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691. SĒJUMS

Bioloģija

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VOLUME 691

Biology

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Recording of Hoopoes *Upupa epops* by means of audio playback in the conditions of Latvia: a preliminary evaluation

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Abstract

Playback is often used to improve recording of birds and therefore to gain more precise data on their numbers and distribution. The aim of this study was to evaluate the potential suitability of this method for survey of Hoopoes *Upupa epops* in Latvia. Playback was carried out in mornings between the end of April and beginning of July in a total of 36 routes, of which 33 routes (137 points) were used to analyse changes in Hoopoe activity. Hoopoes responded to playback at 11 (8 %) of the points. The results suggested that the most appropriate time for playback surveys on Hoopoes was in mornings in May. It is possible that the response behaviour of Hoopoes depends also on local breeding densities. More studies concerning their territorial behaviour are needed to fully evaluate the efficiency of the playback method for studying population size and distribution of the Hoopoe.

Key words: Hoopoe, playback, *Upupa epops*, territory.

Introduction

It is generally well known that birds respond to imitation of their songs or calls. In the simplest case playback is used to improve the recording rate of birds (e.g. Yahner, Ross 1995; Rimmer et al. 1996). In Latvia, this method is most widely used to record Woodpeckers *Piciformes* (Bergmanis 1993) and Owls *Strigiformes* (Avotiņš 1990). Playback is often used also to study the behaviour of birds – the song itself (e.g. Martín-Vivaldi et al. 1999a; Sorjonen, Merilä 2000) and also other aspects of communication and behaviour (e.g. Prescott 1987; Krams 2001; Martin, Martin 2001).

Smith (1996) suggests that the birds that are pressed for time and have to form new social arrangements are more responsive to playback. He also points out that first of all this statement concerns migrants. This description matches also the Hoopoe *Upupa epops*. The research by Martín-Vivaldi et al. (1999a; 2000; 2004) carried out in Spain shows that Hoopoes respond to playback. I used playback mainly to test if the playback method could be used to improve data on the population size and distribution of Hoopoes in Latvia. The main objective of this paper is to present the first evaluation of this method.

Hoopoe *Upupa epops* belongs to the order *Coraciiformes* and has a very simple song consisting of repeated phrases with two to five (usually three) notes in each phrase (Cramp 1994). Song is produced only by males (Martín-Vivaldi et al. 1999b). In Latvia, Hoopoes start singing almost immediately after arriving from wintering grounds in the end of April (Latvian Ornithological Society, unpublished data). The singing activity reaches its

maximum in the prelaying period and decreases after the onset of laying (Martín-Vivaldi et al. 1999a).

Materials and methods

The recording used in the playback survey was taken from the CD-set "Tous les Oiseaux d'Europe" (Roché 1996). All but the first 16 seconds of the Hoopoe *Upupa epops* track was deleted using Cool Edit Pro 2.0 software. The final sound track contained five distinct phrases of song, each consisting of three notes, and two alarm calls – after the first and the fourth phrase. One side of 60-minute audio tapes was recorded with repetitions of this track.

Playback study was carried out in 2003 and 2004. Seven observers covered a total of 36 playback routes (including a number of separate points). Routes that were covered more than once were counted as different routes. The number of playback points was recorded for only 33 of the routes. These 33 routes contained 137 playback points. Most of these points (95) were located in the district of Riga (Fig. 1) where the breeding density of Hoopoes was thought to be the highest (Transehe, Sināts 1936, Priednieks et al. 1989; Lipsbergs 2000). Six playback points were in the district of Valka, seven in the district of Talsi and 29 in the district of Dobele. There had been no records of Hoopoes in the district of Dobele for at least the last five years. Playback was carried out mostly in areas of small gardens, which probably are a highly important breeding habitat for Hoopoes (Latvian Ornithological Society, unpublished data).

Playback was carried out between the end of April and the beginning of July (Fig. 2). Most of the playback points were surveyed in May, which is the month of the highest Hoopoe activity level (Latvian Ornithological Society, unpublished data). During the day, playback was carried out from 5:21 until 14:06 (local time). In one exceptional case

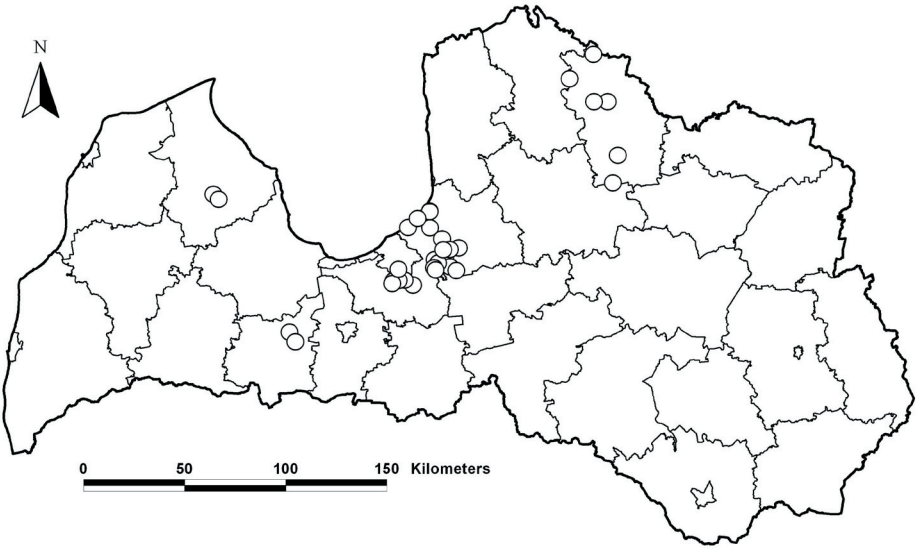

Fig. 1. Distribution of routes in the territory of Latvia in which Hoopoe *Upupa epops* playback survey was carried out. The circles on the map represent the central point of each route.

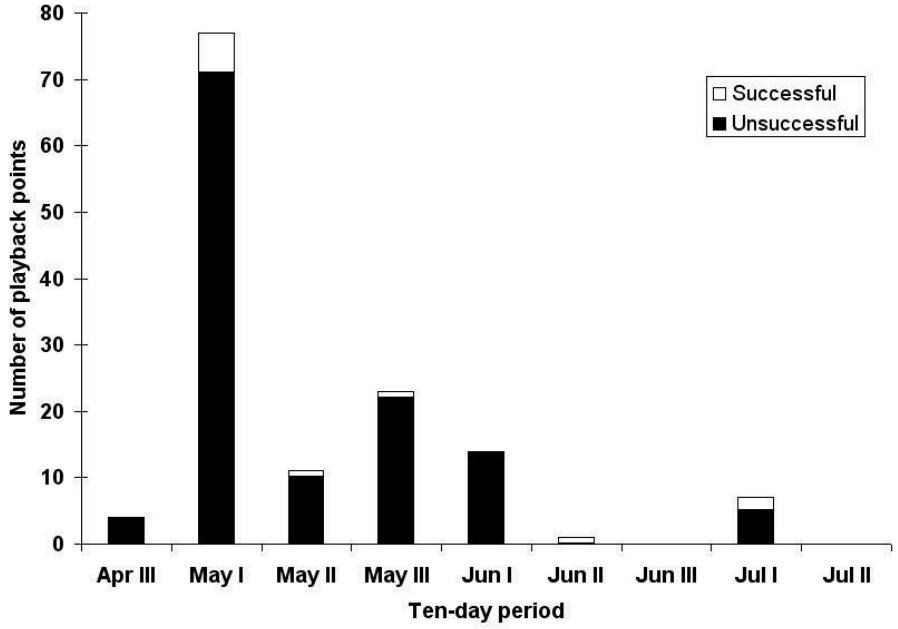

Fig. 2. The number of points in which playback on Hoopoes *Upupa epops* was carried out in ten-day periods. The points in which response of Hoopoe was achieved are considered successful (see details in the text).

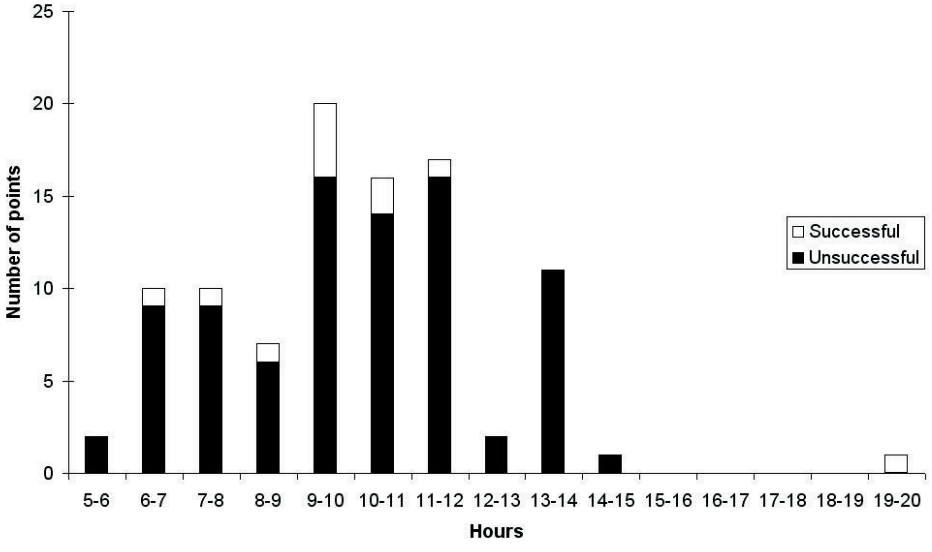

Fig. 3. The number of points in which playback on Hoopoes *Upupa epops* was carried out in terms of time of the day (local summer time). The points in which response of Hoopoe was achieved are considered successful (see details in the text).

playback was carried out also at 20:00 (Fig. 3).

Portable tape recorders were used for playback, with loudness at full volume. At all points a series of 20 distinct phrases was played back (ca. 1 min) followed by listening for a response for about a minute. If there was none, the tape was played for the second and the third time following the same procedure. On average, the time spent in each playback point was about six to seven minutes. As the survey was carried out mainly in areas populated by humans, it was not always possible to follow the described scheme of playback.

Birds from different pairs responding to playback at the same point were counted as separate cases. If the same pair (birds within the same territory) was observed more than once, these were also considered as separate cases of successful playback. Birds were considered responding to the playback if they approached the observer, produced alarm calls or started singing (or any combination of these). One case, when Hoopoe stopped singing after the playback, was not considered successful.

In most cases, irrespective of response, weather conditions (air temperature, cloud cover, precipitation and the strength of wind), precise time of playback and the number of playback series were recorded. In most cases, playback points were mapped, but in the beginning of the study only the successful playback points were mapped. If Hoopoe responded to playback, the series of playback after which it happened, precise time of response and distance to the bird from observer were noted. The exact location of Hoopoe after playback was mapped, mostly using GPS receiver. All data were filled in specially designed forms.

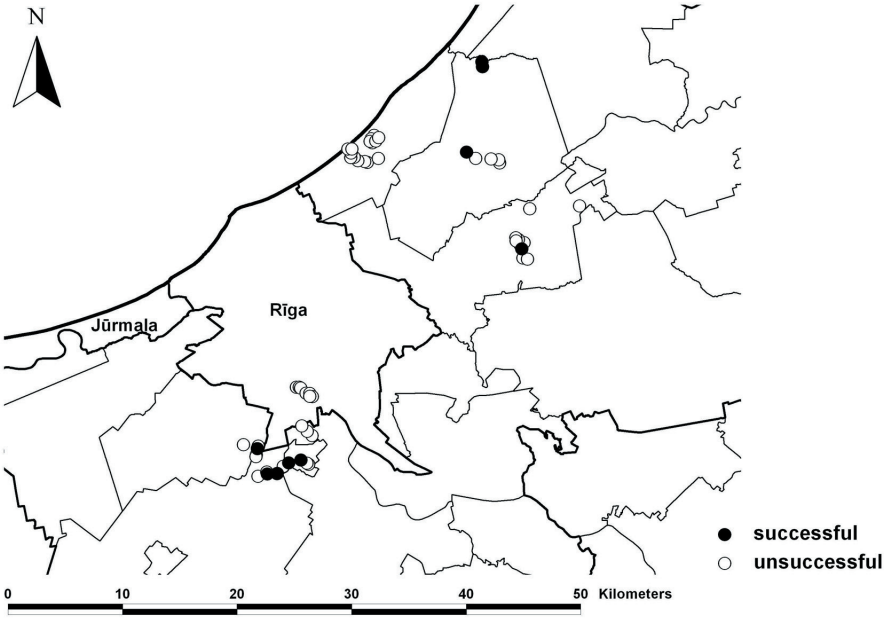

Fig. 4. The points in which playback on Hoopoes *Upupa epops* was carried out in the district of Rīga. The points in which response of Hoopoe was achieved are considered successful (see details in the text). The thick line marks the borders of Rīga, Jūrmala and the district of Rīga. The borders of parishes are marked with the thin line.

Results

Hoopoe responded to playback at 11 points (8 % of the total). Two of these points almost precisely overlap with other two points, but they were counted as separate points as they were surveyed at different times. All successful points lie within the district of Riga (Fig. 4): three of them in military training grounds near Ādaži, one in the nature reserve 'Garkalne forest', and the remainder (n = 7) in the vicinity of Baloži, town south of Riga. Altogether, 22 cases of playback were successful.

Hoopoe males were singing before playback in only two of the successful cases. In one case, which was not considered successful, a Hoopoe male ceased singing after the playback. In all but one case the birds were not seen before playback.

In 59 % (n = 13) of the successful cases Hoopoes responded to playback after the first playback series, in 36 % of the cases (n = 8) after the second, and in one case (5 %) – after the third series. In all cases, when birds from different pairs were attracted to the same playback point, it is not known if the response was triggered by the behaviour of the first bird or by the playback.

Hoopoes approached the observer in 82 % of the successful cases. The mean approaching distance was 31.3 m (0 - 77 m; SD = 24.88, n = 18).

Male Hoopoes responded to playback in 11 cases (50 % of the successful cases), while in three cases (14 %) pairs were apparently responding. In eight cases (36 %) the sex of bird was unknown as no song was heard, and birds of both sexes are very similar in appearance (Cramp 1994).

The percentage of the successful playback points was used to measure changes in Hoopoes' response activity to playback. With unreliable data excluded (see "Discussion"), the results show that the Hoopoe activity reaches its maximum seasonally in May (Fig.

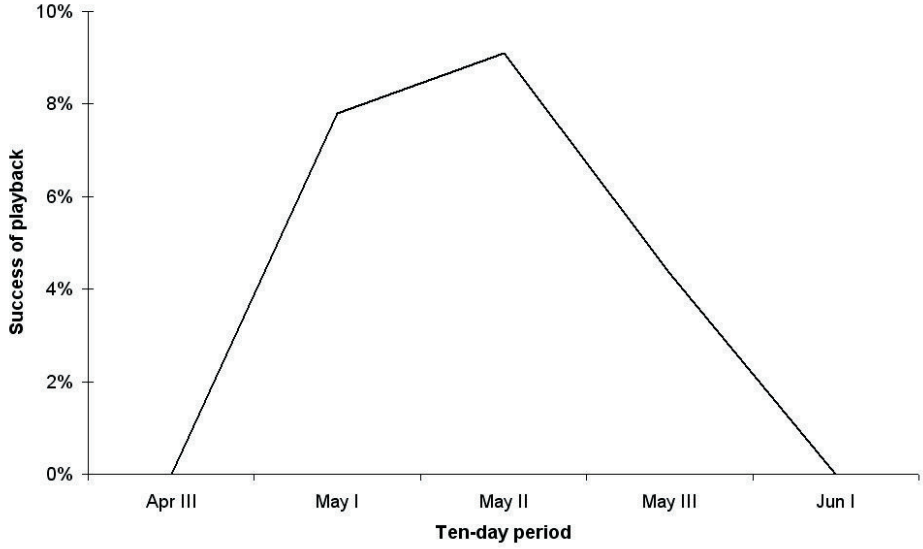

Fig. 5. Percentage of successful playback points (response achieved; see details in the text) from the total as the indicator of seasonal changes in the activity level of Hoopoes *Upupa epops*.

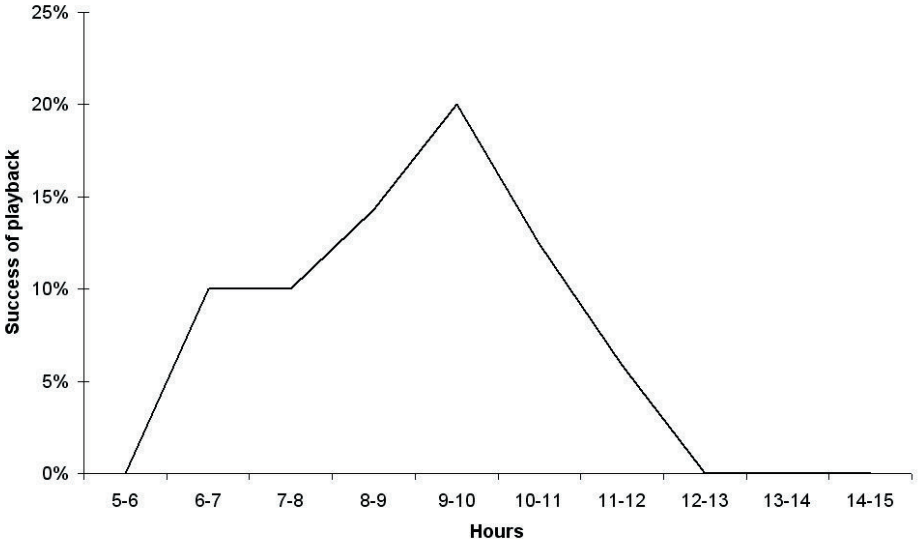

Fig. 6. Percentage of successful playback points (response achieved; see details in the text) from the total as the indicator of diurnal changes in the activity level of Hoopoes *Upupa epops*. Hours are presented according to local summer time.

5). In most cases, Hoopoes were responding between 6:00 and 12:00 (local time). An exceptional case of playback at 20:00 was also successful, but it was not taken into account when describing the diurnal changes of Hoopoe response activity (Fig. 6).

The distance between the bird and observer before playback, estimated approximately, is known only in three cases. Although knowing this distance might be useful in judging the bird's ability to hear the recording, the acquired data are not reliable, not only because of the rough estimate of the distance but also since the Hoopoe's song sounds farther than it really is. Therefore these estimates of distance might be overrated. In one particular case the distance (140 m) in which Hoopoe obviously responded to playback was noted precisely.

Discussion

The current study confirms that Hoopoes in Latvia can be recorded using audio playback, which can increase the chances of recording them, compared to 'passive' surveys, as in most of the cases the birds would have been unnoticed unless playback was performed. However, there are certain limitations of this study regarding assessing the efficiency of this method: sample size, representativity of the time of season and the time of day, as well as the insufficient knowledge about the species territorial and social behaviour.

Some knowledge has been gained on the most appropriate time for playback surveys of Hoopoes in the conditions of Latvia, both in terms of season and time of day. However, due to the rather small sample size, some exceptional records may still distort the overall result. For example, the results of this study show high levels of Hoopoe activity in mid-June and July. Both of these peaks are obviously caused by the small amount of data

(only one playback point in mid-June and seven points in July). A rise of activity in July might be due to an increase in singing activity of Hoopoe males after the end of breeding attempts (Martín-Vivaldi et al. 1999a). However, this maximum should not exceed the activity before pair formation, in the end of April and beginning of May. If both of these exaggerated peaks of activity are omitted, the most appropriate time to carry out playback surveys on Hoopoes in Latvia appears to be in May (Fig. 5).

