureum* (Janka) Hub.-Mor., *Glaucium flavum* Crantz, *Stachys maritima* Gouan, *Astrodaucus littoralis* (M. Bieb.) Drude, *Othanthus maritimus* (L.) Hoffmanns. & Link, *Plantago arenaria* Waldst. & Kit., *Linum tauricum* Willd. subsp. *bulgaricum* (Podp.) Petrova].

Ex situ conservation ensures long-term maintenance of the endangered species collections which could be used as initial material sources for cultivation (*Global Biodiversity Strategy* 1992; *Biodiversity Action Plan for the Conservation of Natural Resources* 2001; *IPGRI Technical Bulletin* no. 7 2001). *In vitro* cultivation was recognised as one of the important tools in the *ex situ* conservation and sustainable use of biodiversity (Bramwell 1990). It offers an easy, rapid and space-efficient way for banking of plant species.

Moreover, the collection and usage of seeds for *in vitro* cultivation does not affect the integrity of the habitats. However, this method is not widely practiced for wild plants in Bulgaria.

The aim of this study was to initiate *in vitro* cultures from seeds of some rare and endangered sandy dune plant species and to investigate the possibility of transferring the regenerated plantlets to *ex vitro* conditions.

Material and methods

Mature seeds from *P. maritimum, S. argentea, A. littoralis, C. erecta, J. albicaulis* subsp. *kilaea, O. maritimus, A. uechtritziana, S. thymifolia, P. arenaria, P. arenarium, V. purpureum, G. flavum, S. maritima,* and *L. tauricum* subsp. *bulgaricum* were used as initial material for *in vitro* cultures, as a part of collection for the Millennium Seed Bank, Royal Botanic Gardens (Kew, UK). The material was collected in summer–autumn 2005 from natural populations along the Bulgarian South Black Sea Coast, with the permission of the Ministry of Environment and Waters, according to the regimes and regulations valid for the protected areas, and in agreement with Bulgarian legislative documents. Sterilization was carried out by consequent soaking in 70 % ethanol (1–2 min) and 100 % commercial bleach (<5 % active chlorine) for 15–20 min and 40 min for *P. maritimum*, followed by triple washing with sterile water. Specified procedures were performed for some of the species prior to sterilization (Table 1). For facilitation of the germination of *J. albicaulis* subsp. *kilaea* and *S. maritima* its outer seed coat was peeled off before sterilization. *Othanthus maritimus* germinated only after soaking overnight in wet filter paper at room temperature. A 24-hour treatment with 0.05 mg/ml of gibberellic acid (GA_3) was applied to *L. tauricum* subsp. *bulgaricum* seeds. *P. maritimum, A. littoralis, P. arenarium,* and *C. erecta* needed additional fungicide treatment (Benlate®) in 1 % solution for 10 or 20 min (*P. maritimum*) to overcome the high contamination of the seeds.

All species were germinated on water agar (8 g/l Plant Agar, Duchefa) and then cultivated on MS media (Murashige & Skoog 1962), 6.5 g/l agar, supplemented with naphtaleneacetic acid (NAA) and/or benzylaminopurine (BAP) in various concentrations (Table 1) and 30 g/l sucrose. All media were adjusted to pH 5.75 prior to sterilization and autoclaved at 121 °C/1 atm for 20 minutes. The cultures were maintained in VitroVent® containers (Duchefa) with 125 ml medium, at 16/8 h photoperiod (3000 lux) and 23 ±1 °C.

The obtained plantlets were transferred *ex vitro* on wet Bentonite® (bentonite supplied with micro and macro elements) in covered plastic pots.

Results and discussion

The results from the *in vitro* cultivation of the fourteen investigated species (six of them with specified threat status) are summarised in Table 1. Successfully initiated cultures of eleven species are shown on Fig. 1.

Sterilization rate varied within the different species. In some cases, simple techniques were enough to achieve excellent disinfection. However, modified sterilization procedures applied to some of the species, according to their seed characteristics and level of contamination, had variable success.

Cionura erecta, *P. arenaria* and *V. purpureum* showed high germination rate. *Plantago arenaria* and *V. purpureum* germinated very fast (24–48 hours), whereas *C. erecta* needed at least four weeks. Fast germination was observed for *P. maritimum* as well. However, its shoots developed very slowly (Fig. 1A). *Pancratium maritimum* was proved to be *in vitro* recalcitrant and its growth could be improved by modification of the auxin concentration in the media (Dragassaki & al. 2003). Fast growing shoots were obtained after germination *in vivo* in wet Bentonite® (Fig. 2A). They could be used as a source of explants for initiation of *in vitro* cultures. In contrast with *P. maritimum*, for *L. tauricum* subsp. *bulgaricum* seeds poor germination was followed by fast growth of the shoots (Fig. 1I). The dormancy of these seeds was overcome by treatment with GA_3 which is widely practiced to stimulate the germination of many plant species (George 1996).

