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Initial responses of explants from rare and endangered coastal plant species during initiation of tissue culture

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Abstract

The strategy of minimum interference was used in the present study to establish *in vitro* cultures of 29 rare and endangered plant species from the coastal zone of Baltic sea in the territory of Latvia by using preferentially seeds as initial plant material. In respect to germination behaviour all species fell into three groups. Seeds of eight species showed uniform germination without any visible signs of dormancy. For 13 species sporadic germination was observed. The remaining species were designated as in an undefined or incompletely understood state. Species-specific morphogenic responses were found during development of plants on hormone-free agarized medium. Annual plants flowered, gave seeds and completed the life cycle within 6 months of tissue culture. Clonal species exhibited pronounced clonal development according to a particular growth form. Axillary bud formation was not stimulated on a hormone-free medium. Root formation was a characteristic for the majority of species. Initiation of tissue culture with shoot explants was performed for seven species. The obtained results form the basis for development of suitable methods for conservation of rare and endangered plant species in tissue culture.

Key words: Baltic Sea, coastal zone, conservation of genetic resources, rare and endangered plant species, tissue culture.

Introduction

In addition to micropropagation plant tissue culture provides a means for conservation of genetic resources. This is especially important for rare and endangered native plant species as the method allows establishing cultures from a minimum amount of starting plant material with possible further multiplication (Benson 2000). In combination with another *in vitro* technique, so-called slow growth, it is possible to establish long-term collections of germplasm with minimal resources (Watt et al. 2000).

Two different strategies are used in respect to tissue culture of rare and endangered plant species. The first startegy can be designated as the minimum interference approach aimed at preserving the genetic identity of source plants. Preferentially using seeds as material for culture establishment, this can be done without damaging of the source plants. This strategy can be effectively used for preservation of particular genotypes, especially using the slow growth procedure. The second strategy is aimed at increasing genetic variation through callus culture with extensive use of synthetic growth regulators

(Seliskar, Gallagher 2000; Wang et al. 2003; Wang et al. 2004; Wang et al. 2005). Sometimes this strategy is also used for mass multiplication needs through a callus culture (Dhar, Joshi 2005).

Coastal habitats are characterized by heterogeneous environmental conditions and have a unique flora that includes many rare and endangered species (Ievinsh 2006). In Latvia more than one third of Red Data Book plant species are located in the coastal zone of the Baltic Sea.

Development of *in vitro* techniques for collecting and preserving threatened plant species has been successfuly started at the National Botanic Garden of Latvia recently (Kļaviņa et al. 2004). In the present study, the strategy of minimum interference was used to establish *in vitro* cultures of several rare and endangered plant species from the coastal zone of the Baltic Sea in the territory of Latvia. Primary growth responses in tissue culture of 29 plant species are reported here.

Materials and methods

During summer of 2005 seeds from 29 rare and threatened plant species from the coastal zone of the Baltic Sea in the territory of Latvia were collected (Table 1). Tissue culture was initiated immediately after collection or within three days. For every species 10 to 50 seeds were used for culture establishment. Seeds were surface sterilized with commercial bleach ACE for 10 to 20 min followed by three washes with sterile deionized water. Sterilized seeds were germinated in 19 × 110 mm test tubes on agar-solidified (6 g l^{-1}) half-diluted Murashige and Skoog medium (pH 5.8) under a 16-h photoperiod provided by a fluorescent light with a photon flux density 10 to 15 μmol m^{-2} min^{-1} at 22 to 25 °C. If the seeds failed to germinate within a prologed period of time different treatments were used to break dormancy including cold stratification at 5 °C, darkness, and thermoperiod.