The results seem to better illustrate the diurnal changes in the response activity of Hoopoe. Apart from one successful case of playback at 20:00, the activity of Hoopoes appears to be highest in the morning hours, reaching its maximum between 9:00 and 10:00 (Fig. 6). Despite the small sample size, this maximum closely fits that recorded in Spain: 8:00 to 9:00 (Martín-Vivaldi et al. 1999a). However, there were no attempts of playback made after 14:06 in this study.

In further playback studies on Hoopoe in Latvia, at least three playback series, one minute each (ca. 7 min of discontinuous playback), are necessary at each point to maximize the possibility of recording response. Shorter playback times may lead to underestimated numbers of birds.

In the future the recording used for playback should be changed by excluding the alarm calls and indistinct song phrases. Although alarm calls should not greatly influence the playback results, as these calls are commonly a part of territorial disputes (Ķerus, unpublished data), the comparison with other studies might be easier if only song is used.

In order to fully assess how successful the playback method is for Hoopoe, and how to improve it, the response behaviour of this species to playback need to be understood.

Playback was successful in territories (near Ādaži and Baloži; Fig. 4) that are known to support relatively high densities of Hoopoes (Latvian Ornithological Society, unpublished data). Nevertheless, there were several unsuccessful playback points in locations where Hoopoes had been observed either before or after the survey (Latvian Ornithological Society, unpublished data). There might be several reasons for this and none of them can be evaluated directly from this study. According to Smith (1996), birds living in an unstable social situation (forced to form new relationships with mates and neighbours in a short time) are likely to be more responsive to playback. This may imply that breeding density is an important factor contributing to the response level of birds: if the population is not saturated, the intensity of territorial relationships may be less and there may be no need to engage in territorial disputes. Only one of the successful playback points (in the nature reserve 'Garkalne forest') might not be attributed to the effect of high breeding density, but in this case playback was carried out at a close range (33 m) to an occupied nest.

Another problem to understanding the potential use of the playback method for studying habitat requirements, population size and distribution of Hoopoes in Latvia is posed by a question: is the Hoopoe response of to playback actually associated with its territory? Martín-Vivaldi et al. (2000) suggest the contrary. They refer to their own findings (Martín-Vivaldi et al. 1999a) and a summary by Cramp (1985): "Size and nature of territories in west Palearctic uncertain; only defence of nest-site and immediate vicinity perhaps involved." This issue requires more study. Breeding densities are known to be significantly higher in the Iberian Peninsula than in the northern periphery of Hoopoe breeding range in which Latvia lies (Hagemeijer, Blair 1997). Differences in the amount of the available resources and breeding densities lead to differences in territory size (del Hoyo

et al. 2001), which means that the level of territory defence behaviour might also differ between the countries. Before drawing any conclusions about the territories of Hoopoes in Latvia, studies of territoriality by analysing successful playback cases in connection with known nest-sites should be carried out.

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Pupuķu *Upupa epops* konstatēšana ar provocēšanas metodi Latvijas apstākļos: pirmais novērtējums

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Kopsavilkums

Provocēšanas metodi izmanto, lai palielinātu putnu konstatēšanas iespējas un iegūtu iespējami precīzāku informāciju populācijas lieluma, izplatības u.c. novērtēšanai. Šī pētījuma mērķis bija noskaidrot, cik sekmīga un lietderīga ir provocēšanas metode pupuķu *Upupa epops* konstatēšanai Latvijas apstākļos. Provocēšanai izmantoja dabā veiktu pupuķu tēviņa dziesmas ierakstu. Veikti 36 provocēšanas maršruti. Lai novērtētu pārmaiņas pupuķu aktivitātes līmenī, izmantoti 33 maršruti (137 punkti). No tiem, 11 punkti (8 %) bija sekmīgi – pupuķis reaģēja uz ierakstu. Provocēšanu veica dienas pirmajā pusē, no aprīļa beigām līdz jūnija sākumam. Pētījums pierāda, ka Latvijā provocēšanas metodi var izmantot pupuķu konstatēšanai. Iegūtie rezultāti apliecina, ka optimālais laiks pupuķu provocēšanai ir rīta stundas maijā, kad pupuķu aktivitāte ir vislielākā. Iespējams, ka provocēšanas sekmes ir atkarīgas arī no pupuķu blīvuma konkrētajā vietā, jo provocēšana bija sekmīga tikai tajās vietās, kur pupuķi sastopami lielākā blīvumā nekā citur. Lai spriestu par provocēšanas rezultātu iespējamo izmantošanu pupuķu skaita un izplatības novērtēšanai, nepieciešami papildus pētījumi par pupuķu teritoriālo uzvedību.

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Assessment of compensatory predation and re-colonisation using long-term duck nest predator removal data

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Abstract

Removal of predators has been often found ineffective to increase duck nest success. Most often failures were explained by compensatory predation by other predator species and/or rapid re-colonisation of the target area by new individuals by the same predator species. We used 13-year data of removing marsh harriers *Circus aeruginosus*, corvids *Corvidae* and American minks *Mustela vison* to test whether (i) removal of an individual predator species increased duck nest success; (ii) removal of an individual predator species decreased subsequent duck nest depredation rates by the same predator species; (iii) removal of one predator species increased subsequent proportion of duck nests depredated by other predator species. We removed 1 590 predators and followed the fates of 3 019 duck nests. Predator removal was measured using a concept of predator-free days, expressed as the number of days of active duck nests during exposure to the removed predator's search if the removal would not happen. Predators were removed from the main duck breeding area and from it's surroundings which altogether formed the entire predator removal area. Harrier removal was positively correlated with the apparent duck nest success ($P < 0.05$) and negatively with subsequent harrier predation rate ($P < 0.05$). However, this was true considering harriers removed from the entire predator removal area, but not when they were removed only from the duck breeding area, thus suggesting that arrival of new harriers from the surroundings was an important factor in determining nest success in the much smaller duck nesting area. Removal of corvids and American mink were not correlated with duck nest success nor the subsequent predation rates of the same species. Mink removal was positively correlated with the proportion of nests depredated by harriers ($P < 0.05$) suggesting that harriers were compensatory predators after mink removal. Re-colonisation and compensatory predation after removing certain predator species may occur in many predator communities thus causing waste of management efforts. We suggest ways of how to evaluate past and ongoing management programmes and to plan future programmes with the aim of providing early diagnostics of a predator problem.

Key words: American mink, compensatory predation, corvids, duck nest success, marsh harrier, predator control.

Introduction

Predator removal has been a common tool in managing breeding performance of game birds (Chapsky 1957; Parker 1984; Beauchamp et al. 1996; Grant et al. 1999). Today, ethical

considerations place legal constraints on the use of catching devices and killing of predator species (Duebbert, Kantrud 1974; European Commision 1992; European Commission 1996; Jackson 2001). Also, from scientific and economic points of view, this method calls for careful examination before launching wide predator control programmes (Balser et al. 1968; Sargeant et al. 1995; Côte, Sutherland 1997). There have been successful predator removal attempts (Balser et al. 1968; Lynch 1972; Duebbert, Kantrud 1974; Duebbert, Lokemoen 1980; Jackson 2001), but often these efforts have not been rewarding (Parker 1984; Clark et al. 1995; Sargeant et al. 1995; Manchester, Bullock 2000). However, the real proportion between successful and unsuccessful studies probably will remain unknown, because successful results are more often published than unsuccessful ones (Macnab 1983; Beauchamp et al. 1996).

When predator management has not been successful, but all planned activities were carried out with sufficient longevity of efforts and number of removed animals, one possible reason of failure could be a compensatory predation by another predator species or a group of predators (Parker 1984; Clark et al. 1995; Beauchamp et al. 1996). Alternatively, failures can be explained by re-colonisation of the territory by new individuals of the target predator species (Sargeant et al. 1995).

Compensatory predators may seem to be a good explanation, because predator control is often directed towards locally predominant predator species (Clark et al. 1995) or a group of predators, i.e. only avian or mammalian (Parker 1984; Sargeant et al. 1995). Although mammalian and avian predators use different senses and search tactics to locate nests (Clark, Nudds 1991; Pasitschniak-Arts, Messier 1995), these predators can apparently find and depredate the same nests.

Presently, the effects of compensatory predation are poorly documented. In Norway, after the removal of corvids, compensatory predation on willow ptarmigan *Lagopus lagopus* and black grouse *Tetrao tetrix* nests by ermine *Mustela erminea* was suspected (Parker 1984). In Scotland, Jackson (2001) tested if the level of wader nest depredation by hedgehogs *Erinaceus europaeus*, after their removal by fencing, could be maintained by common gull *Larus canus*, but the gull predation rate did not increase in the fenced areas. In Scotland, removal of carrion crows *Corvus corone corone* and common gulls failed to prevent a decline in moorland breeding waders and compensatory predation by red foxes *Vulpes vulpes* was suspected (Parr 1993). In North Dakota, USA, after removal of raccoons *Procyon lotor*, striped skunks *Mephitis mephitis* and red foxes, smaller mammalian predators exhibited compensatory predation on grassland songbird nests (Dion et al. 1999). However, the above findings originate from well-planned short-term experiments but not from long-term management programmes. Although many predator removal programmes exist, reports on their evaluation are generally lacking (Harding et al. 2001).

We used 13-year data from a complex predator control programme to test various hypotheses about changes in duck nest success and the local predator community after predator removal. In theory, any predator removal must result in less prey taken, therefore, both compensatory predation and/or re-colonisation may take place if the removal of a predator species does not increase duck nest success and decrease subsequent nest depredation rates by the removed predator species. We assessed potential re-colonisation effects by using predator removal data only from the target area (duck breeding area where nest success data were obtained) and from the entire predator removal area, which included also the surroundings of the target area. If predator removal from only the entire

predator removal area increases duck nest success in the target area, an inference about presence of re-colonisation can be made because predator removal only in target area is apparently insufficient due to the arrival of new predators from the surroundings.

Compensatory predation was evaluated by testing all possible pairwise relationships between removal of a predator species and the subsequent proportion of nests depredated by other predator species. A species was identified as a compensatory predator if it's proportion of depredated nests increased after removal of an other predator species and when removal of the latter was not correlated with duck nest success.

Materials and methods

Study area

The study was carried out on Lake Engure, Latvia (57° 15' N, 23° 07' E), a shallow eutrophic wetland encompassing 3 500 ha. About 40 % of the lake is covered by emergent vegetation, mainly common reed *Phragmites australis*, narrow-leaved cattail *Typha angustifolia* and bulrush *Scirpus* spp. A detailed description of vegetation in the study area was given by Auniņš et al. (2000). The lake hosts internationally important numbers of breeding waterfowl and is a part of a larger Ramsar Site (Vīksne 2000).

We used predator removal and duck nesting data from 1985 to 1997, which were collected as a part of a long-term duck population study (Blums et al. 1996). Predator control has been ongoing in the area since the 1960s but we did not analyse earlier data because of incomplete nest records and because significant transformations of duck nesting habitat took place between 1981 and 1984. Control of avian predators was terminated in 1998, primarily because one of the target species, marsh harrier, is a protected species in the European Union (European Commission 1992) and Latvia aimed to join the Union in the nearest future.

Duck nest data were obtained from the island archipelago and surrounding reedbeds (30 ha, hereafter referred to as the target area) in the central part of Lake Engure. We used two sets of predator removal data: predators removed only from the target area (30 ha, see above) and predators removed from the entire predator removal area (ca 1 600 ha, including large spaces of open water). The target area was situated nearly in the centre of the entire predator removal area.

Duck nest data

Mallard *Anas platyrhynchos*, northern shoveler *Anas clypeata*, garganey *Anas querquedula*, tufted duck *Aythya fuligula* and common pochard *Aythya ferina* were the common breeding duck species in the lake. Gadwall *Anas strepera*, European wigeon *Anas penelope* and ferruginous duck *Aythya nyroca* were present in much smaller numbers and were excluded from the analyses when duck species were studied individually.

The general field procedures were described by Blums et al. (1996). Annually, duck nests were located during two to three complete nest censuses. Nests were monitored weekly until hatching, depredation or abandonment. We obtained the following data for each nest: duck species, clutch initiation date, termination date, nest fate and, in case of depredation, the predator species responsible (see below). Clutch initiation date was estimated by back-dating the total number of eggs in the nest, and by determining the stage of incubation (Westerskov 1950). Many nest hatching dates and some depredation

dates were recorded directly, but otherwise, nests were arbitrarily assumed terminated at the middle of the interval between the date of recording termination and the previous visit (see Mayfield 1961 for a similar approach).

We identified predators based on detailed examination of eggshells (if present), nest material dislocation and signs found in the nest surroundings. This method may not be always reliable (Sargeant et al. 1998; Lariviére 1999) but there are three reasons why we believe that possible misinterpretations were reduced to a minimum. First, field workers were trained in distinguishing between nest predators. Experience was gained from offering duck eggs to captive predators and cases when different predators were disturbed during duck nest predation in field. Secondly, the three most common predators present in the study area leave significantly different cues at depredated duck nests, making their identification relatively easy (Opermanis et al. 2001). Thirdly, in unclear cases, we used the category of unknown predator.

Predator control

The diverse predator community at Lake Engure included mammalian and avian predators, but the predominant predators were relatively few species (Opermanis et al. 2001). Predator species found at least once responsible for depredation of duck nests or killing females were racoon dog *Nyctereutes procyonoides*, red fox, ermine, American mink, wild boar *Sus scrofa*, rats *Rattus* spp., goshawk *Accipiter gentilis*, marsh harrier, eagle owl *Bubo bubo*, common gull, herring gull *Larus argentatus*, raven *Corvus corax*, hooded crow *Corvus corone cornix* and magpie *Pica pica*.

The objective of the predator removal programme was to maintain high and stable duck nest success. Therefore predator control efforts we redirected on the predominant species causing substantial duck nest losses: marsh harrier (further referred to as harrier), corvids (raven, hooded crow, magpie) and American mink (further referred to as mink). Predators were removed by shooting, poisoning and live-trapping prior to and throughout the duck breeding season. All traps were live traps. Net traps baited with artificial duck nest were used for marsh harrier, the Scandinavian trap was used for corvids and tunnel traps for mink. At least daily visits reduced suffering of animals to a minimum. From 1985 to 1992, eggs collected from terminated gull nests and injected with α-chlorolose were offered to avian predators. There was no evidences that other species except target predators were affected, excluding 23 herring gulls and three common gulls. Live-trapped predators were dispatched, but 222 (26 %) marsh harriers were re-located to another coastal lake approximately 60 km SE from Lake Engure. At all times the removed predator species, date, and location were recorded.

Data analysis

We evaluated a predator removal programme rather than conducted a scientific experiment. In most earlier experiments, duck nest success in predator removal areas has been compared with control areas where no predator control was executed with the aim to test differences in nest survival between the areas (e.g. Duebbert, Kantrud 1974; Clark et al. 1995; Sargeant et al. 1995; Beauchamp et al. 1996.. As such a design was lacking in the current study, success of the described predator removal programme could not be tested. Alternatively, we evaluated the functional effectiveness of predator removal using long-term data from a single area. This was done by testing the relationships between

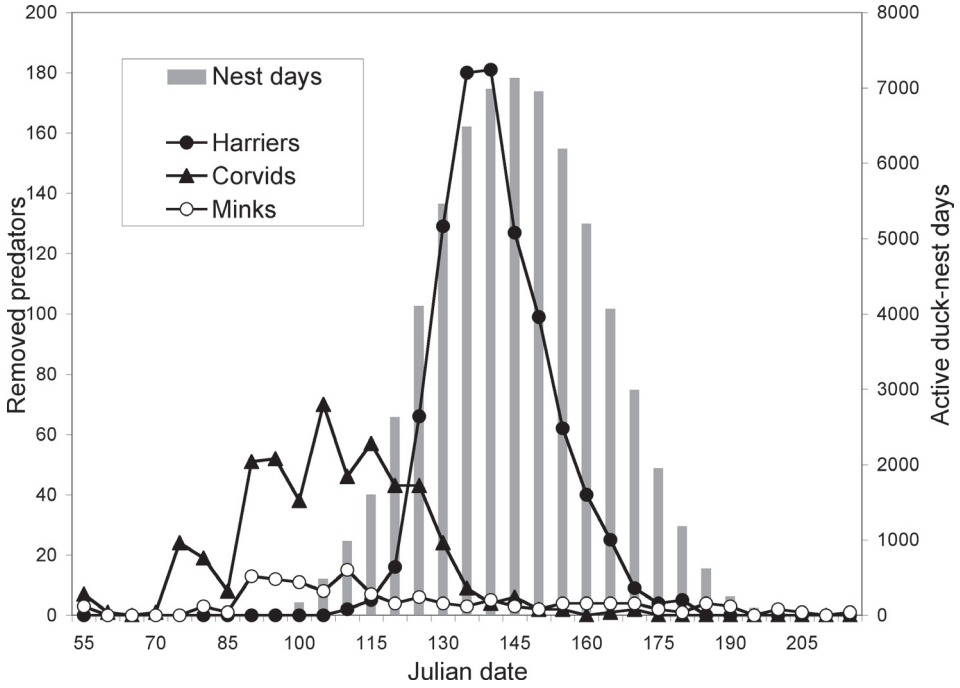

Fig. 1. Times of predator removal and period of duck breeding on Lake Engure, Latvia, from 1985 to 1997. Numbers of removed predators and active duck nest-days were calculated for 5-day intervals. Julian dates are shown on the x-axis.

number of predators removed and duck nest success using a set of breeding seasons. Such an evaluation could be biased, because on Lake Engure, like in many other studies (Balser et al. 1968; Duebbert, Lokemoen 1980; Parker 1984; Sargeant et al. 1995), predator removal was performed before and during the breeding season (Fig. 1). This implies that predators might have been removed after some or many of depredation cases, which were included in estimating apparent nest success for the particular breeding season.

To eliminate the above error, we used a modification of Mayfield's method (1961, 1975) based on nest exposure. The objective of predator removal was to achieve absence of predators in the target area. Predator absence was estimated as the number of days of active duck nests in the target area, which would be exposed to the removed predator's search if the removal did not occur (Fig. 2). Further we refer to this estimate as predator-free days (t). The sum of predator-free days across all individual predator removal cases in a particular breeding season provides a good measure of predator removal, because it accounts for both number of removed predators and prey availability.