Despite of the poor sterilization and/or germination of some of the species (*A. littoralis, C. erecta*, *J. albicaulis* subsp. *kilaea, A. uechtritziana, S. thymifolia, S. maritima*), the obtained seedlings showed vigorous growth. The media composition was selected according to the plant family requirements (Patnaik & Debata 1996; Genkov & al. 1997; Usha & Swamy 1998; Banerjee & al. 1999; Li & al. 2002; Vinterhalter & Vinterhalter 2005).

Table 1. ***In vitro*** **cultivation of sandy–dune plant species.**

Species (Family)	Habitat type	Threat status	Sterilization	Sterilization rate	Germination rate	Media BAP/NAA [mg/l]	*In vitro* regeneration rate
Pancratium maritimum (Amaryllidaceae)	PFD	Endangered (RDB)	B+S	+++	++	0.1/0.9	+
Astrodaucus littoralis (Apiaceae)	PFD	Endangered (RDB)	B+S	+	+	4/0.15	+++
Peucedanum arenarium (Apiaceae)	PWD	–	B+S	++	–	–	–
Cionura erecta (Asclepiadaceae)	PFD	–	B+S	+++	+++	2 or 4/0.15	+++
Jurinea albicaulis subsp. *kilaea (Asteraceae)*	PFD	–	peeling + S	+++	+	1/0.5	+++
Otanthus maritimus (Asteraceae)	PFD	Endangered RDB	soaking + S	++	++	1/0.5	+
Aurinia uechtritziana (Brassicaceae)	PFD	BC	S	+++	+	0.2/0	+++
Silene thymifolia (Caryophyllaceae)	PWD	–	S	+	+	1/0.5 or 0.2/0	+++
Scabiosa argentea (Dipsacaceae)	PFD	–	S	+	–	–	–
Stachys maritima (Lamiaceae)	PWD	Rare (RDB)	peeling + S	+++	+	hormone–free	+++
Linum tauricum subsp. *bulgaricum (Linaceae)*	PFD	Rare (RDB)	GA3 + S	+	+	0.2/0	+
Glaucium flavum (Papaveraceae)	PFD	–	S	+	+	–	+
Plantago arenaria (Plantaginaceae)	PFD	–	S	+	+++	1/0.5 or 0.2/0	+++
Verbascum purpureum (Scrophulariaceae)	PFD	Rare (RDB); BC	S	+++	+++	1/0.5 or 0.2/0	+++

Abbreviations: PFD, Pontic fixed dunes; **PWD,** Pontic white dunes; **RDB,** *Red Data Book of Bulgaria*; **BC,** Bern Convention; **B,** presterilization with Benlate®; **GA_3,** gibberellic acid; **S,** standard sterilization procedure; rate of sterilization/germination/regeneration: + poor; ++ moderate; +++ excellent.

The *in vitro* obtained cultures had different regeneration potential during subcultivation. Some of the species were easily propagated by internode (*C. erecta, S. thymifolia, S. maritima, P. arenaria*) or axillary shoot cultures (*A. littoralis*, *J. albicaulis* subsp. *kilaea, A. uechtritziana, P. arenaria*). *Verbascum purpureum* (Fig. 1K) was successfully subcultivated by excised hypocotyls.

Direct regeneration was achieved for all of the species. The addition of the auxin (NAA) in the media stimulated callus induction for *S. thymifolia* and occurrence of vitrificated shoots and fused petioles of *V. purpureum* (Fig. 1L). *Plantago arenaria* cultures developed rooted shoots on NAA-containing media (Fig. 1J) and in the presence of BAP flowered continuously.

Most of the obtained regenerants were difficult to adapt *ex vitro* due to their weak root system and dysfunctional stomata, except for the plantlets of *C. erecta* which grew easily on wet Bentonite® (Fig. 2B). *S. thymifolia* and *S. maritima* plantlets were also adapted *ex vitro* (Fig. 2C, D).