Several species with no available seeds were tested for a possibility to establish tissue culture using shoot meristems. Explant tissues were surface sterilized with half-diluted

Table 1. Rare and endangered species used in the present experiments for initiation of tissue culture. RDB, Red Data Book of Latvia (2003); 1, endangered species; 2, vulnerable species decreasing in number; 3, rare species

Species	Status RDB	Location	Habitat type	Date of collection	Germination or seed status
Alopecurus arundinaceus Poir.	3	57°50' N, 24°20' E	Coastal meadow	Jul 12	sporadic
Alyssum gmelinii Jord.	3	56°18' N, 20°59' E	Dunes	Jul 27	sporadic
		57°34' N, 21°42' E		Sep 5	sporadic
Angelica palustris (Besser) Hoffm.	1	56°59' N, 23°53' E	Coastal meadow	Aug 18	sporadic
Atriplex calotheca (Rafn) Fr.	3	57°20' N, 23°08' E	Shore	Sep 22	dormant
Blysmus rufus (Huds.) Link	2	57°20' N, 23°08' E	Coastal meadow	Jun 28	dormant
		57°20' N, 23°08' E		Sep 22	dormant
Carex ligerica J. Gay	2	57°50' N, 24°20' E	Coastal meadow	Jul 12	sporadic

(continued)

Species	Status RDB	Location	Habitat type	Date of collection	Germination or seed status
Carex reichenbachii Bonnet	3	57°19' N, 23°08' E	Dune forest	Jun 6	dormant
		57°36' N, 21°59' E		Sep 5	dormant
Centaurium littorale (Turner) Gilmour	2	57°37' N, 22°02' E	Coastal meadow	Sep 5	sporadic
Cephalanthera rubra (L.) Rich	1	57°36' N, 21°57' E	Dune forest	Sep 5	dormant
Eryngium maritimum L.	1	57°14' N, 21°25' E	Dunes	Sep 6	dormant
Euphorbia palustris L.	2	56°58' N, 23°33' E	Coastal lake	Aug 18	explants
Glaux maritima L.	1	57°20' N, 23°08' E	Coastal meadow	Jun 28	explants
		57°20' N, 23°08' E		Sep 22	sporadic
Gypsophila paniculata L.	2	56°18' N, 20°59' E	Dunes	Jul 27	sporadic
Hydrocotyle vulgaris L.	2	57°19' N, 23°08' E	Salt marsh	Jun 28	explants
		57°15' N, 23°08' E	Coastal lake	Jun 28	explants
		57°19' N, 23°08' E	Salt marsh	Sep 22	dormant
Juncus balticus Willd.	3	57°00' N, 23°56' E	Dune slacks	Aug 18	uniform
		57°26' N, 21°39' E	Coastal lake	Sep 5	uniform
Juncus gerardii Loisel.	2	57°19' N, 23°08' E	Salt marsh	Jun 28	explants
		57°19' N, 23°08' E	Salt marsh	Sep 22	uniform
Lathyrus maritimus (L.) Bigelow	2	56°18' N, 20°59' E	Dunes	Jul 27	uniform
		57°15' N, 21°25' E		Sep 6	uniform
Linaria loeselii Schweigg.	3	57°36' N, 21°57' E	Dunes	Sep 5	sporadic
Phleum arenarium L.	1	57°34' N, 21°43' E	Coastal meadow	Sep 5	uniform
Plantago maritima L.	1	56°30' N, 21°02' E	Coastal lake marsh	Aug 24	uniform
		56°30' N, 21°02' E		Sep 18	uniform
Puccinellia capillaris (Lilj.) Jaksen	1	57°00' N, 23°56' E	Shore	Aug 18	sporadic
Schoenus ferrugineus L.	3	57°16' N, 23°09' E	Coastal meadow	Jun 28	dormant
		57°16' N, 23°09' E		Sep 22	dormant
Silene borysthenica (Geuner) Walters	2	56°18' N, 20°59' E	Dunes	Jul 27	uniform
		57°36' N, 21°57' E		Sep 5	uniform
Spergularia salina J. et C. Presl		157°52' N, 24°21' E	Shore	Jul 12	sporadic
Tofieldia caliculata (L.) Wahlenb.	1	57°34' N, 21°43' E	Dune forest	Sep 5	uniform
Tragopogon heterospermus Scweigg.	3	56°18' N, 20°59' E	Dunes	Jul 27	sporadic
		57°34' N, 21°42' E		Sep 5	sporadic
Trifolium fragiferum L.	1	56°29' N, 21°02' E	Coastal lake	Jul 27	sporadic
		57°00' N, 23°56' E	River gulf	Aug 18	sporadic
Triglochin maritimum L.	2	57°20' N, 23°08' E	Coastal lake marsh	Jun 28	sporadic
		57°50' N, 24°20' E	Coastal meadow	Jul 12	sporadic
		57°20' N, 23°08' E	Coastal meadow	Sep 22	sporadic
Tripolium vulgare Nees	1	56°30' N, 21°02' E	Coastal lake marsh	Sep 18	uniform