We used three response variables to test the effects of t. To test the effectiveness of single predator species predator removal, we used the apparent yearly duck nest success. This estimate is more appropriate than Mayfield's nest success estimate in situations when all initiated nests are found and active nests have an equal probability of being found (Johnson, Shaffer 1990; Johnson 1991); both these conditions were met (Blums et al. 1996). To test the response of predator species after removing one individual, we calculated

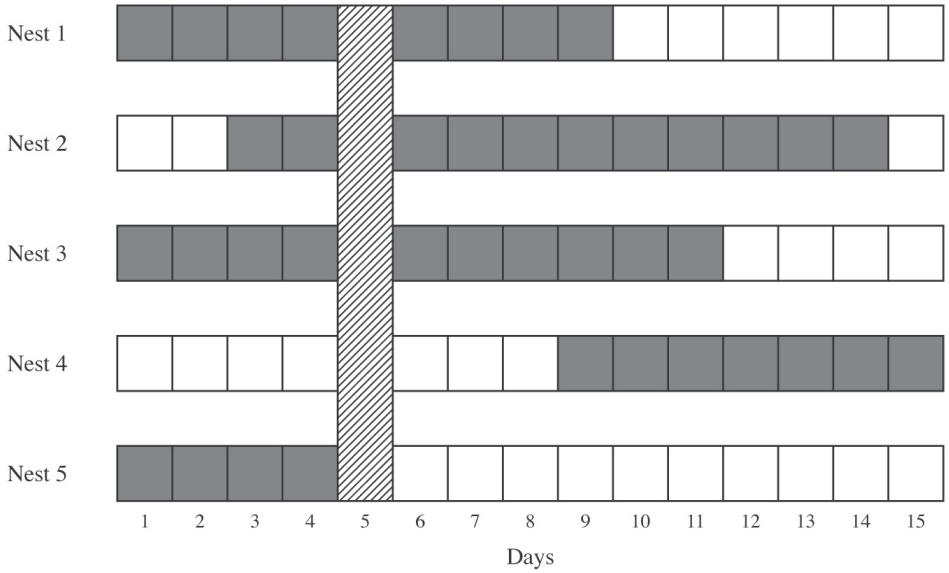

Fig. 2. Calculation of predator-free days (t) for an individual predator removal case. Striped column indicates predator removal day. Grey area indicates active nest days. This simulation assumes that there are only 5 nests in the area and the time span shown represents the whole breeding season. Consequently, t = 5 (nest 1) + 10 (nest 2) + 7 (nest 3) + 7 (nest 4) + 0 (nest 5) = 29 days. The day of predator removal was always included in the estimate of predator-free days.

predation rate (R) for the period subsequent to the removal as:

$$R_{predator} = n_{predator} / t \times 100,$$

where $n_{predator}$ is the number of depredation cases by a given predator species. To test for compensatory predation, we calculated proportion of nests depredated by a given predator species (P%) after removing one individual of another predator species as:

$$P\%_{predator} = n_{predator} / n_{all\ predators} \times 100.$$

Evaluation of single predator species removal effects is difficult when predators of several species are removed simultaneously. The effect of one predator's removal may be influenced by the effect of another's removal before, simultaneously, or later. Because of this uncertainty, we tested our assumptions using two different methods: (i) using yearly totals (t) and means (R and P%) and (ii) using data matrices based on individual predator removal cases when all years were pooled.

We used the bivariate Spearman Rank Correlation to test the degree of association between t and the response variables described above. One-tailed tests were used because it seemed logical that removed predators cannot further affect nest success, i. e. yearly duck nest success should increase, R of the same predator species should decrease and P% by other predators should increase.

We used Multivariate General Linear Model Contrasts (SPSS Inc. 1999) to test the effects of predator removal on subsequent changes in R and P%. Two models based on data matrices were fitted where individual predator removal was a case and predator species was predictor variable. Correspondingly, $R_{harrier}$, $R_{corvids}$, R_{mink} and $P\%_{harrier}$, $P\%_{corvids}$,

Table 1. Number of duck nests recorded and predators removed on Lake Engure, Latvia, 1985 - 1997. *All species, present in the target area only

Year	Duck nests (number)*	Predators removed (number)					
		Marsh harriers		Corvids		American minks	
		Target area	Entire removal area	Target area	Entire removal area	Target area	Entire removal area
1985	180	44	96	15	18	0	1
1986	187	45	109	10	10	1	5
1987	155	70	144	22	25	6	9
1988	175	51	104	36	53	2	7
1989	191	44	65	66	91	3	9
1990	210	54	63	58	81	9	16
1991	251	65	90	34	44	8	14
1992	295	53	74	25	42	4	12
1993	360	57	58	46	70	14	16
1994	318	26	26	24	24	8	9
1995	280	8	8	34	34	10	13
1996	200	64	64	16	16	1	3
1997	217	49	49	2	2	14	16

$P\%_{mink}$ were response variables. We used square-root and logarithmic transformations to normalise the above variables. Deviation Contrasts were used to test whether the mean $R_{predator}$ after removal of the same predator species was smaller than the grand mean $R_{predator}$, and whether the mean $P\%_{predator}$ after removal of other predator species was larger than the grand mean $P\%_{predator}$.

Results

Number of predators removed

During 13 years, 950 harriers, 510 corvids, and 130 minks were removed and 3 019 duck nests were monitored (Table 1). Harriers were removed during the duck nesting season, but corvids and minks mainly before and in the early duck nesting season (Fig. 1). Nevertheless, despite active predator control, on average 28.8 % of nests were depredated annually: 15.1 % by harriers, 5.0 % by corvids, 3.4 % by mink, 3.8 % by unknown predators, and 1.5 % by uncommon predators (see above). Including other reasons than prediation of nest failure than predation, the mean proportion of unsuccessful nests was 37.9 % (Table 2). Depredated nests by unknown and uncommon predators, abandoned and flooded nests were excluded from subsequent analyses.

Did predator removal affect duck nest success?

Removal of harriers and all predator species pooled were positively correlated with the apparent duck nest success (Table 3). Both relationships were significant only when predator from the entire predator removal area were used. Removal of harriers was also

Table 2. Number of nests and apparent nest success of five duck species in the Lake Engure study area, Latvia, from 1985 to 1997

Species	Number of nests	Nest success (%)		
		Mean ± SE	Min	Max
Mallard	1286	55.9 ± 4.1	33.3	80.8
Tufted duck	653	67.7 ± 3.0	46.0	83.6
Common pochard	499	60.3 ± 4.7	27.5	77.7
Northern shoveler	322	63.7 ± 4.6	29.6	89.1
Garganey	197	62.9 ± 5.7	44.4	94.7

correlated with nest success of tufted duck (target area $r_S = 0.74$, $P < 0.01$; entire removal area $r_S = 0.83$, $P < 0.01$) and mallard (entire removal area $r_S = 0.61$, $P < 0.05$). Removal of other predators was not correlated with overall duck nest success or nest success of any individual duck species.

Did predator removal affect subsequent predation rates?

Removal of harriers was negatively correlated with the subsequent harrier predation rate but this was not observed in corvids and mink (Table 3). The relationships were statistically significant using predator data from the entire predator removal area and marginally significant using predator data from the target area.

The mean harrier predation rate in the period subsequent to removing one harrier was significantly lower than the average harrier predation rate (grand mean). This was true both using harrier removal from the target area (Contrast estimate = - 0.13, SE = 0.04, P = 0.002) and the entire removal area (Contrast estimate = - 0.14, SE = 0.02, P < 0.001).

Effect of compensatory predation

We found a positive correlation suggesting compenastory predation only between mink removal and the subsequent proportion of duck nests depredated by harriers, which was statistically significant for mink removal data from the target area ($r_S = 0.62$, $P < 0.05$) and marginally significant using data from the entire removal area ($r_S = 0.49$, $P = 0.05$).

The mean proportion of duck nests depredated by harriers in the period subsequent to removing one mink was significantly greater than than the average proportion of nests depredated by harrier (grand mean). These differences were consistent in the target area (Contrast estimate = 0.36, SE = 0.14, P = 0.008) and in the entire predator removal area (Contrast estimate = 0.31, SE = 0.10, P = 0.003). The proportion of depredated nests did not significantly increased for the other predator species after removal of another species.

Discussion

The present study showed that only harrier removal was correlated with duck nest success, indicating that harrier removal at the existing level of intensity had a functional relationship with duck nest success. This was also found for all predator species pooled, but it was obvious that the number of all predators removed was influenced by the number of harriers removed (Table 1). Another reason explaining the effectiveness of harrier removal

Table 3. Correlations between predator removal intensity and duck nest success and between predator removal intensity and subsequent predation rates at Lake Engure, Latvia, from 1985 to 1997. Bivariate Spearman Rank Correlation coeffectives are shown. Significant correlations are indicated: * P = 0.05, ** P < 0.05

Variables	Predator	Removal location	
		Target area	Entire removal area
Sum of yearly predator-free days vs yearly duck nest success	Harrier	0.30	0.57**
	Corvids	0.04	0.16
	Mink	-0.33	-0.20
	All predators	0.12	0.48*
Sum of yearly predator-free days vs mean yearly predation rate	Harrier	-0.50**	-0.53**
	Corvids	0.00	-0.08
	Mink	-0.31	-0.26

was the clear predominancy of harriers on duck nests: they alone were responsible for ca 53 % of all duck nest depredations in the target area, while this percentage for corvids and minks was 17 % and 12 %, correspondingly. In addition, corvide appeared in the study area rather seasonally and restricted to the early breeding season. Most corvids, except magpies and some regularly foraging ravens, disappeared from the study area in early June with an increase of vegetation height and density. Thus corvids caused losses mainly to early nests (see also Opermanis et al. 2001). Thus, if ever duck nest success improved in the early season due to corvid removals, it would have had little effect on yearly nest success. Unlike corvids, harriers imposed severe hunting pressure on duck nests throughout the season. On Lake Engure the hourly probability that a harrier crossed the airspace from which a duck nest in the study area could be spotted was 0.69 (Opermanis 2001). The importance of harriers as duck nest predators was confirmed by the statistics from the years after the cessation of harrier control: the overall duck nest success dropped to 51.9 % in 1999, 23.0 % in 2000 and 32.1% in 2001 (Opermanis, upublished data).

Only harrier removal intensity was correlated with subsequent predation rates. Obviously, harrier removal was effective due to a large number of removed animals, which overscored the re-colonisation of the target area by new individuals. However, it was surprising that it was possible to remove on average 73 harriers per year. According to Schipper (1977), the mean marsh harrier hunting range size during breeding is 4.5 km^2 (range: 2.5 to 8.0 km^2). Assuming maximum density, there could be six to seven breeding pairs in the predator control area (16 km^2). The number of removed birds many times exceeded the possible number of resident birds, suggesting that Lake Engure holds a high density of non-breeding birds. In 1999, during the peak duck breeding season between 20 May and 18 June, 30 harriers were captured and released in the study area, but only two birds were recaptured during this period, suggesting that many birds apparently spent a relatively short time at the lake. The above observations show that harrier removal, even at the given intensity and scope, did not fully prevent the re-colonisation. However, in other locations, removing much fewer harriers might result in increased duck nest success.

The fact that harrier removal was correlated with duck nest success and subsequent predation rates only when predator data was used from the entire removal area (Table

3), supports the raised idea about intensive re-colonisation of target area by new harrier individuals as others were removed. Thus this study supports earlier recommendations that predator removal in areas adjacent to a target area should be conducted as well (e.g. Sargeant et al. 1995). This could be especially important when dealing with avian predators.

For corvids and minks, a correlation between removal and subsequent predation rate was not found, probably because of insufficient removal intensity, both in the target area and surroundings (Table 1), and due to lover relative importance of these species as duck nest predators. However, we found a positive relationship between removal of minks and the proportion of duck nests depredated by harrier. This suggests that the potential benefit arising from mink removal was lost due to compensatory predation by harrier. Apparently this compensatory predation did not much affect management results at Lake Engure where harriers were removed as well, but this may be the case in other waterfowl breeding areas where only mink removal is carried out.

The present study raised suggestions for future experimental work and for long-term predator removal programme. The objective of the first is to obtain clear answers on questions asked, while the objective of the second is to maintain or increase duck nest success on a long-term basis. In a thoroughly planned scientific experiment any outcome will provide important results, but for management programmes, a continuous decrease in nest success will be considered a failure.

An experiment can allow the sacrifice of a given amount of duck nests in favour of good scientific results. This is normally acceptable, because experiments are relatively short, i.e., one to five years (Duebbert, Kantrud 1974; Duebbert, Lokemoen 1980; Parker 1984; Parr 1993; Clark et al. 1995; Jackson 2001). To continue studies on compensatory predation, an ideal experimental study should exclude one predator species from a study area while the other predator species are left undisturbed; this arrangement would show if the removal (absence) of this single predator species increases duck nest success. If this is not done, the re-colonisation ability of the species removed and potential compensatory predators could be evaluated as suggested above. Other predator species can be removed during later seasons.

Typically, predator management programmes, which should be based on findings from experimental studies (Macnab 1983; Clark, Nudds 1991), are operated longer than experiments. Unless there is a good reason to believe that duck nests suffer from a single predominant predator species, the entire predator complex should be treated to avoid compensatory predation (Balser et al. 1968). Unfortunately, data from such programmes may not be well-suited for stringent statistical analyses because of simultaneous predator removal and the overlapping of removal effects. For large management programmes it is problematic to establishsufficiently large control areas either because such are not available or because it would at least double monitoring costs. Nevertheless, there is a clear need to evaluate the effectiveness of such management programmes, of which recording of predator removal and duck nesting data must be integral parts. Unfortunately, scientific experiments are carried out by professional biologists, but many management programmes are implemented by site managers with only a general knowledge in animal ecology. Predator management is usually only one of a wide range of activities listed in site management plans, thus often responsible managers lack time, money or experience to care about proper monitoring of management success. The experience problem can

be eliminated through transfer of knowledge from scientists to broader environmental managers. To facilitate data collection and adjust the level of complexity, simpler schemes could be planned; e.g., instead of asking managers to identify duck nest predators from nest remains, which is complicated, they could record predator activity through direct (avian predators) and indirect (mammalian predators) observations (e.g. Johnson et al. 1989).

In the present study we lacked data on the numerical response of predator species to removal efforts; we analysed only the functional response of predator removal in terms of duck nest success and predation rates. In future programmes, we recommend recording the number of predators removed, number of predators observed, and duck nest success, which should provide a sufficient basis for assessment of management effectiveness, including the presence of potential compensatory predation and re-colonisation effects.

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Kompensējošās plēsonības un plēsēju rekolonizācijas novērtējums izmantojot ilglaicīgus pīļu ligzdu postītāju skaita regulācijas datus

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Kopsavilkums

Plēsēju skaita samazināšana bieži neuzlabo pīļu ligzdošanas sekmes. Visbiežāk to izskaidro ar citu plēsēju sugu kompensējošo plēsonību vai arī ar teritorijas ātru rekolonizāciju ar tās pašas sugas īpatņiem. Mēs izmantojām 13 gadu datus par niedru lijas *Circus aeruginosus*, vārnveidīgo putnu *Corvidae* un Amerikas ūdeles *Mustela vison* skaita samazināšanu Engures ezerā, lai pārbaudītu (1) vai katras atsevišķās plēsēju sugas īpatņu skaita samazināšana ietekmēja pīļu ligzdošanas sekmes; (2) vai katras atsevišķās plēsēju sugas īpatņu skaita samazināšana izraisīja tās pašas sugas postījumu biežuma samazināšanos; (3) vai vienas plēsēju sugas īpatņu skaita samazināšana izraisīja citas plēsēju sugas postījumu biežuma palielināšanos. Pavisam 1590 plēsējus likvidēja vai arī transportēja prom no ezera un izsekoja 3019 pīļu ligzdu likteņiem. Plēsēju skaita samazināšanu mērīja, ieviešot parametru "no plēsējiem brīvās dienas". To izteica kā to dienu skaitu, kuru laikā pīļu ligzdas pētījumu rajonā būtu plēsēja barības meklējumu padraudētas, ja vien šis plēsējs nebūtu likvidēts. Plēsējus ķēra gan pētījumu rajonā, kur tika novērotas pīļu ligzdas, gan arī šī rajona apkārtnē. Niedru lijas skaita samazināšana pozitīvi korelēja ar pīļu ligzdošanas sekmēm ($P < 0,05$), bet negatīvi ar turpmāko niedru lijas postījumu biežumu ($P < 0,05$). Tomēr šī likumsakarība bija spēkā tikai iekļaujot likvidēto niedru liju skaitu gan no pīļu ligzdošanas vietām, gan arī no to apkārtnes. Tas lika domāt, ka pīļu ligzdošanas sekmes ietekmēja arī niedru liju rekolonizācija no plašākas apkārtnes. Vārnveidīgo putnu un Amerikas ūdeles skaita samazināšana nekorelēja ne ar pīļu ligzdošanas sekmēm, ne ar to pašu sugu turpmāko postījumu biežumu. Amerikas ūdeles skaita samazināšanas intensitāte pozitīvi korelēja ar niedru lijas izpostīto ligzdu proporciju ($P < 0,05$). Tas nozīmē, ka niedru lija varēja veikt kompensējošo postīšanu pēc Amerikas ūdeles skaita samazināšanas. Kompensējošā plēsonība un rekolonizācija pēc kādas plēsēju sugas skaita regulēšanas var notikt daudzās plēsēju sabiedrībās, tādējādi apsaimniekošanai patērētās pūles un ieguldītie līdzekļi var nedot cerēto rezultātu. Mēs esam ieteikuši veidus, kā izvērtēt bijušās un notiekošās plēsēju skaita regulācijas problēmas, kā arī to, kā plānot nākotnes projektus, lai iepriekš minētās problēmas tiktu diagnosticētas savlaicīgi.

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Effect of environmental factors on the propagation of deciduous azalea by cuttings. I. Influence of stock plant management on rooting and carbohydrate status

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Abstract

The aim of this study was to estimate the effect of stock-plant forcing in a greenhouse during spring as a management technique for stock plants on rooting and on changes of carbohydrate contents of cuttings from deciduous azalea cultivar 'Polārzvaigzne' during the rooting period. Forcing of the stock plants enabled to start propagation of deciduous azalea by cuttings one month earlier. Conditions in the greenhouse promoted accumulation of carbohydrates in the shoots of forced stock plants compared to control plants. However, the stock-plant forcing affected neither the number of rooted cuttings nor the duration of time necessary for maximum rooting. The patterns of carbohydrate changes were similar in forced and control cuttings. The amount of reducing sugars, total soluble sugars, and starch increased significantly in the cuttings after severing, while the level of sucrose generally fluctuated over time. After root emergence the content of reducing sugars, sucrose, and starch decreased initially in the stem base and later in the leaves of cuttings. The content of starch was better related to the rooting process than the content of total soluble sugars.

Key words: adventitious root formation, *Rhododendron*, soluble sugars, starch, stock plant forcing, vegetative propagation.

Introduction

Rhododendron L. is one of the most popular ornamental woody plant genera planted in parks and gardens. Despite the opportunities offered by new micropropagation techniques, cuttings are still widely used to propagate deciduous azalea cultivars. However, there are several problems with cuttings related to both the rooting process and their survival.

Management of stock plants is often used to improve the rooting potential of difficult-to-root species. Some of the most widely-used methods include reducing of irradiance or etiolation, modifying mineral nutrition, winter pruning, as well as combination of different methods (Howard 1994; Hartmann et al. 2002). Reduced irradiance results in some anatomical changes such as decreased lignification and reduced cell differentiation; thereby increasing initiation of root primordia (Ernsten, Hansen 1986; Bassuk, Maynard 1987; Maynard, Bassuk 1991; Hansen, Potter 1997; Voltolini, Fachinello 1997). Fertilisation of stock plants promotes the supply of cuttings with mineral elements, improving adventitious rooting, but often resulting in decline of survival (Henry et al. 1992; Rowe et al. 1999; Spanos et al. 1999). Stock plant pruning can help to maintain high rooting

potential due to promotion of juvenile-like growth of plants (Cameron et al. 2001). Also, the growing of plants in a greenhouse conditions is included in pre-treatment of the stock plants. The forcing of stock plants at higher-than-normal temperatures and humidity not only promotes shoots development and allows to start propagation by softwood cuttings earlier, but also affects the morphology and physiology of cuttings, promoting rooting and further development of cuttings (Samostchenkov 1985; Polikarpova, Pilugina 1991). However, generally the benefit from each treatment is genotype dependent.

Some studies have focused on changes of carbohydrate concentrations during rooting of tree cuttings (Welander 1994; Murai et al. 1999; Pellicer et al. 2000), due to their importance as a source of energy and building blocks of macromolecules during the root development (Dey, Harborne 1997; Heldt 2005). Woody plant species differ in their requirements for propagation conditions, in relation to the rooting process and physiological processes including carbohydrate metabolism (Haissig 1990; Welander 1994; Hartmann et al. 2002; Rowe et al. 1999). The management of stock plants can influence the carbohydrate status of cuttings as well (Rowe et al. 1999; Hoad, Leakey 1996). Hartmann et al. (2002) described the general characteristics of changes in carbohydrate levels during the root formation, as follows. Starch hydrolysis during rooting releases soluble sugars, which can be used for root formation. The bases of cuttings acts as sinks attracting assimilates. Application of auxins for stem cuttings enhances both adventitious root formation and the movement of assimilates towards the auxin source. Although carbohydrates are essential for root formation of cuttings, they are not the cause of rooting, nor do they initiate it. However no extensive research on the carbohydrate status in deciduous azaleas during rhizogenesis has been conducted.