The first trials presented a promising propagation of psammophytes, some of them rare, endangered and medicinal plant species (*P. maritimum, A. littoralis, C. erecta, J. albicaulis* subsp. *kilaea, O. maritimus, A. uechtritziana, S. thymifolia, S. argentea, S. maritima, P. arenaria,* and *V. purpureum*). The obtained *in vitro* cultures could be further investigated for genetic stability, synthesis of biologically active substances, and vitality during long-term storage and possibility for acclimatization. The *in vitro* germination of four of the species was found to be low or impossible (*Peucedanum arenarium, S. argentea, L. tauricum* subsp. *bulgaricum, G. flavum*). Therefore, other sources of explants could be used for cultivation, preferably from *in vivo* collection.

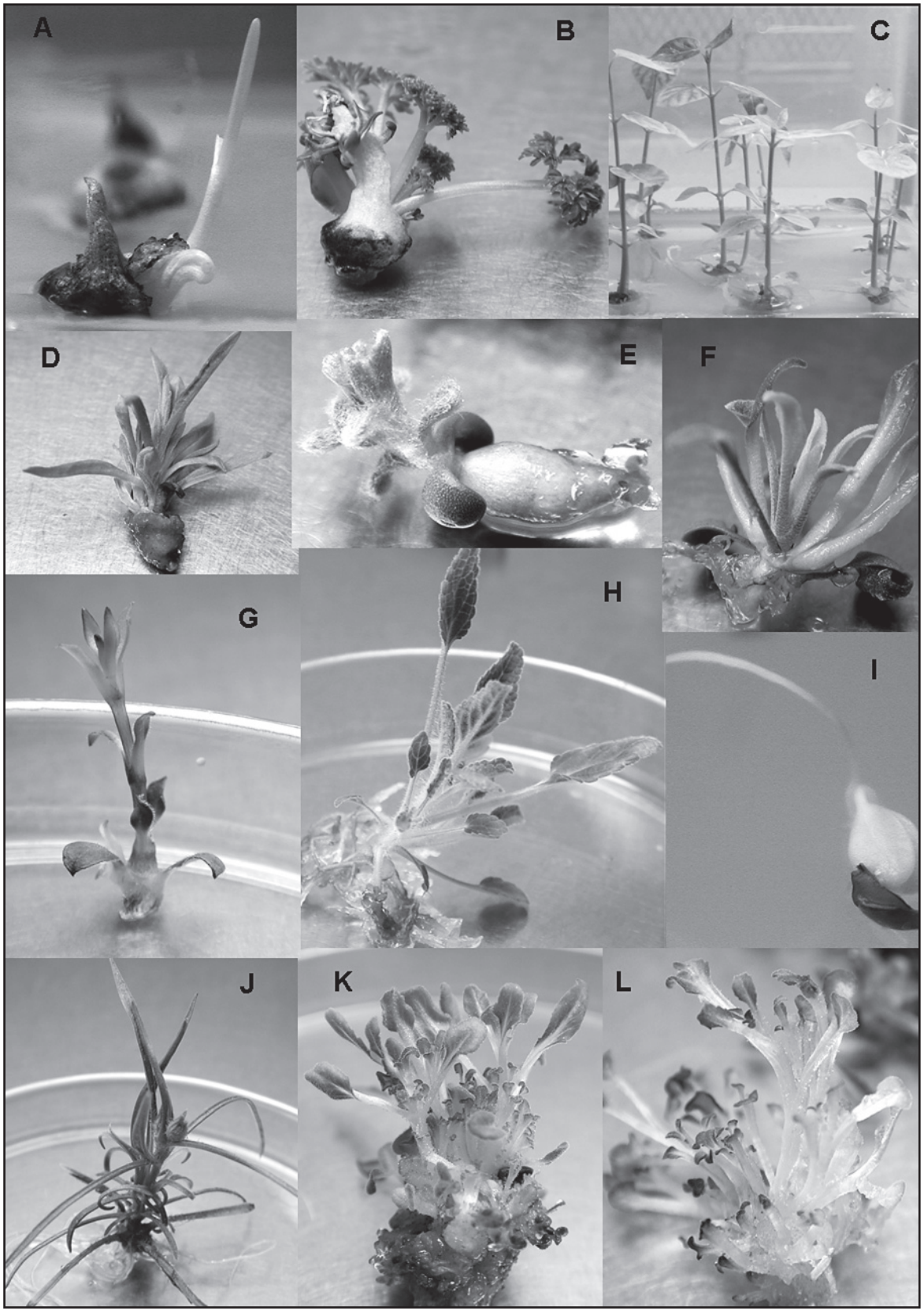

Fig. 1. *In vitro* cultures of: *Pancratium maritimum* (**A**), *Astrodaucus littoralis* (**B**), *Cionura erecta* (**C**), *Jurinea albicaulis* subsp. *kilaea* (**D**), *Othanthus maritimus* (**E**), *Aurinia uechtritziana* (**F**), *Silene thymifolia* (**G**), *Stachys maritima* (**H**), *Linum tauricum* subsp. *bulgaricum* (**I**), *Plantago arenaria* (**J**), *Verbascum purpureum* (**K**, **L**).