Table 2. Species of rare and endangered coastal plants with uniform seed germination without dormancy. Seeds were surface sterilized with ACE and germinated in test tubes on agar-solidified half-diluted Murashige and Skoog medium under a 16-h photoperiod, 22 to 25 °C. *, immature seeds

Species	Duration of seed storage (days)	Start of germination (days)	Germination (%)	Seedling establishment from germinated seeds (%)
Juncus balticus	1	7 - 24	95	96
Juncus gerardii	1	2 - 40	35	100
Lathyrus maritimus	1	12 - 104	83*	80
Phleum arenarium	3	4 - 56	97	91
Plantago maritima	6	2 - 17	94	94
Silene borysthenica	3	4 - 11	78	79
Tofieldia calyculata	3	15 - 95	97	28
Tripolium vulgare	2	3 - 76	86	90

Table 3. Species of rare and endangered coastal plants with sporadic seed germination in conditions of tissue culture. Seeds were surface sterilized with ACE and germinated in test tubes on agar-solidified half-diluted Murashige and Skoog medium under a 16-h photoperiod, 22 to 25 °C. *, mature seeds; ** immature seeds

Species	Duration of seed storage (days)	Start of germination (days)	Germination (%)	Seedling establishment from germinated seeds (%)
Alopecurus arundinaceus	80	3 - 73	30	67
Alyssum gmelinii	1	15 - 47	63	20
Angelica palustris	1	11 - 70	50	55
Carex ligerica	1	27 - 42	23	67
Centaurium littorale	3	12 - 76	15	33
Glaux maritima	1	2 - 24	29	50
Gypsophila paniculata	1	12 - 34	63	40
Linaria loeselii	3	116	7	100
Puccinellia capillaris	1	-	-	-
	42	9 - 75	30	100
Spergularia salina	1	131 - 133	73	100
Tragopogon heterospermus	1	-	0*	-
	2	12 - 35	100**	100
Trifolium fragiferum	1	15 - 81	82	78
Triglochin maritimum	1	8 - 26	67	50

ACE and placed in 19 × 110 mm test tubes in the same conditions as described above for seeds. In the case with *Euphorbia palustris* and *Cephalanthera rubra* up to 0.5 mg l^{-1} of 6-benzylaminopurine was added to the medium.