The objective of this study was to estimate the influence of stock-plant forcing on adventitious rooting and on the carbohydrate status of deciduous azaleas cuttings. Cuttings from the cultivar 'Polārzvaigzne' were evaluated for rooting and analyzed for concentrations of soluble carbohydrates and starch in the leaves and the stem bases of cuttings during the rooting period.

Materials and methods

As stock plants 7-year-old bushes of deciduous azalea cultivar 'Polārzvaigzne' (♀ *Rhododendron* × *kosterianum* C.K. Schneid. × ♂ *Rh. roseum* Rehd.) propagated by cuttings were used.

At the end of February 1999 thirty stock plants were potted individually in 40 l plastic pots with a medium consisting of equal volumes of peat and semi-decomposed pine needles and were placed into a greenhouse for forcing, without additional heating and light. As a control stock plants (n = 30) maintained outdoors in the bed were used. Forced cuttings (FC) from the forced stock plants were collected on 28 May 1999, while control cuttings (CC) from the bed stock plants were collected on 29 June 1999.

Softwood cuttings were trimmed 6 to 8 cm in length. Five or six leaves were left on each cutting. The leaves were trimmed to approximately one-third of the original leaf area. The base of each cutting was wounded by removing a 1.0 to1.5 cm strip (in length) of bark on one side of the stem and then treated with 0.5 % (w/w) indole-3-butyric acid powder in talc. The cuttings were inserted in a plastic flat (30 × 60 × 10 cm) filled with a medium consisting of equal volumes of peat and semi-decomposed pine needles. A total of 1700

cuttings for each variant were planted in 34 flats (50 cuttings per a flat). The cuttings were placed under a polyethylene tent in a non-mist semi-shadow greenhouse (40 to 90 μmol m^{-2} s^{-1}) under natural photoperiod with temperatures of 23 ± 4 °C (day) and 15 ± 4 °C (night).

Cuttings were sampled for measurement at 3 or 4-day intervals within 14 weeks. Samples were taken in three replications between 10:00 and 11:00 hours. At each sampling, five cuttings were randomly selected. The leaves and the basal 2.0 cm segment of the stem were separately removed and used for analysis. The cuttings were considered as rooted if there was at least one root ≥ 5 mm long. Rooting percentage was calculated, as a proportion of all cuttings in the sample. The samples were rinsed with water, fixed in water vapour and dried at 60 °C for 48 h. Dry leaves were milled by laboratory-mill and the stems were scrupulously cut in approximately 0.4 mm thin slices and bruised in the pestle together with granulated glasses to powdery consistence. The concentrations of reducing sugars, sucrose and starch were determined.

For carbohydrate analysis 0.25 g of leaf tissue and 0.10 g of stem tissue (dry mass) was used. To determine the concentrations of reducing sugars (glucose and fructose) and sucrose, dry plant material was ground with 10 ml 96 % (v/v) ethanol and 50 ml hot water was added. The extraction was performed in a water bath at 75 °C for 45 min. After the extraction, the proteins, lipids and tannins were precipitated by alkaline 2 ml $Pb(CH_3COO)_2$ for 1 h in warm solution. To precipitate the remaining lead (Pb) ions, an equivalent amount of saturated Na_2SO4 was added. Then the solution was brought to 100 ml with distilled water and filtered. In the extracts the amount of reducing sugars was measured using the copper-iodometric titration method (Shaffer, Somogyi 1933; Strong, Koch 1974). For determination of sucrose, the total amount of sugars was measured first. Three ml of 8 % (w/v) oxalic acid was added to 25 ml filtrate. The solution was placed in a water bath at 100 °C for 10 min to induce sucrose hydrolysis. After cooling, 1 N NaOH was used for neutralization and the solution was brought to 50 ml with distilled water. Then the total amount of sugars was determined as previously described for reducing sugars. The content of sucrose was calculated by subtracting the reducing sugar content from the total sugar content and multiplying the result by 0.95. To determine starch content, dry plant material was ground with 10 ml 80 % (w/v) $Ca(NO_3)_2$ solution and boiled for 3 to 5 min to pass starch into the colloidal solution. The amount of starch was determined by the Berthram method of bichromate-sodium thiosulfate titration (Strong, Koch 1974). Carbohydrate concentration was expressed as a percentage of dry mass attributable to sucrose, reducing sugars and starch.

Statistical analyses were performed using SPSS 11.0 for Windows.

Results

Forcing of the stock plants by planting them into a greenhouse enabled to start propagation of deciduous azalea by cuttings one month earlier – the control cuttings were collected for rooting on 29 June, and the cuttings from forced stock plants were already collected on 28 May.

There was no statistically significant effect of stock-plant forcing on rooting of cuttings (Fig.1). While the increase of rooting percentage tended to be slightly more pronounced in the forced cuttings, the first rooted cuttings in both variants were established only on

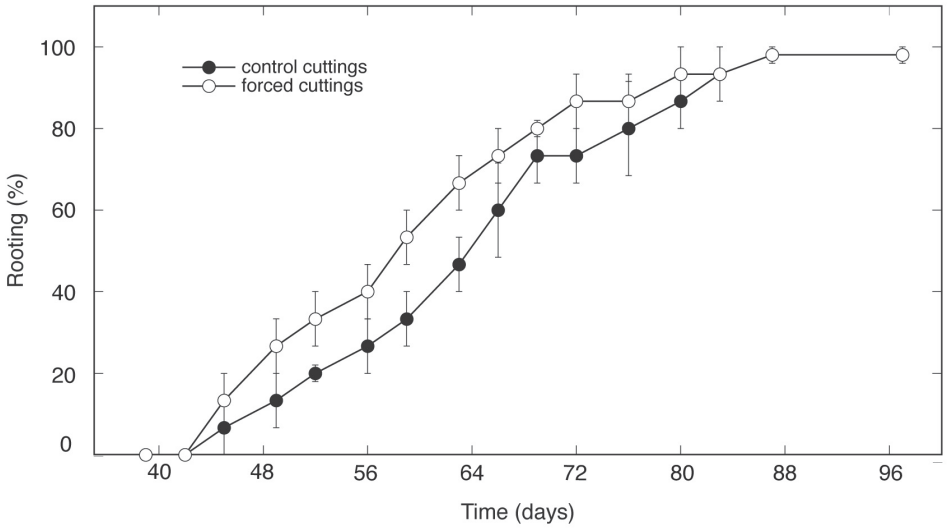

Fig. 1. Effect of stock plant forcing on rooting percentage of deciduous azalea cultivar 'Polārzvaigzne'. Deciduous azalea cultivar cuttings from outdoor-raised stock plants (control cuttings) were collected on 29 June 1999, and cuttings from greenhouse-forced stock plants (forced cuttings) were collected on 28 May 1999. Mean values ± SE are shown, n = 3. Root emergence was observed visually.

day 45. Also, maximum rooting (98 %) for forced and control cuttings was achieved nearly simultaneously (day 87). Thus, the stock-plant forcing affected neither the number of rooted cuttings nor the duration of time necessary for maximum rooting.

The different environmental conditions used for cultivation of stock plants influenced the amount of sugars and starch in shoots. Before day 0, the leaves of cuttings from forced stock plants contained more sugars than cuttings from control plants (Fig. 2 and 3). However, in the stem bases of forced cuttings all carbohydrates had higher amounts than in control cuttings.

Also, during the next three days the changes in contents of carbohydrates differed between the variants in the leaves of cuttings. The levels of all carbohydrates increased in the leaves of control cuttings, while in forced cuttings only the content of reducing sugars increased, but the level of sucrose and starch declined. Subsequently, until two weeks after propagation the starch level decreased in cuttings of both variants, while the amount of reducing sugars continued to grow.

In total, during the following course of rooting the trends of changes in carbohydrate amounts were similar in the leaves and the stem base of both cutting variants. During the first half of the rooting period the amount of starch increased in both stems and leaves of azalea cuttings, followed by a decrease during the second half (Fig. 2A and 3A). The level of sugars continuously decreased after the initial stage of rooting in the stems of cuttings, reaching extremely low levels at the end of the experiment (Fig. 2A).

In general, both the amount of starch and total soluble sugars in the stems during rooting were higher in azalea cuttings taken from forced stock plants in comparison with control cuttings (Fig. 2A). The differences in soluble sugar content were due to both reducing sugars and sucrose (Fig. 2B).

In contrast, in the leaves of cuttings, the effect of stock-plant forcing was less pronounced (Fig. 3). During the first half of the rooting period not only the amount of starch, but also the level of reducing sugars and total soluble sugars, significantly increased in the leaves of azalea cuttings. During the period of intensive root formation (since the day when the first root emerged) the levels of all soluble sugars decreased. However, in leaves the decrease of sugars was considerably delayed and less dramatic than in the stem base of cuttings. In the leaves the decline of starch content started only at the end of experiment when maximal rooting was achieved.

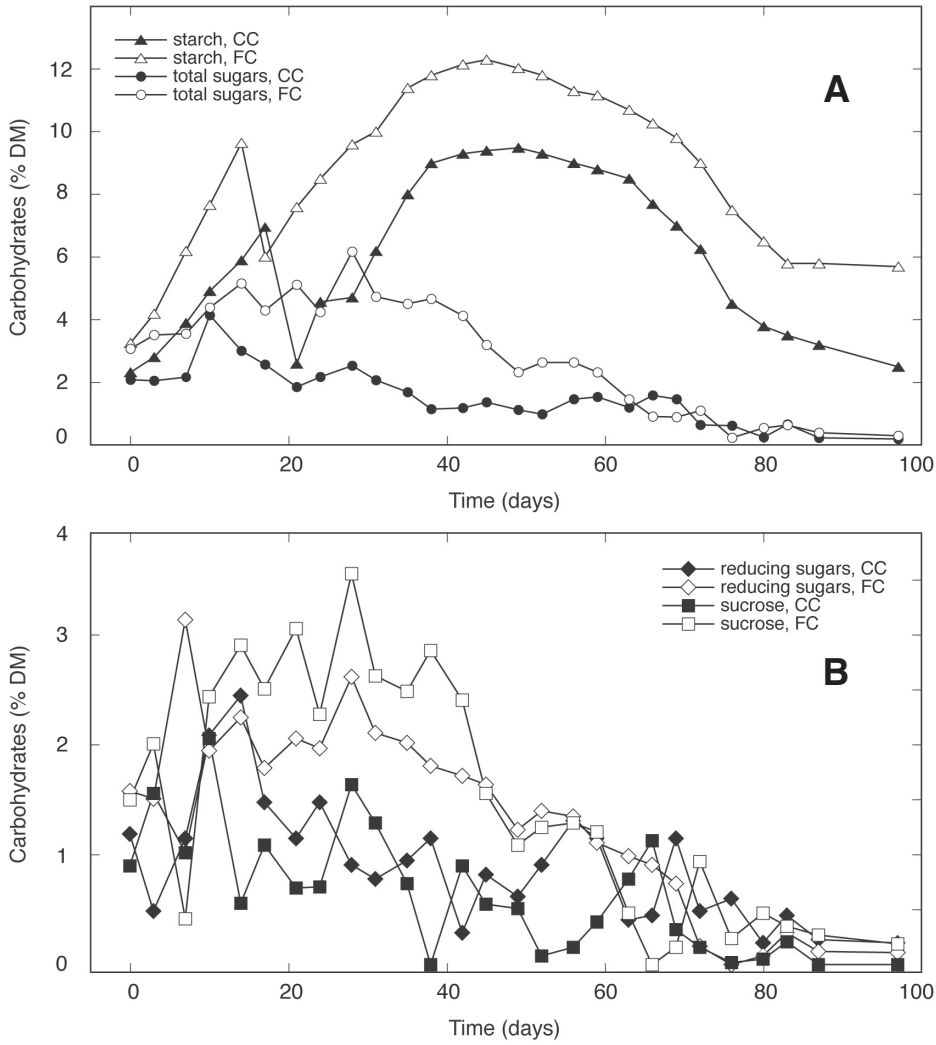

Fig. 2. Changes of sugar and starch amounts in the 2-cm stem base of azalea cuttings during rooting. A, starch and total soluble sugars; B, reducing sugars and sucrose. Deciduous azalea cultivar 'Polārzvaigzne'. cuttings from outdoor-raised stock plants (CC) were collected on 29 June 1999, and cuttings from greenhouse-forced stock plants (FC) were collected on 28 May 1999. Mean values are shown, n = 3.

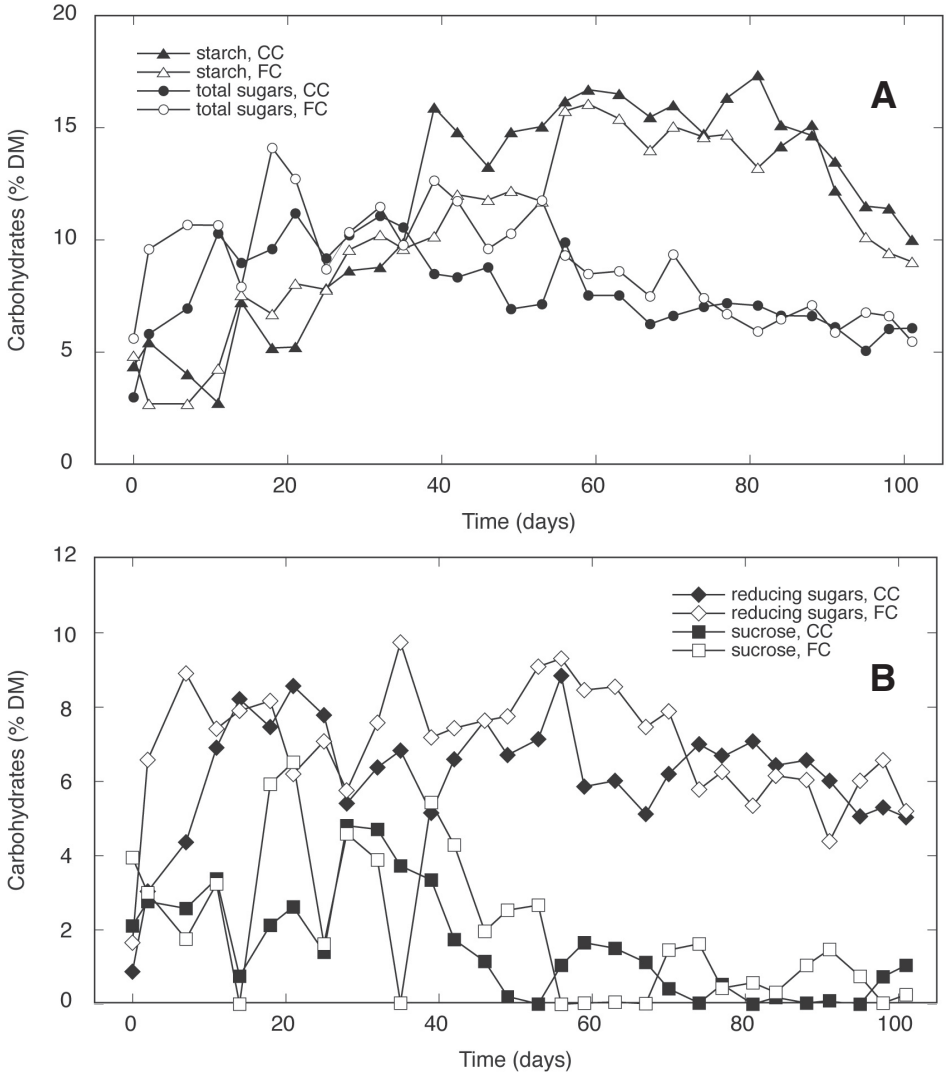

Fig. 3. Changes of sugar and starch amounts in the leaves of azalea cuttings during the rooting period. A, starch and total soluble sugars; B, reducing sugars and sucrose. Deciduous azalea cultivar 'Polārzvaigzne'. cuttings from outdoor-raised stock plants (CC) were collected on 29 June 1999, and cuttings from the greenhouse-forced stock plants (FC) collected on 28 May 1999. Mean values are shown, n = 3.

Discussion

In the present study, stock-plant forcing enabled to start propagation by cuttings one month earlier. Similar results have been obtained with pear cultivars, apple rootstocks and others cultures (Tarasenko, Omeltchuk 1985; Polikarpova, Pilugina 1991). However, in a study with apple rootstocks, root formation in cuttings from forced stock plants

occurred within 14 days, while the cuttings of control variants rooted in 26 to 36 days. Furthermore, the forced cuttings often showed a higher percent of rooting than control cuttings (Polikarpova 1990). The results of the present study did not consistent with these results (Fig. 1). These differences can be explained by the fact that in the conditions of the present experiments, maximum rooting was achieved even with control plants. In previous studies, stock-plant forcing was observed to have a greater positive effect on the rooting of difficult-to-root plants (Polikarpova, Pilugina 1991).

Generally, the patterns of carbohydrate changes widely vary among the cuttings of different species. Similar carbohydrate levels as in the present study were found for leafy cuttings of *Rhododendron catawbiense* 'Roseum Elegans' (Davis, Potter 1987): in the base of cuttings both the starch and the sucrose amount increased much after excision and later declined during root development, but the glucose level fluctuated variably. Also, in experiments with red maple (*Acer rubrum* 'Red Sunset'; Smalley et al. 1991) the total soluble carbohydrate level rose, after an initial decrease, till the start of rooting and decreased afterwards, but, in this case, changes in the starch concentration did not demonstrate an evident pattern. In contrast, the level of reducing sugars in *Betula pubescens* cuttings was observed to remain nearly constant during rooting, although the sucrose amount increased by several times (Welander 1994).

In the present study, the differences in environmental conditions used for stock plant management significantly affected the initial levels of sugars and starch in azalea cuttings (Fig. 2, 3). Higher initial levels of carbohydrates in forced stock plants might reflect better conditions for photosynthesis in the greenhouse. It has been shown that leaves of forced shoots have several anatomical changes that positively affect photosynthesis (Polikarpova 1990). In plant leaves starch is deposited in the form of transitory starch, which is degraded during the following night (Heldt 2005). Since the samples in the present experiment were collected in the morning the observed level of starch in the leaves most probably reflects changes in the basal content of starch. It is possible that leaves of the forced stock plants accumulated more starch during a day than leaves of the control plants.

After detachment the shoots experience water stress, which may cause stomatal closure and subsequent reduction of photosynthesis (Smalley et al. 1991). This may explain the decline of starch concentration in the leaves during the first two weeks of rooting (Fig. 3A). The effect of stock-plant forcing on changes in carbohydrate levels during the first days of the experiment could be related to differences in the anatomical structure of shoots of forced stock plants. Growth under greenhouse conditions leads to a raised density of palisade parenchyma, volume of intracellular space as well as the number of stomata (Polikarpova 1990). These changes may result in greater sensitivity of forced cuttings to water stress and a subsequent decline of the starch amount, as well as an increase of the reducing sugar amount in leaves of these cuttings. In orange trees, it was observed that during water stress, the content of starch and sucrose decreased in leaves, while the content of reducing sugars slightly increased (Vu, Yelenosky 1989). The changes of carbohydrate levels in the leaves of forced cuttings found in the present study conform to these results.