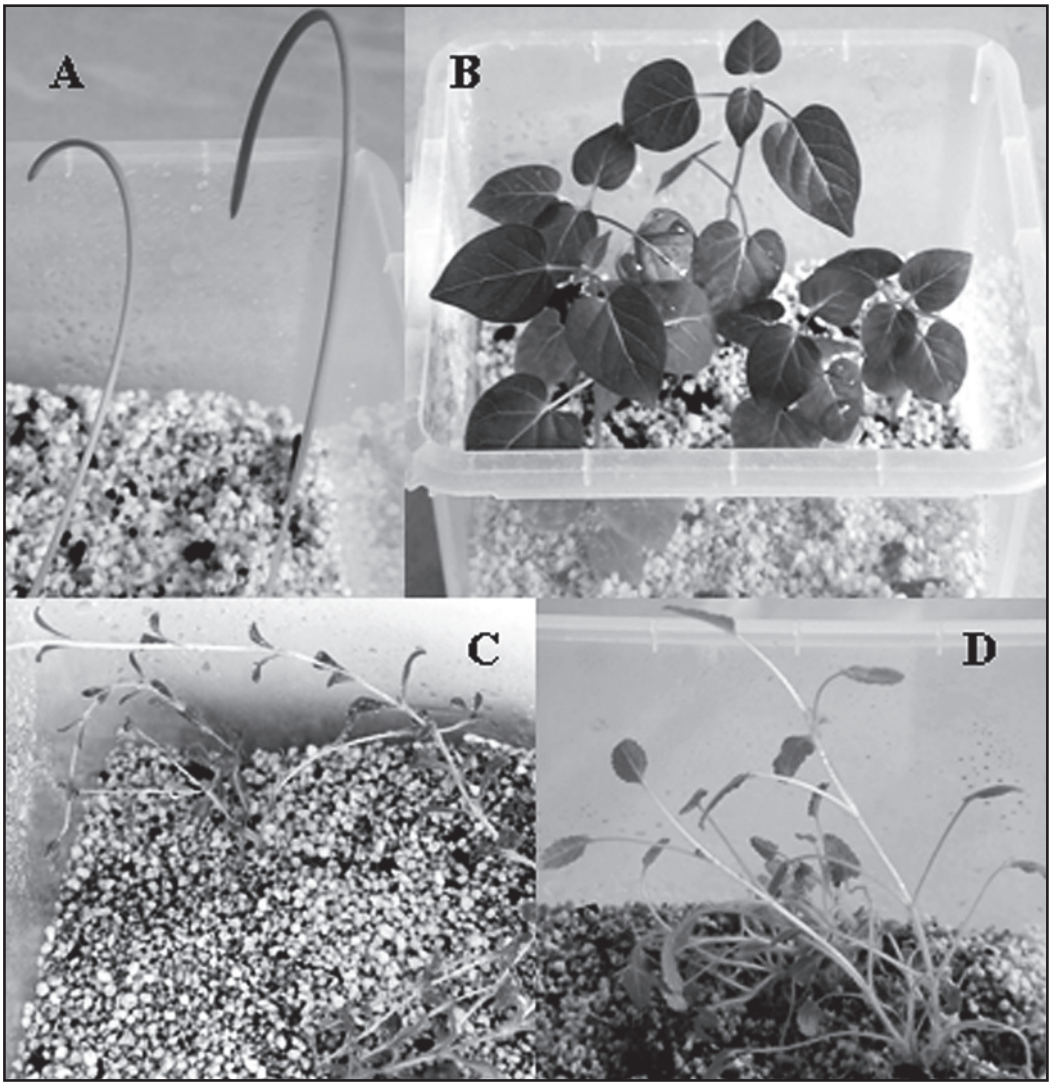

Fig. 2. *In vivo* germination of *Pancratium maritimum* (**A**); *ex vitro* adaptation of: *Cionura erecta* (**B**); *Silene thymifolia* (**C**); *Stachys maritima* (**D**).

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