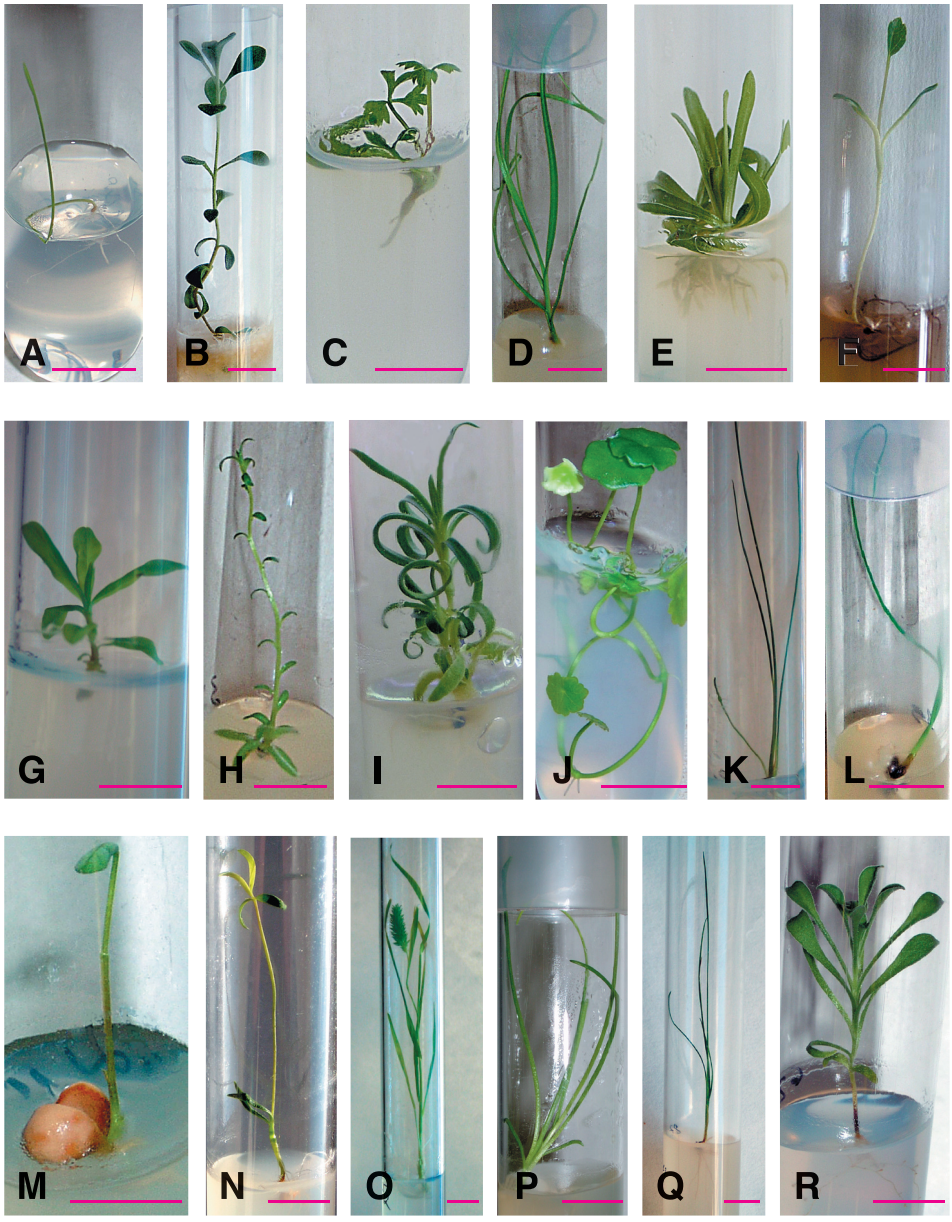

Fig. 1. Primary morphogenic responses of rare and endangered coastal plant species of the Baltic Sea in tissue culture. A, *Alopecurus arundinaceus*; B, *Alyssium gmelinii*; C, *Angelica palustris*; D, *Carex ligerica*; E, *Centaurium littorale*; F, *Eryngium maritimum*; G, *Euphorbia palustris*; H, *Glaux maritima*; I, *Gypsophila paniculata*; J, *Hydrocotyle vulgaris*; K, *Juncus balticus*; L, *Juncus gerardii*; M, *Lathyrus maritimus*; N, *Linaria loeselii*; O, *Phleum arenarium*; P, *Plantago maritima*; Q, *Puccinellia capillaris*; R, *Silene borysthenica*. Bar represents 1 cm. All species shown were established in tissue culture from seeds except *Euphorbia palustris* and *Hydrocotyle vulgaris* for which shoot explants were used.

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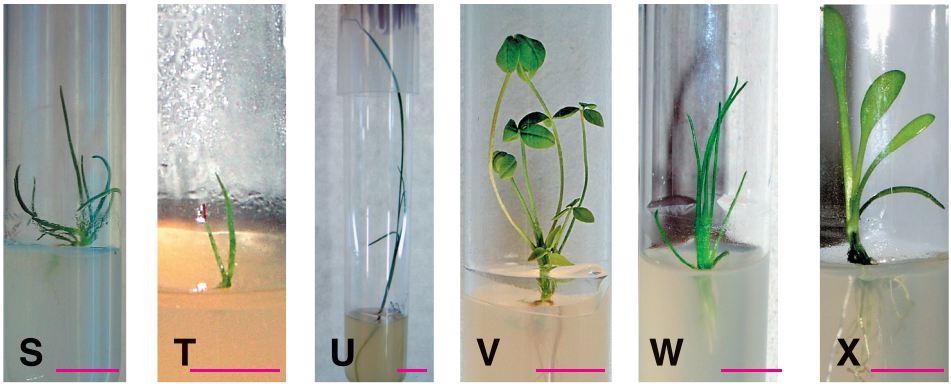