After severing, cuttings produce wound periderm and callus, as well as form root initials and root primordia (Hartmann et al. 2002). These processes may help to explain the intensive utilization of starch in the stem base of cuttings from both variants during the third week of propagation. However, as the rooting of rhododendrons is a long process (Kondratovics, Megre 1999; Hartmann et al. 2002) an increase in the starch concentration

continues and a high level of reducing sugars remaines in both leaves and the stem base of the cuttings (Fig. 2 and 3). The high accumulation of starch, particularly in the base of cuttings, suggests that the early stages of adventitious root development do not request large energy resource. The results indicated that, during the subsequent stage of root development, root formation was supported by starch accumulated in the basal part of the stem at first, and the starch pool in the leaves is used for further root development. Growing roots act as a sink for assimilates that are utilised very rapidly there. This can explain why all of the determined carbohydrate levels declined during intensive root development.

Newly synthesized carbohydrates are generally very important for root growth and development of leafy cuttings (Hoad, Leakey 1996; Pellicer et al. 2000). An optimal photosynthesis process is essential for woody plant cuttings with a long rooting period, to compensate the used carbohydrate store during the rhizogenesis period (Aminah et al. 1997; Pellicer et al. 2000). The results of our study also confirm these conclusions and suggest that the concentration of starch in cuttings could be used as an indicator of the rooting process.

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Vides faktoru ietekme uz vasarzaļo rododendru pavairošanu ar spraudeņiem. I. Mātesaugu apstrādes ietekme uz apsakņošanos un ogļhidrātu saturu

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Kopsavilkums

Pētījuma mērķis bija novērtēt vasarzaļo rododendru mātes augu pavasara steidzināšanas siltumnīcā ietekmi uz spraudeņu apsakņošanos un ogļhidrātu satura izmaiņām tajos apsakņošanās laikā. Mātes augu steidzināšana deva iespēju uzsākt pavairošanu mēnesi agrāk, nekā izmantojot kontroles augus. Attīstībai labvēlīgie apstākļi siltumnīcā veicināja ogļhidrātu uzkrāšanos steidzināto mātes augu dzinumos. Tomēr mātes augu steidzināšana būtiski neietekmēja ne apsakņoto spraudeņu daudzumu, ne arī apsakņošanās procesam nepieciešamo laiku. Gan steidzinātajiem, gan kontroles spraudeņiem ogļhidrātu satura izmaiņas apsakņošanās gaitā bija visumā līdzīgas. Spraudeņos būtiski pieauga reducējošo cukuru, kopējais šķīstošo cukuru un cietes daudzums, bet saharozes saturs bija salīdzinoši svārstīgs. Reducējošo cukuru, saharozes un cietes daudzuma samazināšanos spraudeņiem novēroja tikai pēc adventīvo sakņu parādīšanās – vispirms spraudeņu pamatnē, bet vēlāk arī to lapās. Cietes satura izmaiņas spraudeņos raksturoja spraudeņu apsakņošanās procesu labāk nekā kopējā šķīstošo cukuru daudzuma izmaiņas tajos.

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Effect of environmental factors on the propagation of deciduous azalea by cuttings. II. Influence of an extended growth period on bud-break, overwinter survival and carbohydrate levels of rooted cuttings

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Abstract

The influence of stock-plant forcing in a greenhouse during spring, as well as an expanded photoperiod and ground heating during autumn and winter, on post-rooting growth and overwinter survival of cuttings was tested using cuttings from the deciduous azalea cultivar 'Polārzvaigzne'. The stock-plant forcing and extended lighting during the autumn supported the development of new shoots in the post-rooting period. During autumn, all starch stored in the leaves of cuttings was transported to perennial parts of plant. During winter, the use of starch depended on temperature. A high temperature (10 °C) promoted depletion of starch in the stem of cuttings if it could not be replenished. Additional light and heating promoted the retention of leaves and the photosynthesis ability of cuttings. Overwinter survival was affected by environmental conditions during winter mainly, while stock-plant forcing did not significantly influence survival. An extended light period together with additional heating in winter improved overwinter survival of cuttings, but delayed their further growth in spring..

Key words: carbohydrate, extended photoperiod, overwinter survival, *Rhododendron*, stock-plant forcing, vegetative propagation.

Introduction

One of the problems in practical vegetative propagation of woody plants is the loss of even successfully rooted cuttings during the first winter or spring. This is also true for many of the splendid cultivars of deciduous azaleas (Berg, Heft 1991). These problems also occur in other woody plant genera (*Acer* L., *Betula* L., *Cornus* L., *Corylopsis* L., *Hamamelis* L., *Magnolia* L., *Stewartia* L., *Viburnum* L.) propagated by cuttings (Smalley et al. 1987).

Poor overwinter survival may be caused by an insufficient level of stored carbohydrates in cuttings (Perkins, Bassuk 1995; Hartmann et al. 2002). Carbohydrate reserves depend on the initial level of carbohydrates at severing of cuttings, carbohydrate depletion or accumulation during rooting as well as on the ability to replenish them during the post-rooting period (Perkins, Bassuk 1995; Hoad, Leakey 1996). Hence, the post-rooting growth and development of new shoots during the current season is very important, because leaves of new shoots promote accumulation of photosynthates (Smalley et al. 1987). However,

growth after rooting during the current season is not typical for rhododendrons.

To promote post-rooting growth, different methods including management of stock plants and various treatments of cuttings can be used. For example, light exclusion as pre-treatment of stock plants can affect bud break and subsequent growth. Stem banding (localized light exclusion) usually promotes growth effectively, but the influence of whole plant etiolation may be variable (Maynard et al. 1990; Sun, Bassuk 1991a; Sun, Bassuk 1991b; Blakesley et al. 1992). Also, stock-plant forcing as pre-treatment of stock plants can improve post-rooting growth. Stock-plant forcing in the greenhouse during spring promotes shoot development and allows to start propagation by cuttings earlier, leading to a head-start in cutting advancement and ensuring additional time for establishment of rooted cuttings (Samostchenkov 1985; Polikarpova, Pilugina 1991). Moser (1991) noted that this method can be used for deciduous azaleas. Treatments that may promote shoot growth of cuttings after rooting include foliar spray with plant growth regulators (gibberellins, cytokinins; McConnel, Herman 1980; Ernsten, Hansen 1986; Maynard et al. 1990), leaf defoliation (English 1981), provision with high nutrition levels (Johnson, Hamilton 1977, Rieckerman et al. 1999), warm temperatures (Dixon 1980), and long day or night interruption (Smalley et al. 1987; Rieckerman et al. 1999). In addition, auxins, which are used widely to favour adventitious root formation, prevent bud break and new shoot growth (Maynard et al. 1990; Sun, Bassuk 1991a). Berg and Heft (1991) observed that an extended growth season with an extended photoperiod and additional heating in a greenhouse during autumn can improve development of rooted cuttings as well as overwinter survival of azalea cuttings, probably due to improved carbohydrate supply.

The objective of this study was to examine the effects of stock plant forcing, together with extended light and additional heating in autumn and winter, on post-rooting growth and overwinter survival of rooted cuttings. The levels of sugars and starch in the cuttings were measured due to their importance as energy reserves.

Materials and methods

As stock plants, 7-year-old bushes of deciduous azalea cultivar 'Polārzvaigzne' (♀ *Rhododendron* × *kosterianum* C.K. Schneid. × ♂ *Rh. roseum* Rehd.) propagated by cuttings were used. The stock plants were grown as described previously (Apine, Kondratovičs 2005). Forced cuttings from forced stock plants were collected on 28 May 1999, while control cuttings from bed stock plants were collected on 29 June 1999. The cuttings were prepared and propagated as described previously (Apine, Kondratovičs 2005).

The cuttings were placed under a polyethylene tent in a non-mist semi-shadow greenhouse (40 to 90 μmol m^{-2} s^{-1}) under a natural photoperiod with temperatures of 23 ± 4 °C (day) and 15 ± 4 °C (night). After 6 August 1999, when the day length became shorter than 16 h, an extended light period was provided to maintain a 16-h photoperiod for half of the cuttings from both variants (variants with additional light). The supplementary light (25 - 35 μmol m^{-2} s^{-1}) treatment was white fluorescent tubes, suspended 80 cm above the middle of the plant block (1.2 m width). The tubes were set above the polyethylene tent. During autumn and winter, flats with cuttings in the greenhouse were covered by a twofold polyethylene tent. After 20 October 1999, to maintain a stable temperature during winter, the soil was heated: for the variants with additional light to 13 ± 4 °C, for the

variants without additional light to 10 ± 2 °C. As a control, four flats from each variant were overwintered in the greenhouse without extended light and heating (temperature fluctuated from -9 °C to +4 °C). The variants without additional light and heating were assessed only during the spring because the cuttings of these variants soon lost their leaves.

Bud-break and new shoot growth were estimated during first season on 1 October 1999 and during the second season on 4 April 2000. The survival of overwintered cuttings was determined after the beginning of growth during the spring of the second season in 2000. Four replications (four flats, approx. 45 cuttings per flat) from each variant were used to determine the percentage bud break, new shoot lengths and survival of cuttings. The mean of new shoot length and percentage of cuttings with new shoots were calculated. Cuttings that did not start to grow in the period before 15 May 2000 were defined as dead. The percentage of overwintered cuttings was calculated as the proportion of cuttings that continued to develop over all cuttings in the sample.

For determination of carbohydrates in the leaves, the cuttings were sampled at 7- to 10-day intervals from 1 October until 27 December 1999 and twice per month until 30 March 2000. On 15 March 2000, before bud break, the concentration of starch in stems of cuttings was determined. In both cases the samples were taken in three replications between 10:00 and 11:00 hours. At each sampling, five cuttings were randomly selected. The samples were rinsed with water, fixed in water vapour and dried at 60 °C for 48 h. Dry leaves were milled by laboratory-mill, and stems were scrupulously cut in approximately 0.4 mm thin slices and bruised in the pestle together with granulated glasses to powdery consistence. The concentrations of reducing sugars, sucrose and starch were determined as described previously (Apine, Kondratovičs 2005).

Statistical analyses were performed using SPSS 11.0 for Windows. The data were analyzed using ANOVA. Pair-wise comparisons were made by testing for the least significant difference (LSD) at a 95 % confidence level. Pearson correlation coefficients were generated to describe the relationships among several carbohydrates.

Results

Post-rooting growth

Both the forcing of stock plants and the extended photoperiod during the end of the growth season significantly ($P \leq 0.0001$) stimulated the formation of new shoots (Fig. 1). However, the forced cuttings showed the best results in both variants (with and without additional light) compared to the control cuttings. Development of new shoots on rooted cuttings occurred from mid-August till October, but during the subsequent period till spring the development had terminated. At the end of October the lengths of new shoots were 7.7 ± 0.1 cm for the forced cuttings with additional light, 4.0 ± 0.2 cm for the forced cuttings without additional light and 5.0 ± 0.6 cm for the control cuttings with additional light (data not shown).

From mid-October the leaves of cuttings from variants without additional light and heating started to senescence, and at mid-November the cuttings lost leaves. The leaves of cuttings from variants with additional heating but without additional light gradually began to turn yellow only from the end of November and the leaves abscised at the end of December. However, all new shoots of cuttings from these variants retained their leaves.

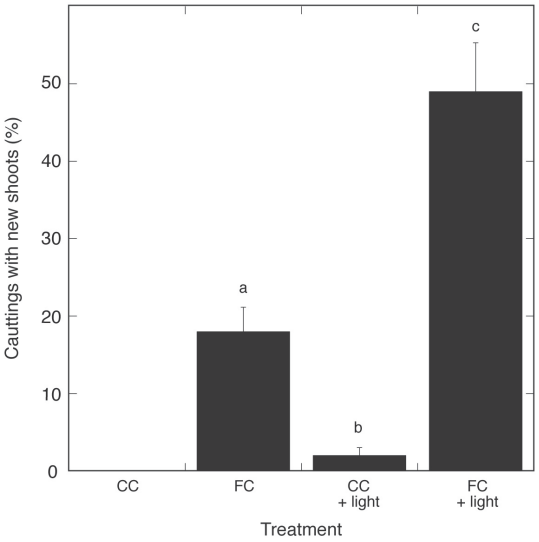

Fig. 1. Effect of stock plant forcing and extended photoperiod on the formation of new shoots on deciduous azalea cultivar 'Polārzvaigzne' stem cuttings. Measurments were made on 1 October 1999, 126 days after the start of the experiment. Mean values are shown, n = 4. Columns with different letters are significantly different at the $P \leq 0.05$ level..

The cuttings from variants with additional heating and light retained almost all their leaves till the end of experiment (data not shown).

Carbohydrate status in the leaves

The carbohydrate status in the leaves of rooted cuttings was assessed in all of the experimental variants. The additional light regime only insignificantly affected the time course of sugar and starch content in the leaves, therefore, for reason of clarity, only data from the experimental variants with additional light and heating are shown in Fig. 2.

In October there were significant differences in the amounts of starch, reducing sugars and total soluble sugars between the variants (Fig. 2A). The control cuttings contained 11.5 % starch, while forced cuttings contained only 6.9 % starch. In the following period, the starch level gradually decreased in both variants. However, a significant difference in respect to starch content was observed between the forced and control cuttings. While starch was depleted almost completely and fluctuated from 0.3 % to 0.5 % in the leaves of control cuttings, the level of starch remained relatively high (1.6 % to 3.8 %) in the leaves of forced cuttings. After February, the starch level gradually increased in the cuttings of variants subjected to extended light (which kept leaves). Differences in the amount of total soluble sugars between experimental variants during October and November were due to higher content of reducing sugars in the leaves of control cuttings in comparison with forced cuttings.

Similar to starch, during autumn and winter the amount of reducing sugars continually decreased in the leaves of cuttings from both variants, while the amount of sucrose increased slightly (Fig. 2B). During winter the levels of sucrose and reducing sugars were quite stable for all of the experimental variants. But at the end of the study period, during

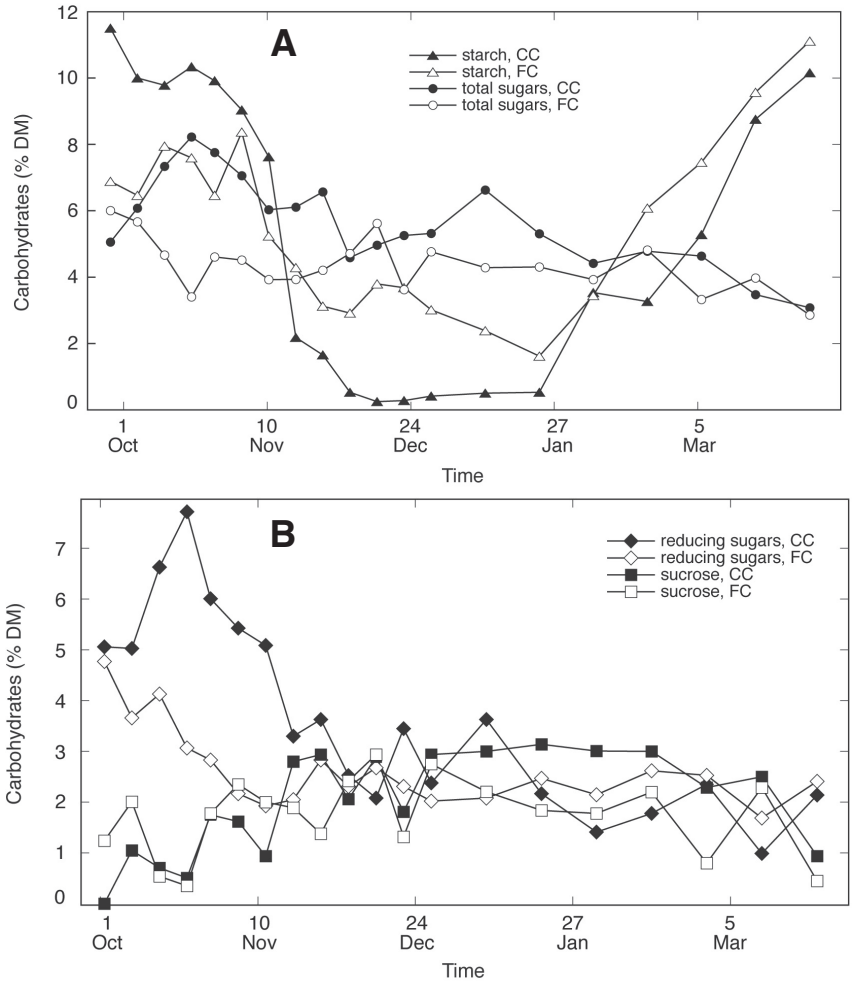

Fig. 2. Changes of carbohydrate concentrations in the leaves of rooted cuttings of deciduous azalea cultivar 'Polārzvaigzne' during the overwintering period. A, starch and total soluble sugars (TSS); B, reducing sugars and sucrose. Cuttings were taken from outdoor-raised stock plants on 29 June 1999 (control cuttings, CC) and from greenhouse-forced stock plants on 28 May 1999 (forced cuttings, FC). Mean values of variants subjected to an extended photoperiod and additional heating are shown, n = 3.

March the amount of soluble sugars tended to decrease in both control and forced cuttings mainly due to a decline of the level of sucrose.

Assessment of overwintering

The environmental conditions during the overwintering period influenced survival more significantly than stock-plant forcing in spring. The overwinter survival of rooted cuttings was assessed after bud-break. The cuttings from variants with additional light and heating had the best overwinter survival compared to the cuttings from the other variants (Fig. 3).

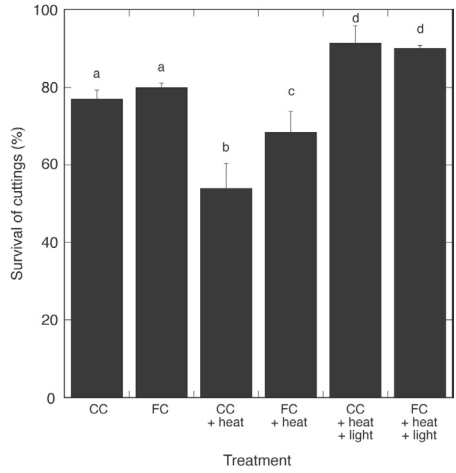

Fig. 3. Effect of experimental conditions on the overwinter survival of deciduous azalea cultivar 'Polārzvaigzne' stem cuttings. Cuttings were evaluated on 1 April 2000. Mean values are shown, n = 4. Columns with different letters are significantly different at the $P \leq 0.05$ level..

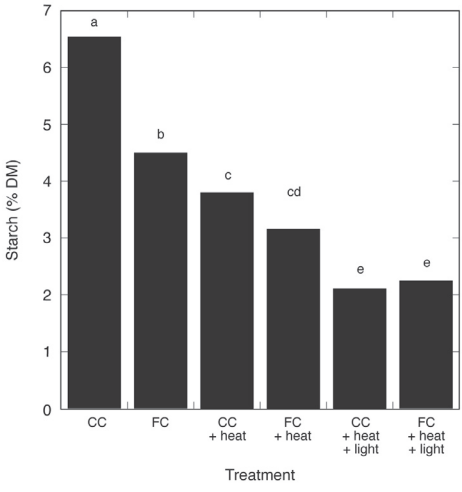

Fig. 4. Effect of experimental conditions on formation of new shoots after overwintering of deciduous azalea cultivar 'Polārzvaigzne' stem cuttings in the spring of the second season spring. Cuttings were evaluated on 1 April 2000. Mean values are shown, n = 4. Columns with different letters are significantly different at the $P \leq 0.05$ level..

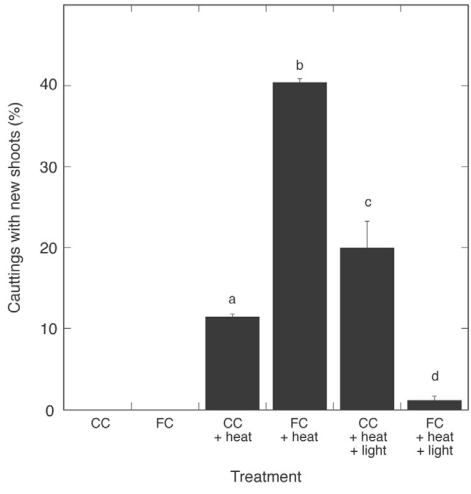

Fig. 5. Effect of experimental conditions on the starch concentration in the stems of rooted cuttings of deciduous azalea cultivar 'Polārzvaigzne' after overwintering. Samples were taken on 15 March 2000, 291 days after the start of the experiment. Mean values are shown, n = 3. Columns with different letters are significantly different at the $P \leq 0.05$ level..

All cuttings that formed new shoots before winter and the cuttings that had retained their leaves throughout the winter survived (data not shown). In general, the conditions during the overwintering period affected survival of cuttings significantly ($P \leq 0.001$), while the effect of stock plant forcing was not significant.

During spring, bud-break was first observed in the variants with additional heating and without extended light, but the development of new shoots in cuttings from the variants with additional light and heating was significantly weaker (Fig. 4). The lengths of the new shoots were 3.6 ± 0.8 cm in control cuttings from the variant with additional light and heating; 2.4 ± 0.6 cm in control cuttings from the variant with additional heating but without light; 2.5 ± 1.2 cm in forced cuttings with additional light and heating and 2.3 ± 0.7 cm in forced cuttings with additional heating but without light. Bud-break for control and forced cuttings from the variants without additional light and heating was initiated later, but during their development they reached the growth achieved by the cuttings that overwintered with additional heating but without additional light (data not shown).

Both stock-plant forcing ($P \leq 0.05$) and environmental conditions during winter ($P \leq 0.001$) significantly affected the starch level in the stem of cuttings. The highest amount of starch was found in the cuttings that overwintered without additional light and heating, and the lowest – in the cuttings that were provided with additional light and heating (Fig. 5). The control cuttings from the experimental variants that entered dormancy contained considerable more starch than forced cuttings from these variants.

Discussion

The present study clearly showed that pre-treatment of stock plants and the treatments of rooted cuttings significantly affected their further development. In general, the stock-plant forcing promoted formation of new shoots during the post-rooting period, but did not significantly affect the condition of leaves and survival of cuttings. Additional light positively influenced all parameters: new shoot growth, condition of leaves and survival of cuttings. Additional heating promoted retention of leaves, especially together with additional light, while the effect on the survival was conflicting. Heating (10 °C) alone without additional light diminished the survival of cuttings, but together with additional light increased survival. All of these observed effects on growth and survival were also reflected in changes of carbohydrates status of cuttings.

The extended photoperiod and especially the stock-plant forcing, which ensured extra time for post-rooting growth, promoted bud-break and new shoot growth of cuttings (Fig. 1). Poor bud-break was noticed for control cuttings, because of unfavourable conditions for these cuttings. The control cuttings were used for propagation one month later than forced cuttings, but progressing in the growth season reduces the ability for bud break (Smalley at al. 1987). The treatment of cuttings with additional light and heating significantly improved the condition of leaves of rooted cuttings, possibly due to a more beneficial ratio of growth promoters to growth inhibitors (Smalley at al. 1987; Hartmann et al. 2002). The retention of leaves maintains plant capacity for photosynthesis, which allows to replenish the carbohydrate reserves. Also the development of new shoots is very important for the development of the cuttings (Turetskaya, Polikarpova 1968; Polikarpova 1990; Hartmann et al. 2002), promoting supplement of carbohydrate stores due to an increase of the leaf area (Smalley at al. 1987). The importance of new shoots for photosynthesis and provision with photosynthates can explain the higher starch level in forced cuttings compared to the starch level in control cuttings during the December.

A decline of the starch amount in leaves and an interruption of new shoot growth suggest that the provided supplementary light could not compensate the decrease of

natural irradiance and day lengths (Fig. 2). During late autumn and December, the reason for a reduced starch level could be both a decrease of photosynthesis and transport of assimilates to perennial parts of the plant. In softwood cuttings the leaves are an important store of assimilates. Our results suggest that the rooted cuttings completely utilized the carbohydrates accumulated in leaves either for development or stored them in the perennial parts.

Although the overwinter survival was affected mainly by the conditions during winter, the stock-plant forcing-related effect of new shoot growth during the post-rooting period on overwinter survival was evident (Fig. 3). In several studies, it was established that cuttings developing new shoots overwinter better (Jermakov, 1975; Loach, Whalley, 1975; Goodman, Stimart, 1987). In Acer rubrum, all cuttings breaking bud were observed to survive the overwintering period (Smalley et al. 1987). Also, in our study, all cuttings with new shoots survived and in spring they grew more rapidly than the cuttings without new shoots. As all new shoots maintained leaves and photosynthetic capacity obviously they were able to replenish carbohydrate stores, which helped to initiate growth.

The results of the present experiment showed a correlation between the overwintering conditions and the starch amount in the stem of cuttings (Fig. 5). The cuttings subjected to low temperature during the overwintering period could reduce the amount of carbohydrate used in respiration. The condition of additional heating without additional light promoted the depletion of carbohydrates in cuttings without the ability to replenish the stores, which resulted in the lowest survival rate of these cuttings. The treatment of cuttings with additional light and heating during winter, despite the lowest level of starch in the stems, showed the best overwintering, because these cuttings maintained photosynthetic capacity. However the poor growth of new shoots of these cuttings in spring suggests that the natural seasonal dynamics of plants was disturbed due to prevention of the dormancy period.

While sufficient carbohydrate storage is important for winter survival of cuttings (Smalley at al. 1987; Perkins, Bassuk 1995), in our study the starch concentration in stems of the cuttings was not related to survival ability. Further studies should consider also the accumulation of carbohydrates in roots. The importance of roots for storages has been shown in several studies using both intact plants (Loescher et al. 1990; Nguyen et al. 1990; von Fricks, Sennerby-Forsse 1998) and cuttings (Smalley et al. 1987; Hartmann et al. 2002).

Generally, stock-plant forcing of deciduous azalea enhanced post-rooting growth of cuttings, and an extended photoperiod and additional heating in winter improved the survival of the cuttings. However, the natural seasonal dynamics were disturbed and the development of plants was delayed in spring. To improve the conditions for rooting and survival of cuttings, stock-plant forcing during spring and an extended photoperiod with heating during the autumn are recommended, although, during the winter a dormancy period with the respective temperature should be provided so that the natural seasonal dynamics are not disturbed.

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Vides faktoru ietekme uz vasarzaļo rododendru pavairošanu ar spraudeņiem. II. Paildzināta augšanas perioda ietekme uz apsakņoto spraudeņu attīstību, pārziemošanu un ogļhidrātu satura izmaiņām

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Kopsavilkums

Pētījuma mērķis bija novērtēt, kā vasarzaļo rododendru mātes augu pavasara steidzināšana siltumnīcā un papildus apgaismojums un augsnes apsilde rudenī ietekmēja apsakņoto spraudeņu tālāko attīstību un pārziemošanu. Mātes augu steidzināšana pavasarī un papildus apgaismojums rudens periodā veicināja jauno dzinumu attīstību jau tajā pašā rudenī. Spraudeņu lapās uzkrātā ciete rudenī tika transportēta uz pārziemojošajām auga daļāmm. Rezerves ogļhidrāta cietes izmantošana ziemas laikā bija atkarīga no temperatūras. Salīdzinoši augsta temperatūra (10 °C) veicināja uzkrātās cietes patēriņu. Papildus apgaismojums un apsilde veicināja lapu saglabāšanos spraudeņiem un, līdz ar to, arī spraudeņu fotosintēzes iespējas. Vides apstākļi ziemošanas laikā ietekmēja spraudeņu izdzīvošanu, bet mātes augu steidzināšana pārziemošanu būtiski neietekmēja. Papildus apgaismojums kopā ar augsnes apsildīšanu ziemošanas laikā uzlaboja spraudeņu izdzīvošanas iespējas, bet aizkavēja to attīstību pavasarī.

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Main migratory direction of Marsh Harrier *Circus aeruginosus*: an analysis of recovery data of specimens ringed in Latvia from 1925 to 2004

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Abstract

This paper describes the main migratory directions of the Latvian Marsh Harrier *Circus aeruginosus* population and provides an overview of recovery data. The study was based on analysis of recovery data of Marsh Harrier that were ringed in Latvia from 1925 to 2004 and subsequently recovered. In total, of the 744 Marsh Harriers that were ringed only 47 (6.30 %) were recovered. The majority of recoveries of Marsh Harrier were due to hunting 46.8 % (n = 22), 25.5 % (n = 12) were found dead and 27.7 % (n = 13) were captured in the area of ringing. Frequency analysis of direction and recovery distribution mapping showed that the Latvian population of Marsh Harrier more frequently had a SSW migration direction, suggesting a Mediterranean passage across Italy.

Key words: Marsh Harrier, migration, recovery, ringing.

Introduction

Diurnal birds of prey *Falconiformes* represent a diverse group of highly mobile, wide-ranging predators, whose populations occur across a broad range of western Palearctic habitats (Cramp, Simmons1980). Each year a number of these birds migrate long distances, in many instances across and among entire continents, en route to their breeding and wintering grounds. Most raptor species are diurnal migrants. Only occasional cases of nocturnal migration of Harrier have been reported (Russel 1991).

The migratory behaviour of birds is controlled by endogenous factors and influenced by various environmental factors. Important exogenous factors are weather and topography (Alerstam 1976). Although raptors often migrate across broad fronts, individuals of many species congregate along established corridors on migration, particularly along specific geographic features including mountain chains, coastal plains, isthmuses, and peninsulas (Bildstein 1998).

The mapping of migration patterns, especially those of long-distance migrants, has long been a major focus of study in the biology of migration. The first ringing in Latvia was made in 1909. Systematic ringing was started in 1925 (Kazubiernis 1989). The first report on the recovery of a Marsh Harrier *Cicer aeruginosus* (hereafter MaH) that was ringed in Latvia came from 1928, when a MaH ringed at Lake Engure was recovered (shot) later in the same year and in the same area. Comparatively little is known about Latvian

population of Harrier migration patterns, routes and destinations. The aim of this study was (i) to describe the main migratory directions of Latvian Marsh Harrier population, and (ii) to provide an overview of recovery data.

Materials and methods

This study was based on ringing data of the Marsh Harrier accumulated in Latvia from 1925 to 2004. Only recovery data were used. Historically, MaH ringing in Latvia has been carried out by two different methods. The first included parent tracing, nest searching and ringing of chicks at a suitable age e.g. a sufficient size of tarsus and toes to keep the ring stable. The second way was linked with predator control programmes in waterfowl breeding areas. This method was applied in the 1960s up to 1998 at Lake Engure. Annually from April to June, about 70 to 80 MaH were captured (Lipsberg 1983; Opermanis et al. 2005). MaH, both full grown and immatures, were trapped on dummy waterfowl nests using a snap-net trap and released in the same area or more than 50 km from the trapping place. Since adult birds trapping were carried out not only in the MaH breeding period, but also during the spring migration, a part of the ringed birds were migrants from northern populations.

The migration map and recovery statistics were produced following Fransson's (2001) described approaches and using ArcView 9 software (ESRI 2004a; ESRI 2004b).

Latitude and longitude co-ordinates of ringing and recovery sites given by the Latvian Ringing Centre were used. The Mercator projection map was used. In this projection, a straight line follows in a constant compass bearing (loxodrome), while the earth's surface is distorted and areas in the North are enlarged compared with areas close to the equator. North, East South and West directions marked as 0°, 90°, 180° and 270° respectively. Four different symbols were used to indicate different stages relating to migration. When translocation co-ordinates were given as start point and co-ordinates of releasing as an end point, these cases were excluded from the ringing-recoveries map and from frequency display. Lines between ringing and accordant recovery places sites were used.

A frequency display based on loxodrome directions for MaH was produced and the median and modal class directions were calculated. When displayed in the figure, directions within five-degree intervals were pooled. To avoid impact of records from postbreeding dispersion and cases when birds from northern populations were ringed, directions more than 270° and less than 90° were not included in calculating the frequency statistic. Recovery data from wintering, spring migration and autumn migration periods were used.

Recovery data for MaH were summarized and included the number ringed, number recovered, proportion recovered and longest travel. The distances of travel were calculated as the shortest distance between the ringing and finding site.

Results

In total, of the 744 MaH that were ringed only 47 (6.3 %) were recovered. The majority of recoveries of MaH were made by hunting 46.8 % (n = 22), 25.5 % (n = 12) were found dead and 27.7 % (n = 13) were captured in the area of ringing. A distance of 2473 km was the longest from the area of ringing in that a MaH was recovered. The frequency display based

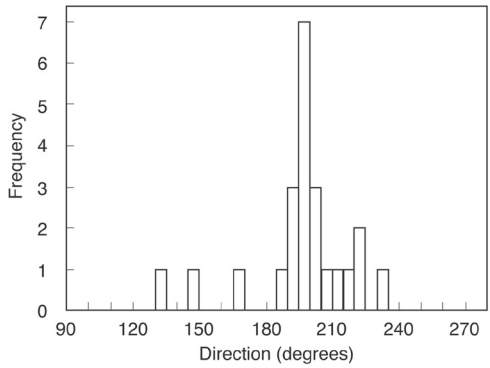

Fig. 1. Frequency distribution of migration direction based on recoveries of Marsh Harrier *Circus aeruginosus* ringed in Latvia 1925 - 2004 (n = 23). Degrees show compass bearing directions between ringing and recovery areas (00 – North, 90° – East, 180° – South and 270° – West, respectively).

on loxodrome directions showed that the central tendency (mode) of migratory direction of MaH was determined as 195° and the median observation of migratory direction of MaH in autumn was 196° (Fig. 1). The migration of MaH was directed SSW, passing through Central Europe and Italy (Fig. 2).

From the 47 recovered MaH, 25 were relocated before releasing. All MaH with subsequent recoveries were ringed from April 29 to July 21, and included adults, immature and juvenile birds. The timescale of recoveries and ringing age of recovered MaH are given in Table 1.

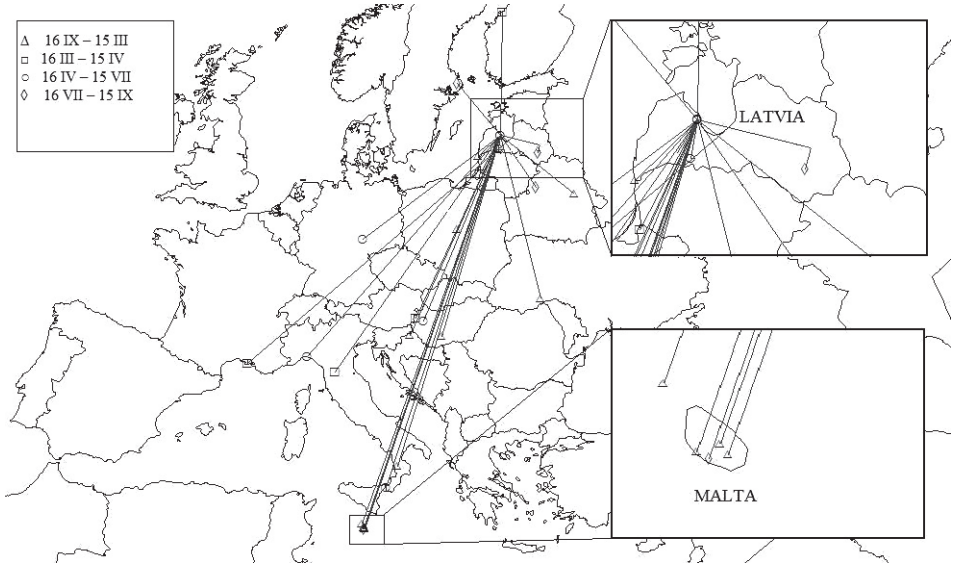

Fig. 2. Recovery of Marsh Harrier *Circus aeruginosus* ringed in Latvia 1925 - 2004 and recovered during 16 July to 15 September (n = 4); 16 September to 15 March (n = 11); 16 March to 15 April (n = 5); 16 April to 15 July (n = 7)..

Table 1. Age of Marsh Harrier *Circus aeruginosus* ringed and recovered in Latvia 1925 - 2004

Age	Recoveries in ringing year (number)	Recoveries in next year (number)	Recoveries after two years and later (number)	Recovered in total (number)
Pull	3	3	5	11
At least 1-year old	4	4	9	17
Second-year birds	1	1	-	2
At least 2-years old	-	1	1	2
Full grown	4	3	8	15

Discussion

Latvian Harrier population can be defined as annual migrants. According to Terrill and Able (1988) this term should be applied to populations (species or geographically defined breeding populations of a species) in which all individuals migrate from their breeding sites on an annual basis. A few MaH individuals occasionally have been found wintering (LOB 1999), particularly in SW Latvia. However, the counted numbers of individuals per year are very few and their area of origin is not clearly known.

Frequency analysis of directions (Fig. 1) and recovery distribution mapping (Fig. 2) showed that the Latvian population of MaH more frequently used a migration direction involving Mediterranean passage across Italy. Since five recoveries came from Malta, it is reasonable to believe that MaH leave Sicily and migrate to Africa across Malta. Wintering in Africa has been documented by Cramp and Simmons (1980) and Fransson and Pettersson (2001). The West European (e.g. British, Netherlands etc. North Sea countries) population has been referred to as migrants across France and Iberia, and the Central/East European population as migrants across central Mediterranean. The easterly breeding populations are described as migrants over land bridges between Europe and Africa at the eastern edge of the Mediterranean Sea (Spaar, Bruderer 1997). In Malta, one female was recovered on 4 March, one male and one female in the second half of September, and one female and one male in the first decade of October, suggesting the Italy was being used in both autumn and spring migrations. However, a possible overwintering in Mediterranean isles is discussed in Misbah (1982). In contrast, Garcia and Arroyo (1998) described that European Montagu's Harrier *C. pygargus* had two main migratory routes across the Mediterranean – the Gibraltar and the Sicily Channel (strait between Sicily and Africa). Their study showed that the passage frequency was higher in the post-breeding than the pre-breeding migration in the Gibraltar Strait, but the opposite occurred in the Sicily Channel. The authors discussed the possibility that Montagu's Harrier have a partially circular migration. In this respect, the recovery of MaH from the Mediterranean coast in South France (Fig. 2) in first decade of April suggests a possible owerwintering in South Europe or using Gibraltar as western land bridge between Europe and Africa.

The number of recoveries used in my analysis was insufficient to identify the correct wintering areas of MaH, and artifacts can disfigure interpretation. As argued by Fransson (2001), even if a few individuals remain in Europe, the chance of receiving a report from these is much higher than from those wintering in Africa. Also, differences in hunting

traditions and hunting legislation between countries in the Harrier flyway might generate differences in recovery probability, e.g. all recoveries from Malta came from shot specimens.

Since 1925 when systematic ringing was started till 1986 in Latvia, 441 MaH were ringed. The recovery rate for MaH from 1961 to 1985 was 7.9 % (Kazubiernis 1989). This high recovery rate was due to waterfowl predator control activities at Lake Engure, where the majority of MaH were ringed. Potentially, this predator control negatively affected the MaH population. However, waterfowl management indirectly provided the highest recovery rates in overall ringed *Falconiformes* species in this period in Latvia (Kazubiernis 1989). From the beginning of the 1990s, when species protection legislation was strengthened, MaH trapping and relocation in the breeding period was terminated. Since that time, the recovery proportion fell to 6.3 %.