Fig. 1. *(continued)* Primary morphogenic responses of rare and endangered coastal plant species of the Baltic Sea in tissue culture. S, *Spergularia salina*; T, *Tofieldia calyculata*; U, *Tragopogon heterospermus*; V, *Trifolium fragiferum*; W, *Triglochin maritimum*; X, *Tripolium vulgare*. Bar represents 1 cm. All species shown were established in tissue culture from seeds.

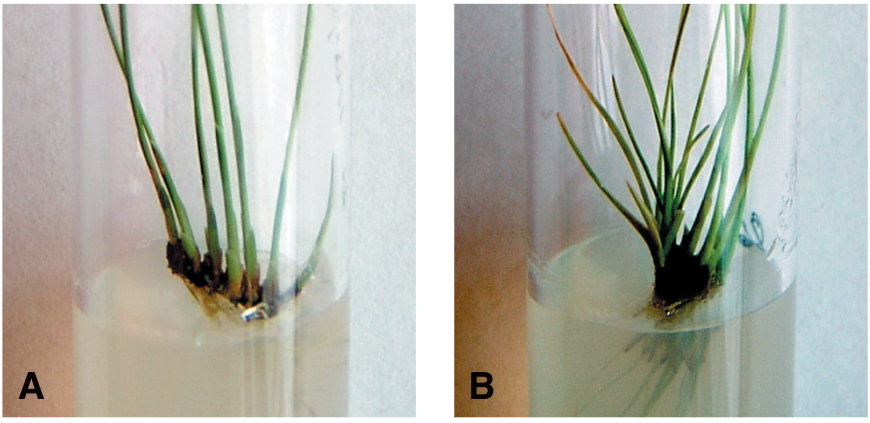

Fig. 2. Clonal growth of *Juncus balticus* (A) and *Juncus gerardii* (B) during cultivation on hormone-free Murashige and Skoog medium for 6 months.

Results

Large variability of seed germination was found for the studied species. In general, in respect to germination behaviour, all species fell into three groups: (i) species with uniform seed germination without any visible signs of dormancy (Table 1, Table 2); (ii) species with sporadic seed germination with a characteristic wide period of start of germination for every particular species (Table 1, Table 3); (iii) species with an undefined or incompletely understood state (most probably, apparent seed dormancy or uncompleted seed maturation; Table 1, Table 4).

In total, seeds of eight species showed uniform germination (Table 2). The majority of seeds from the first group species started to germinate within a week in conditions

Table 4. Species of rare and endangered coastal plants with an undefined or incompletely understood state of seeds. Seeds were surface sterilized with ACE and germinated in test tubes on agar-solidified half-diluted Murashige and Skoog medium under a 16-h photoperiod, 22 to 25 °C

Species	Treatment or status	Start of germination (days)	Germination (%)	Seedling establishment from germinated seeds (%)
Atriplex calotheca	thermoperiod 24 / 5 °C	4 - 100	20	90
Blysmus rufus	thermoperiod 24 / 5 °C		0	-
Carex reichenbachii	2 months at 5 °C	130	0	-
Cephalanthera rubra	darkness, 15, 24 °C	60 - 90	2	necrotic after 2 months
	15, 24 °C		2	
Eryngium maritimum	2 months at 5 °C	110 - 116	20	100
	afterripening +	88 - 100	50	100
	2 months at 5 °C			
Hydrocotyle vulgaris	seeds at different stages of ripeness	-	-	-
Schoenus ferrugineus	seeds at different stages of ripeness	-	-	-

Table 5. Species of rare and endangered coastal plants established in tissue culture by shoot explants. Explants were cultivated on agar-solidified half-diluted Murashige and Skoog medium medium for 6 months. *, indicate infection.