Cramp and Simmons (1980) summarized that autumn migration of MaH, started by juveniles moving away from breeding areas, began in early August. Recovery of three specimens that were ringed as nestlings and recovered in the same year within 50 km from the ringing area suggests that fledglings can remain at the natal area till the third decade of August or even mid September. Recoveries in mid January and in the third decade of January in Poland and Belarus, respectively, suggest that the migration period may be spread over a long period or allow owerwintering in areas relatively close to Latvia. Unfortunately, these two birds were ringed there as at least one year old and full grown, and it was not clear whether they came from the Latvian breeding population.

From MaH that were ringed in other countries and recovered in Latvia, 12 were ringed in Finland, four in Sweden and one in Estonia (Latvian Ringing Centre, unpublished data). The majority (excepting one) were ringed as nestlings. Therefore, this indicated that the flyway of these northerly breeding populations pass over Latvia. Overlapping of migrations of Baltic and Finland MaH populations in a route over Hungary has been discussed by Misbah (1982). Two MaH that were ringed in Latvia in the first and third decades of May as at least one-year old and subsequently recovered in Finland and Sweden (Fig. 2.) provide evidence that part of the captured birds represent northern populations. However, Fransson and Pettersson (2001) showed that most of the Swedish MaH population migrated crossing Denmark. A specimen ringed as a nestling in Latvia and recovered in a 194° direction in South Italy indicates the main migratory direction (Fig. 2), suggesting that breeding populations in the Latvian and the nearest Baltic Sea countries (Finnland and Estonia) have the same flyway. Two recoveries in late spring from Italy in the first decade of May were considered to indicate late migration or possible no breeding (Misbah 1982).

As factors affecting the dispersal behaviour of juveniles can differ from those that govern the dispersal behaviour of adults, especially in long-lived species, dispersal of juveniles during their first winter and adults are usually analysed separately (Fransson 2001). Four specimens ringed as nestlings and recovered in the breeding period in the next or later years came from areas less than 50 km distance from the ringing site, and indicating return in the next years to their natal sites. Recovery of four specimens ringed as immature and recovered in the same year and for one specimen that ringed as immature and recovered in the next year were made in Latvia. Three recoveries, to a more westerly directing (232°) in central Germany, a single recovery from Ukraine (167°) and a single recovery from Sweden (317°; Fig. 2), came from specimens that were ringed as immature

in Latvia and recovered in the next year, indicating that wide dispersal is possible.

Recovery of full grown specimen in the breeding season indicates breeding philopatry in both sexes. One male that was ringed in the Lake Engure as more than two-years old subsequently was recovered in the same area after more than seven years and one female ringed as full grown was recovered after 447 days at a 21 km distance from the ringing area.

In subsequent breeding seasons, four birds from both sexes and ringed at different ages (at least one year old and full grown) were recovered near the capturing area. One female that was trapped in the second decade of May in the Lake Engure was repeatedly captured in the same area after 30 days. Founding areas of recoveries during migration of relocated MaH were not outside of the main direction tendencies. Thus, capture and relocation showed no impact on migration and dispersal.

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Latvijā ligzdojošo niedru liju *Circus aeruginosus* galvenie migrācijas virzieni: laikā no 1925. gada līdz 2004. gadam Latvijā gredzenoto īpatņu gredzenošanas atradumu analīze

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Kopsavilkums

Šajā darbā aprakstīti Latvijā ligzdojošo niedru liju *Circus aeruginosus* galvenie migrācijas virzieni un sniegts pārskats par gredzenu atradumiem. Veiktais pētījums balstīts uz laikā no 1925. līdz 2004. gadam Latvijā gredzenoto niedru liju gredzenošanas datiem. No kopējā šajā laikā apgredzenotā liju skaita (n = 744) saņemti tikai 47 (6,3 %) ziņojumi par gredzenu atrašanu. Atrašanas apstākļu analīze parādīja, ka vairumā gadījumu gredzenotās niedru lijas bija cilvēku nomedītas (46,8 %; n = 22); atrastas beigtas (25,5 %; n = 12) vai gredzenu dati nolasīti, tās atkārtoti kontrolējot ligzdošanas vietās (27,7 %; n = 13). Migrācijas virzienu frekvenču analīze un gredzenošanas atradumu attēlojums kartē parādīja, ka Latvijā ligzdojošā niedru liju populācija galvenokārt migrē dienvidu-dienvidrietumu virzienā un šķērso Vidusjūru, pārlidojot Itāliju.

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Ethylene is involved in *Trichoderma*-induced resistance of bean plants against *Pseudomonas syringae*

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Abstract

Trichoderma strains are effective biocontrol agents against a wide range of fungal plant pathogens. Current findings suggest that, in addition to microbial antagonism, the beneficial effect of *Trichoderma* is related to activation of plant defense responses. The aim of the present investigation was to study the effect of *Trichoderma harzianum* on oxidative enzyme activities and resistance to *Pseudomonas syringae* pv. *phaseolicola* of bean (*Phaseolus vulgaris* L.) plants. Special attention was focused on the role of ethylene as a signal mediating *Trichoderma*-induced responses. Both active and denatured fungal preparations induced sharp and transient increase of ethylene formation in bean leaves. The activity of both peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.10.3.2) increased in bean leaves after the treatment of derooted plants with active or inactivated *Trichoderma* formulations. Treatment with *Trichoderma* resulted in blocking of disease symptoms arising from inoculation with *P. syringae*. To demonstrate if the changes in defense responses and resistance against *Pseudomonas* were ethylene-dependent, bean seedlings were pretreated with 1-methylcyclopropene, a competitive inhibitor of ethylene action. Pretreatment with 1-methylcyclopropene led to a drastic acceleration of disease development both in control and *Trichoderma*-treated plants. The activities of peroxidase and polyphenol oxidase were suppressed by 1-methylcyclopropene treatment, suggesting that ethylene acts as one of the signals in *Trichoderma*-enhanced resistance against *P. syringae*.

Key words: ethylene, induced resistance, peroxidase, *Phaseolus*, polyphenol oxidase, *Pseudomonas*, *Trichoderma*.

Introduction

Trichoderma species are among the most widely used biological control agents against soilborne plant pathogens (Hjeljord, Tronsmo 1998). Until recently, research on *Trichoderma*-plant interactions focused mostly on mycoparasitic aspects of plant protection. Only a few studies so far have dealt with plant defense responses induced by *Trichoderma* (Chang et al. 1997; Yedidia et al. 1999; Yedidia et al. 2000; Martinez et al. 2001). *Trichoderma*-derived xylanase and cellulase are well known inducers of ethylene biosynthesis (Avni et al. 1994; Piel et al. 1997). Recently it was shown that cellulase treatment leads to activation of defense mechanisms in melon cotyledons (Martinez et al. 2001). Considering that *Trichoderma* acts in rhizosphere, the effect of native formulations of the mycoparasite on defense responses in plant leaves has been a neglected aspect in plant resistance studies.

Peroxidase and polyphenol oxidase are oxidative enzymes contributing to defense

against pathogens. The peroxidase activity increases in plants during pathogen infection and has been correlated with resistance (Hammerschmidt et al. 1981; Bashan et al. 1987; Bestwick et al. 1998). High activity of peroxidase increased the ability of transgenic tobacco plants to suppress growth of the pathogenic bacterium *Erwinia carotovara* (Elfstrand et al. 2002). Several previous experiments have demonstrated the importance of polyphenol oxidase-mediated phenolic oxidation in restricting plant disease development. For example polyphenol oxidase-overexpressing tomato plants were shown to exhibit a great increase in resistance to *P. syringae* (Li, Steffens 2002). Compared with control plants, these transgenic lines showed less severity of disease symptoms, with over 15-fold fewer lesions, and strong inhibition of bacterial growth, causing more than an 100-fold reduction of bacterial population in the infected leaves. Consequently, high activities of peroxidase and polyphenol oxidase can be components of resistance against pathogens, including bacteria.

Ethylene is an important factor in the regulation of plant reaction to pathogens. An increase of ethylene formation in pathogen-challenged plants has been related both to defense responses leading to resistance as well as to symptom development during pathogenesis. The role of ethylene in incompatible interactions is mostly contradictory and depends on the pathogen used (Lawton et al. 1995; Hoffman et al. 1999; Thomma et al. 1999). Similarly, regarding to susceptible responses, ethylene insensitivity may lead to both increases and decreases of symptom development (Lund et al. 1998; Hoffman et al. 1999). In addition, recent findings indicate the role of ethylene in systemic acquired resistance (Knoester et al. 2001) and in induced systemic resistance (Pieterse et al. 2001). The fact that ethylene is a known inducer of several pathogen defense-related enzymes, e.g. peroxidase, glucanase, and chitinase, lends support to the regulative role of ethylene in resistance responses (Ishige et al. 1993; Xu et al. 1994; Siefert, Grossmann 1997; Ohme-Takagi et al. 2000).

The aim of the present experiments was to test the hypothesis that treatment of the above-ground parts of *Phaseolus vulgaris* with *Trichoderma* formulations can stimulate resistance against the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola*. Special attention was focused on investigating the role of ethylene and oxidative enzymes in *Trichoderma*-induced defense mechanisms.

Materials and methods

Plant material

Seeds of *Phaseolus vulgaris* L. cv. *Saxa* bush bean were surface sterilized with $KMnO_4$ and sown individually into 10-cm plastic pots containing commercial peat-moss (pH 6.0) with addition of mineral nutrients. Plants at a two-leaf stage with fully grown primary leaves were used for experiments.

Plants were grown in a growth chamber with a photoperiod of 16/8 h in a photosynthetic photon flux density of 120 μmol m^{-2} s^{-1}, 25 ± 2 °C, 50 to 70 % relative humidity.

Treatment and inoculation

Trichoderma harzianum Rifai isolate B-21 (provided by Dr. A. Lielpetere, *Bioefekts*, Riga, Latvia) was grown in darkness on plates with agarized malt extract, containing 50 g l^{-1} malt extract and 15 g l^{-1} agar. For inoculum preparation, the plates were washed with water to

collect spores. The inoculum concentration was determined to be 10^6 germinated spores ml^{-1}. Inactive *Trichoderma* was prepared by autoclaving the inoculum for 40 min at 110 °C, 0.1013 MPa. For treatment, bean plants were derooted and individually incubated in glass flasks containing the appropriate *Trichoderma* inoculum for 4 h. Control plants were incubated in water. After treatment, the plants were further grown in water. Alternatively, for measurement of ethylene production, one leaf of the bean plants was sprayed with the appropriate *Trichoderma* formulation until run-off. Control plants were sprayed with water.

Pseudomonas syringae pv. *phaseolicola* was grown on glucose yeast extract agar medium plates for 2 days at 30 °C. The medium consisted of 20 g l^{-1} glucose, 10 g l^{-1} yeast extract, 20 g l^{-1} $CaCO_3$, 17 g l^{-1} agar. The bacterial cells were harvested, washed twice, and resuspended in 10 mM $MgCl_2$. The inoculum concentration was determined to be 10^8 colony-forming units ml^{-1}.

Plants were inoculated 20 h after the start of the *Trichoderma* treatment one leaf per plant with *Pseudomonas syringae* pv. *phaseolicola*, for infiltration with a suspension of bacteria using a syringe without a needle. As a negative control plants were infiltrated with 10 mmol l^{-1} $MgCl_2$.

Pretreatment with 1-methylcyclopropene

For 1-methylcyclopropene (1-MCP) treatment, bean plants were placed within a 5-l polystyrene chamber 12 h before *Trichoderma* treatment was started. To release 1-MCP into a chamber, 10 mg of SmartFresh™ (AgroFresh Inc., USA) was placed in a vial containing 5 ml of water.

Measurement of ethylene formation

For measurement of the basal rate of ethylene production (Kruzmane et al. 2002), detached leaves were rinsed with water, blotted dry, cut in half, rolled and placed individually in 4-ml screw-capped bottles. After 20 min, 1 ml of headspace gas was analyzed for ethylene concentration using a gas chromatograph Chrom 5 (Czech Republic) equipped with a glass column filled with Al_2O_3 and a flame ionization detector. Helium was used as a carrier gas.

Determination of enzyme activity

For measurement of enzyme activitiy, leaves were rinsed with deionized water, frozen in liquid nitrogen and stored at -80 °C until analysis. For extraction of enzymes, leaf tissues were ground in liquid nitrogen and extracted with 25 mmol l^{-1} HEPES (pH 7.2) for 15 min at 4 °C. The homogenate was filtered through nylon cloth and centrifuged at 15,000 g_n for 15 min. Guaiacol peroxidase (EC 1.11.1.7) activity and diphenolase (EC 1.10.3.2) activity of polyphenol oxidase was measured spectrophotometrically in the supernatant as described previously (Kruzmane et al. 2002).

Standard Bradford assay was used to test the protein concentration in leaf extracts in each sample.

All experiments were repeated two or three times. All calculations (means and standard error) were performed using MS Excel. The Student's *t*-test was used to determine the levels of significance. All chemicals were purchased from Sigma-Aldrich Chemie, Germany.

Results

Effect of Trichoderma *on ethylene formation*

Two treatments were used for analyzing the effect of *Trichoderma* on ethylene formation in bean leaves: (i) one leaf of an intact bean plant was sprayed with an appropriate *Trichoderma* formulation and (ii) bean plants were derooted and individually incubated in *Trichoderma* inoculum. In general, treatment of bean plants with active or inactivated *Trichoderma* formulations resulted in an increase of the rate of basal ethylene production from the leaves (Fig. 1 and 2). However, when derooted seedlings were incubated in *Trichoderma* formulations, ethylene production intensity increased by ten times over that induced in intact seedlings. When *Trichoderma* was applied only to one leaf of intact bean plants, stimulation of ethylene production intensity was observed both in the treated and untreated systemic leaves (Fig. 1). Maximum ethylene production was reached at 4 h and 2 h after treatment with *Trichoderma* for intact and derooted bean plants respectively. In contrast to intact plants, where active formulation was more effective in eliciting an increase in ethylene production (Fig. 1), induction of ethylene production by active *Trichoderma* was less pronounced in leaves of derooted bean plants (Fig. 2). In addition, the dose response experiment revealed that a ten-times diluted *Trichoderma* formulation was more active in eliciting enhanced ethylene production from leaves of derooted seedlings than the undiluted solution (data not shown).

As treatment of derooted plants resulted in an earlier and more pronounced increase

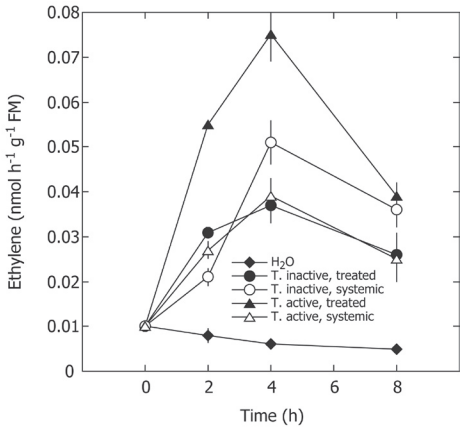

Fig. 1. Time course of ethylene formation in bean leaves treated with heat-inactivated or active culture of *Trichoderma harzianum*. One leaf of bean plants was sprayed with H_2O or appropriate *Trichoderma* formulation. After the indicated intervals of time, both treated and non-treated leaves were detached and placed in bottles to accumulate ethylene during the subsequent 20 min. Values are the means ± SE of two independent experiments with four replicates each.

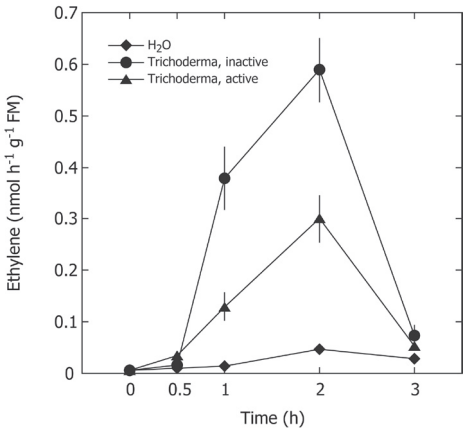

Fig. 2. Time course of ethylene formation in leaves of bean plants incubated in heat-inactivated or active cultures of *Trichoderma harzianum*. Bean plants were cut and immersed with the end of the stems in H_2O or appropriate *Trichoderma* formulation. After the indicated intervals of time, leaves were detached and placed in bottles to accumulate ethylene during the subsequent 20 min. Values are the means ± SE of three independent experiments with three replicates each.

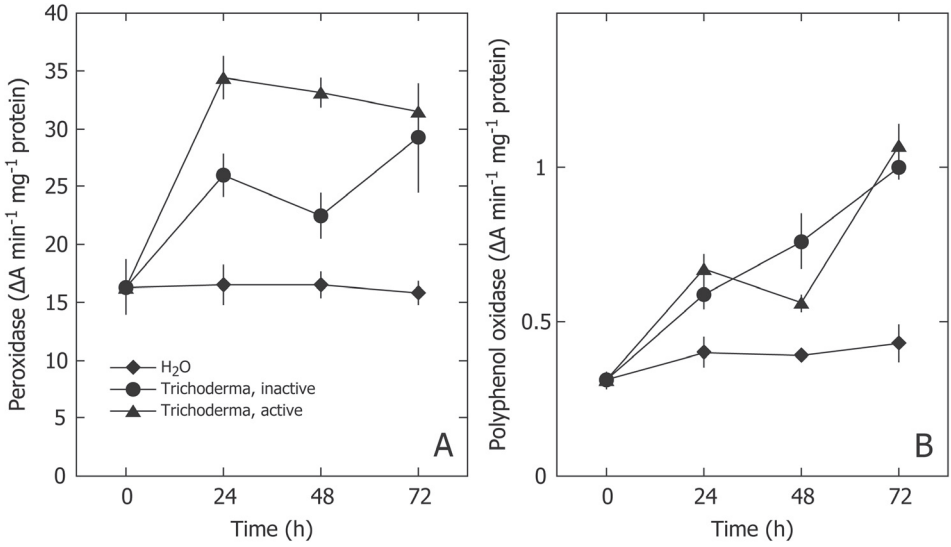

Fig. 3. Time course of peroxidase activity (A) and polyphenol oxidase activity (B) in leaves of bean plants incubated in heat-inactivated or active culture of *Trichoderma harzianum*. Bean plants were cut and immersed with the end of the stems in H_2O or appropriate *Trichoderma* formulation for 4 h. After treatment, plants were further grown in water. After appropriate intervals of time, leaves were detached and used as material for enzyme analysis. Values are the means ± SE of two independent experiments with three replicates each.

of ethylene production than treatment of intact plants, for further experiments only derooted bean plants were used for treatment with *Trichoderma*.

Effect of Trichoderma *on enzyme activity*

The activity of both peroxidase and polyphenol oxidase increased in bean leaves after the treatment of derooted plants for 4 h with active or inactivated *Trichoderma* formulations (Fig. 3). Treatment of plants with active *Trichoderma* resulted in a higher peroxidase activity 24 and 48 h after treatment than that with the inactivated mycoparasite formulation (Fig. 3A). In contrast, the polyphenol oxidase activity did not differ significantly between the active and inactivated formulations (Fig. 3B).

The role of ethylene as an inducer of enzyme activities resulting from *Trichoderma* treatment was tested by pretreatment of bean plants with gaseous 1-MCP, a competitive inhibitor of ethylene action. For active *Trichoderma*-treated plants, 1-MCP pretreatment resulted in a statistically significant decrease of *Trichoderma*-induced enzyme activities in the leaves, with a more pronounced inhibitory effect for polyphenol oxidase (Table 1). In contrast, when bean plants were pretreated with 1-MCP and subsequently treated with inactivated *Trichoderma*, both activities were further stimulated. 1-MCP pretreatment alone did not affect peroxidase activity in H_2O-incubated plants, however, polyphenol oxidase activity was slightly inhibited by the pretreatment.