Species	Sterile explants (%)	Developing explants (%)	Rooting (%)	Coefficient of propagation
Alyssium gmelinii	20	0	-	-
Euphorbia palustris	80	60	0	1.2 - 1.5*
Glaux maritima	56	100	100	2 - 6
Hydrocotyle vulgaris	7	50	88	2 - 4
Juncus gerardii	0	27*	0	-
Silene borysthenica	0	0	-	-

of tissue culture. High germination energy of *Juncus balticus*, *Plantago maritima*, *Silene borysthenica* and *Tofieldia calyculata* was observed. Other species germinated during a longer period of time. Germination of *Lathyrus maritimus* succeeded only in the case when immature seeds were used.

For 13 species sporadic seed germination was observed (Table 3). Seeds for some species started to germinate within a week or two weeks with a low germination energy leading to low number of germinated seeds (*Alopecurus arundinaceus*, *Glaux maritima*). Some species of this group failed to germinate in a few months (*Linaria loeselii*, *Spergularia salina*). Germination of *Tragopogon heterospermus* were observed only in the case of immature seeds. After-ripening of seeds by dry storage at warm temperature (25 °C) was a prerequisite for germination of *Puccinellia capillaris*. Germination of *Spergularia salina* was

only delayed and the seeds successively germinated eight days after seed transplantation on a fresh medium consisting only of water with agar. Transplantation was performed 119 days after initiation of sterile culture.

The other species were designated as being in an undefined or incompletely understood state. Most probably they had dormant or incompletely developed seeds (Table 4). Thus, seeds of *Eryngium maritimum* germinated only after two months of cold stratification and after ripening for two weeks (warm stratification at 24 °C) followed by cold stratification for 2 months. After ripening the seeds were removed from the seed coat. The procedure significantly enhanced the percentage of germination. Dust-like seeds of *Cephalanthera rubra* represent only undeveloped embryo consisting only of few cell layers. However seed germination and protocorm formation with primary leaf scales was observed. Later the tissues became necrotic within two months of cultivation. Seeds of the annual species *Atriplex calotheca* were apparently partially dormant and only a very low percent of germination was achieved after cold stratification. However, in the experiments with seeds collected in previous years, *Atriplex calotheca* germinated sporadically, developed well and finished growth within six months of tissue culture (Kļaviņa et al., unpublished data).

Seeds of *Blysmus rufus*, *Hydrocotyle vulgaris*, *Schoenus ferrugineus* and *Carex reichenbachii* exhibited hard seed coats and were dormant. Low temperature stratification for two months did not result in any signs of germination for these species.

Species-specific morphogenic responses were found during development of plants on hormone-free agarized medium in sterile conditions (Fig. 1). The annual plants *Phleum arenarium*, *Spergularia salina*, and *Atriplex calotheca* flowered, gave seeds and completed the life cycle within 6 months of tissue culture. Some of the seeds of *Spergularia salina* germinated already in the cultivation tube. In general axillary bud formation was not stimulated on a hormone-free medium leading to formation of single shoots. However root formation was characteristic for the majority of the species except *Tofieldia calyculata*. Root formation was considerably depressed for *Tragopogon heterospermus* due to root wounding during transplantation after four months of cultivation leading to callus formation. Many seedlings from sporadically germinated seeds of *Alyssum gmelinii* and *Gypsophila paniculata* were vitrified. Several seedlings of *Glaux maritima* exhibited a characteristic albino phenotype.

The clonal species *Juncus balticus* and *Juncus gerardii* exhibited pronounced clonal development in conditions of tissue culture on a medium without growth regulators within six moths of cultivation (Fig. 2). The phenotype of these plants was similar to that characteristic for plants in natural conditions except that the rhizome internodes were extremely short.

Initiation of tissue culture with shoot explants was performed with seven species (Table 5). Culture establishment from apexes of *Alyssum gmelinii* and *Silene borysthenic*a collected at the end of July failed because active growth had been terminated and the infection rate was relatively high due to hairy leaves. Rosy bacteria infection was found in culture of etiolated buds of *Juncus gerardi* but the presence of the bacteria appeared not to affect growth. Shoot explants of *Euphorbia palustris* did not show any signs of growth on a hormone-free medium and no roots developed. Therefore 6-benzylaminopurine at low concentration (up to 0.5 mg l^{-1}) was added to the cultivation medium which initiated explant development. However no root formation was visible.

Shoot explants of *Glaux maritima* and *Hydrocotyle vulgaris* showed a relatively high

rate of multiplication on a hormone-free medium indicating that propagation of these species can be easily achieved with nodal explants. The clonal plant *Hydrocotyle vulgaris* exhibited extensive development of rhizomes with leaves, inflorescences and roots formed at nodes.

Discussion

To maintain a wide genetic basis it is preferred to establish tissue cultures of rare and endangered plants from seeds (Benson et al. 2000). Therefore, in our experiments seeds in different stages of development and maturity, if available, were collected as the preferred plant material for culture establishment. By using appropriate germination techniques it was possible to establish tissue cultures of 21 out of 29 species from seeds.

Several germination patterns were established for coastal plant species possibly reflecting different environmentally imposed dormancy strategies. Seeds with different genotypes may have a stronger or weaker dormancy potential and the intensity may differ for various populations. Several authors have showed that temporal variation in seed germination depends on hydration intensity, temperature regime, light conditions, as well as on ontogenic experience during dormancy release (Garvin, Meyer 2003; Walck, Hidayati 2004; Zia, Khan 2004; Kagaya et al. 2005). Therefore, in natural conditions seedling emergence occurs sporadically only when environmental conditions necessary for a particular genotype are met. In addition, a number of seeds in each population of the particular species may be programmed to remain dormant even in suitable environmental conditions indicating the existence of multiple-level dormancy (Garvin, Meyer 2003). Thus, in the present study seed coat-dependent maturation was found for *Eryngium maritimum* together with cold stratification-released dormancy.

From the point of view of the minimum interference approach, *in vitro* methods possibly leading to somaclonal variation must be avoided (Benson et al. 2000). Among them, propagation through callus culture (Arene et al. 1993) and the use of high concentrations of growth regulators in order to achieve high rate of multiplication (Karp 1992) are the most dangerous. Therefore, in the present experiments only a minimum amount of cytokinin-like substances was used in the most critical case with *Euphorbium palustre* to initiate tissue culture.

At present, further experiments are being performed to develop suitable methods for slow growth (including cold storage) techniques and subsequent efficient micropropagation of rare and endangered coastal plant species successfuly established in tissue culture.

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Reto un apdraudēto piekrastes augu eksplantu sākotnējā reakcija audu kultūru iniciācijas procesā

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Kopsavilkums

Dotajā pētījumā konsekventi izmantoja minimālās iejaukšanās stratēģiju, lai uzsāktu *in vitro* kultūru 29 retu un apdraudētu sugu augiem no Baltijas jūras piekrastes zonas Latvijas teritorijā, pēc iespējas izmantojot sēklas kā izejas materiālu. Visas sugas varēja iedalīt trīs grupās pēc to dīgšanas veida. Astoņu sugu sēklām bija raksturīga vienmērīga dīgšana bez redzamām miera perioda pazīmēm, bet 13 sugu sēklām – sporādiska dīgšana. Pārējo sugu sēklas apzīmēja kā ar nenoteikti vai nepilnīgi izpētītu statusu. Sugu specifiskas morfoģēniskās reakcijas parādījās uz bezhormonu agarizētas barotnes. Viengadīgie augi sešu mēnešu laikā audu kultūrā uzziedēja, veidoja sēklas un pabeidza dzīves ciklu. Klonālajām sugām bija raksturīga izteikta klonālā attīstība atbilstoši konkrētajai augšanas formai. Aksilāro pumpuru attīstību nenovēroja bezhormonu barotnē. Sakņu veidošanās bija raksturīga lielākajai daļai sugu. Audu kultūru ar dzinuma eksplantu palīdzību veica septiņām sugām. Iegūtie rezultāti veido pamatu piemērotu metožu izstrādāšanai reto un apdraudēto sugu augu saglabāšanai audu kultūras.