Effect of Trichoderma *on disease development in* P. syringae*-inoculated leaves*

Disease development in leaves of bean plants was analyzed 20 days after inoculation with

Table 1. Effect of 1-methylcyclopropene (1-MCP) pretreatment on the activity of peroxidase and polyphenol oxidase in bean leaves. The data represent means of two independent experiments performed in triplicate. Mean values of activity with the same letter within each column are not significantly different at P = 0.05

Treatment	Enzyme activity (%)	
	Peroxidase	Polyphenol oxidase
H_2O	100[a]	100[a]
1-MCP + H_2O	99[a]	87[a]
Trichoderma (active)	175[c]	168[c]
1-MCP + *Trichoderma* (active)	145[b]	103[a]
Trichoderma (inactive)	149[b]	148[b]
1-MCP + *Trichoderma* (inactive)	207[d]	206[d]

P. syringae. Leaves inoculated with $MgCl_2$ were generally symptomless with an occasional wound at the site of inoculation (Fig. 4A). Leaves inoculated with *P. syringae* produced characteristic necrotic lesions at the sites of infiltration. Leaves of water-treated bean seedlings inoculated with *P. syringae* became diseased, with large spreading chlorotic areas developing both on inoculated leaves as well as on non-inoculated leaves (Fig. 4B). Treatment of bean plants with heat-inactivated *Trichoderma* resulted in smaller chlorotic areas on *P. syringae*-inoculated leaves with complete blocking of disease symptoms on non-inoculated leaves (Fig. 4C). Development of disease symptoms was blocked in active *Trichoderma*-treated plants inoculated with *P. syringae* (Fig. 4D). In comparison with control or inactive *Trichoderma*-treated bean plants development of broad, red-coloured zones around lesions at the sites of bacterial inoculation were evident on leaves of active *Trichoderma*-treated plants.

To test for possible involvement of ethylene in *Trichoderma*-induced limitation of disease development, bean plants were pretreated with gaseous 1-MCP 12 h before *Trichoderma* treatment. Blocking ethylene receptors with 1-MCP led to a drastic acceleration of disease development both in control and *Trichoderma*-treated plants. Ten days after inoculation with *P. syringae*, 1-MCP-pretreated plants had already developed intensive disease symptoms on their leaves with large chlorotic areas (Fig. 5C,D). In comparison, at that time 1-MCP non-treated control plants showed only the first signs of disease development (Fig. 5A). Further, 1-MCP-treated plants showed extremely fast disease development, with complete abscission of *P. syringae*-inoculated leaves only a few days later (data not shown).

Discussion

Biocontrol mechanisms of *Trichoderma* have been studied mostly as mycoparasitic interactions, with focus on antibiosis and hydrolytic enzyme activity (Grondona et al. 1997). Here for the first time we report on *Trichoderma*-induced inhibiting effect on the development of bacterial disease symptoms in plant leaves. Based on our results, it is difficult to identify the precise means by which *Trichoderma* formulations act to restrict the pathogenicity of *P. syringae* in bean leaves. It can not be excluded that *Trichoderma*-produced

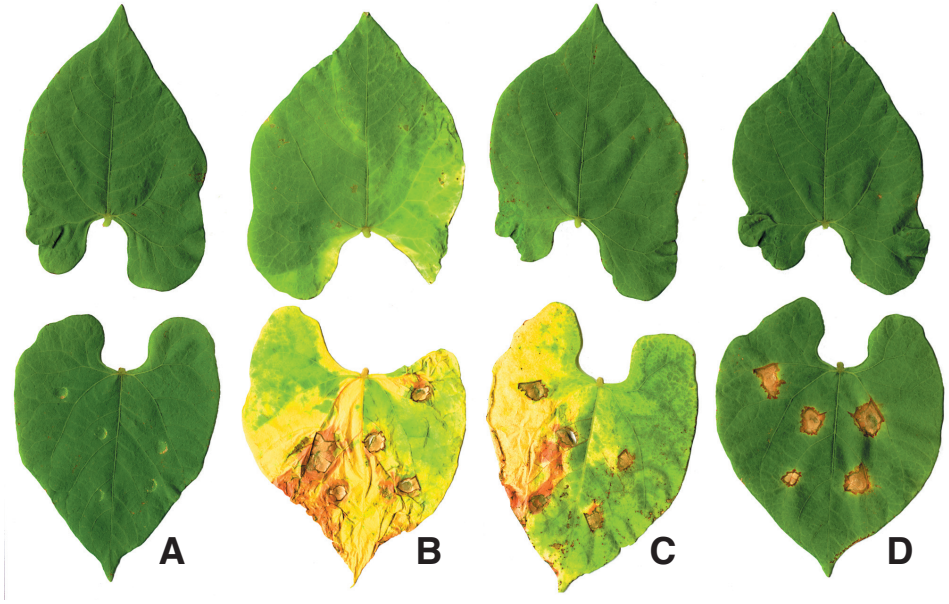

Fig. 4. Effect of heat-inactivated and active *Trichoderma harzianum* on disease development in leaves of bean plants inoculated with *Pseudomonas syringae* pv. *phaseolicola*. A, control plants, 10 mmol l^{-1} $MgCl_2$; B, control plants, *P. syringae*; C, inactivated *Trichoderma*, *P. syringae*; D, active *Trichoderma*, *P. syringae*. Lower row, inoculated leaves; upper row, systemic leaves of the same plants. Bean plants were cut and immersed with the end of the stems in H_2O or appropriate *Trichoderma* formulation for 4 h. After treatment, plants were further grown in water. Inoculation of bean plants with *P. syringae* was performed 20 h after the start of the *Trichoderma* treatment. Photographs were taken 20 days after inoculation. This experiment was repeated three times with ten plants per treatment and the representative symptoms are shown.

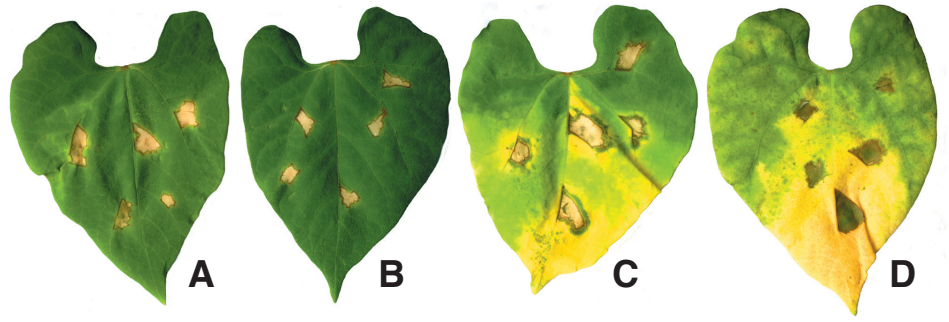

Fig. 5. Effect of 1-methylcyclopropene (1-MCP) pretreatment on disease development in leaves of bean plants treated with active *Trichoderma harzianum* and inoculated with *Pseudomonas syringae* pv. *phaseolicola*. A, no 1-MCP, control plants; B, no 1-MCP, *Trichoderma*-treated plants; C, 1-MCP pretreatment, control plants; D, 1-MCP pretreatment, *Trichoderma*-treated plants. Photographs were taken 10 days after inoculation. This experiment was repeated two times with ten plants per treatment and the representative symptoms are shown.

antibiotics directly suppress the growth of the bacteria (Faull et al. 1994). However, several observations suggest activation of plant defense responses resulting from the activity of the components of the *Trichoderma* formulations. Several cell wall-degrading enzymes in *Trichoderma* culture, e.g. xylanase and cellulase, can induce the formation of plant cell wall-derived oligogalactournoides, which further act as endogenous elicitors (Boudart et al. 1998; Enkerli et al. 1999). The increased activities of peroxidase and polyphenol oxidase can limit disease development through the formation of polymerized phenolic barriers around the sites of infection (Smit, Dubery 1997; Li, Steffens 2002). In this respect, in the present experiments *Trichoderma*-treated plants had noticeably intense red-colored zones around the sites of bacterial inoculation (Fig. 4). In addition, peroxidase and polyphenol oxidase can contribute to synthesis of anti-nutritive, antibiotic, and cytotoxic compounds leading to enhanced resistance against pathogens (Hammerschmidt, Nicholson 1999). We also found that *Trichoderma* increased ethylene formation in leaves of bean seedlings (Fig. 2). Ethylene, in turn, was essential for a delay of disease development (Fig. 5) and was partially necessary for the increase of oxidative enzyme activity (Table 1), suggesting that ethylene acts as one of the signals leading to enhanced resistance against *P. syringae*.

It is evident that *Trichoderma* can affect plant resistance to a pathogen either by inducing the basal level of defense reactions immediately after treatment or by enhancing a capacity for rapid and effective activation of cellular defense responses, which are induced only after contact with a challenging pathogen, a process known as "sensitization" or "priming" (Conrath et al. 2002). In the present experiments, we focused on the direct effect of active and heat-inactivated *Trichoderma* on defense responses.

One of the well-known responses of *Trichoderma*-derived xylanase and cellulase in plant tissues is induction of ethylene biosynthesis (Avni et al. 1994; Piel et al. 1997; Martinez et al. 2001). Our data on increased ethylene production due to *Trichoderma* treatment are in accordance with those reported earlier. Xylanase treatment in tobacco leaves caused an increase of ethylene biosynthesis with a maximum within 3 to 4 h (Avni et al. 1994). Similarly, cellulase from *Trichoderma viride* induced ethylene production in *Phaseolus lunatus* leaves with a maximum at 3.5 h after the treatment (Piel et al. 1997). In contrast, ethylene production from melon cotyledons infiltrated with cellulase from *Trichoderma longibrachiatum* peaked at 24 h after the treatment (Martinez et al. 2001). It is evident that, in comparison with purified cell wall-degrading enzymes, native *Trichoderma* formulations contain several substances that may affect ethylene biosynthesis. The more effective stimulation of ethylene production by diluted *Trichoderma* inoculum compared to undiluted suggests the presence of inhibitors of ethylene formation in the formulation. These inhibitors might be heat sensitive, as inactivated *Trichoderma* was more effective in eliciting ethylene production than active *Trichoderma*.

In our experiments, 1-MCP accelerated the development of bacterial infection both in control and *Trichoderma*-treated bean plants (Fig. 5). Consequently, ethylene is involved in mechanisms leading to delay of the development of infection in bean plants both in natural conditions as well as under the effect of *Trichoderma*. As 1-MCP completely blocked *Trichoderma*-induced polyphenol oxidase activity and partially blocked *Trichoderma*-induced peroxidase activity (Table 1), possibly ethylene-induced enzyme activities after *Trichoderma* treatment are among the putative mechanisms leading to increased resistance against *P. syringae*. However, 1-MCP-increased activities of peroxidase and polyphenol oxidase in heat-inactivated *Trichoderma*-treated bean plants suggests different effects

of ethylene in plants treated with active or inactivated *Trichoderma* extracts. It might be suggested that ethylene-suppressed enzyme activity in inactive *Trichoderma*-treated bean plants gives a lower degree of protection by inactivated *Trichoderma* against *P. syringae*. Our data contradicted those by Martinez et al. (2001), who reported that ethylene was responsible for the induction of peroxidase and chitinase activities in melon cotyledons by heat-denatured cellulase from *Trichoderma longibrachiatum*. The increase of activities of peroxidase and chitinase by active cellulase appeared to be independent of ethylene synthesis in these experiments. This contradiction may been ascribed to the fact that the *Trichoderma* cultures used in our experiments probably contained several substances besides cellulase with a potential effect on defense responses in general and on ethylene formation in particular.

As ethylene is involved in regulating the delay of disease symptoms in control plants (as suggested by the 1-MCP-induced acceleration of disease development, Fig. 5) without a statistically significant effect on peroxidase and polyphenol oxidase activities (Table 1), ethylene may be involved in the regulation of *P. syringae*-induced defense reactions. It is possible that ethylene is necessary for the regulation of the timing of induced defenses after pathogen challenge. Similarly, it has been shown that ethylene is involved in the generation or translocation of the systemically transported signal for rhizobacteria-mediated induced systemic resistance (Pieterse et al. 2001). A faster production of ethylene during the initial phase of infection might contribute to enhanced resistance. Therefore, we suggest ethylene not only as an inducer of basal defense responses, but also as one of the signals regulating priming during *Trichoderma*-induced resistance in bean plants. However, for a more complete understanding of the role of ethylene during natural and *Trichoderma*-induced resistance it is necessary to characterize the defense-related responses induced by *P. syringae* treatment.

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Etilēns piedalās *Trichoderma*-inducētās izturības nodrošināšanā dārza pupiņām pret *Pseudomonas syringae*

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Kopsavilkums

Trichoderma sugas ir efektīvi biokontroles aģenti pret dažādiem sēņu dabas augu patogēniem. Jaunākie pētījumi liecina, ka *Trichoderma* labvēlīgā ietekme uz augiem ir saistīta ne tikai ar mikrobiālo antagonismu, bet arī ar augu aizsardzības reakciju aktivāciju. Dotā darba mērķis bija pētīt *Trichoderma harzianum* ietekmi uz dārza pupiņu (*Phaseolus vulgaris*) oksidatīvo fermentu aktivitāti un augu izturību pret *Pseudomonas syringae* pv. *phaseolicola*. Īpašu uzmanību pievērsa etilēna kā signāla nozīmei *Trichoderma*-inducētajās atbildes reakcijās. Gan aktīvs, gan denaturēts sēnes preparāts izsauca krasu un īslaicīgu etilēna veidošanās pieaugumu pupiņu lapās. Nogrieztu pupiņu augu inkubēšana aktīvā vai neaktīvā *Trichoderma* preparātā izraisīja peroksidāzes (EC 1.11.1.7) un polifenolu oksidāzes (EC 1.10.3.2) aktivitātes pieaugumu. Pupiņu augu apstrāde ar *Trichoderma* bloķēja *P. syringae* izsauktās slimības simptomus. Lai noteiktu, vai aizsargreakciju izmaiņas un izturība pret *Pseudomonas* bija atkarīga no etilēna, veica pupiņu augu priekšapstrādi ar 1-metilciklopropēnu, konkurējošu etilēna darbības inhibitoru. Šāda priekšapstrāde izsauca dramatisku slimības simptomu attīstību gan kontroles, gan ar *Trichoderma* apstrādātiem augiem. Inhibitora ietekmē samazinājās arī *Triochoderma* izsauktais peroksidāzes un polifenolu oksidāzes aktivitātes pieaugums, kas apliecināja etilēna kā signāla nozīmi *Trichoderma* izraisītās izturības pret *P. syringae* nodrošināšanā.

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Intracellular redox state regulates the resistance of *Zymomonas mobilis* alcohol dehydrogenase II to cyanide and oxygen

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Abstract

The variable sensitivity of *Zymomonas mobilis* iron-containing alcohol dehydrogenase isoenzyme (ADH II) to cyanide and oxygen was studied. The cyanide-sensitivity of ADH II was highest in cells grown under conditions of vigorous aeration, in which intracellular NADH concentration was low. Anaerobically grown bacteria, as well as those cultivated aerobically in the presence of cyanide, maintained a higher intracellular NADH pool along with a more cyanide-resistant ADH II. In aerobically grown permeabilized cells, cyanide caused gradual inhibition of ADH II, which was largely prevented by externally added NADH, and, to lesser extent, by NAD. It was demonstrated that cyanide acted as a competitive inhibitor of ADH II, competing with nicotinamide nucleotides. NADH increased both the cyanide resistance and oxygen resistance of ADH II.

Key words: alcohol dehydrogenase, cyanide resistance, oxygen resistance, *Zymomonas mobilis*.

Introduction

There are two cytoplasmic alcohol dehydrogenase (ADH) isoenzymes (EC 1.1.1.1) in the Gram-negative, aerotolerant, ethanol-producing bacterium *Zymomonas mobilis*: a zinc-containing ADH I and an iron-containing ADH II (Kinoshita et al. 1985; Neale et al. 1986; Conway et al. 1987). Both of them are NAD^+-dependent, and together they carry out rapid and efficient synthesis of ethanol, which has attracted the attention of researchers for decades (Rogers et al. 1982; Sprenger 1996; Dien et al. 2003). ADH I is essential in the early stages of culture growth, while ADH II plays a key role later in the fermentation at high ethanol concentrations (O'Mullan et al. 1995). No ADH I-negative mutants have been reported so far, yet ADH II-negative mutants have been obtained and characterized by several authors (Wills et al. 1981; O'Mullan et al. 1995; Delgado et al. 2002). The absence of ADH II results in a prolonged generation time of the mutant strain, as well as impaired growth and ethanol synthesis during the late exponential and early stationary phases of culture (O'Mullan et al. 1995).

However, ethanol synthesis might not be the sole function of ADH II. It seems likely that iron-containing bacterial alcohol dehydrogenases are involved also in respiratory metabolism and oxidative stress response. There is strong evidence from aerobic chemostat experiments (Kalnenieks et al. 2002) that ADH II may function as a component of the

respiratory pathway of *Z. mobilis*. Under vigorous aeration it oxidizes ethanol and supplies the respiratory chain with NADH, while ADH I catalyses ethanol synthesis, thus forming an "ethanol cycle". Notably, iron-containing alcohol dehydrogenases themselves are sensitive to oxygen. Under oxic conditions, ADH II looses much of its activity, apparently due to free radical reactions of oxygen at the active site iron (Tamarit et al. 1997). The same is true for *E. coli* iron-containing isoenzyme ADH-E, homologous to *Z. mobilis* ADH II, which also is highly sensitive to metal-catalyzed oxidation (Membrillo-Hernández et al. 2000). It has been demonstrated that ADH-E needs to be protected against oxidative damage by the chaperone DnaK during aerobic growth (Echave et al. 2002). Besides, ADH-E also acts as a H_2O_2 scavenger and, becoming partially inactivated, protects *E. coli* cells against hydrogen peroxide stress (Echave et al. 2003).

Recently we demonstrated another property of ADH II, traditionally associated with respiratory metabolism – its sensitivity to inhibition by cyanide (Kalnenieks et al. 2003). Inhibition of ADH II at submillimolar cyanide concentrations proceeds gradually, and presumably, reflects slow binding of cyanide to the active site iron. Moreover, the cyanide-sensitivity of ADH II can change. When cells are grown aerobically in the presence of submillimolar cyanide concentrations, ADH II largely looses its sensitivity to cyanide (Kalnenieks et al. 2003). The sensitivity change takes several hours, and the peak of ADH II cyanide resistance coincides with the paradoxical stimulation of aerobic growth in the presence of cyanide (Kalnenieks et al. 2000; 2003). The nature of the variable ADH II cyanide resistance remains an open question. In the present work our aim was to study the mechanism of this phenomenon.

Knowing that cyanide rapidly inhibits the respiratory chain of *Z. mobilis* (Kalnenieks et al. 2000; 2003), a cyanide-dependent shift of intracellular redox cofactor balance could be anticipated as another major effect of this inhibitor. We put forward a working hypothesis postulating that the cyanide-dependent variation of ADH II cyanide-sensitivity was related to the change of the intracellular NADH/NAD^+ ratio. Therefore, in the present work we studied the effects of NADH and NAD^+ on the kinetics of ADH II inhibition by cyanide.

Materials and methods

Bacterial strain and cultivation

Z. mobilis ATCC 29191 was maintained and cultivated at 30 °C in a growth medium containing glucose (50 g l^{-1}), yeast extract ('Difco'; 5 g l^{-1}) and mineral salts, as described previously (Kalnenieks et al. 1993). Aerobic chemostat cultivation was carried out in a 'Labfors' fermenter ('Infors') with 1.5 l culture volume, air flow at 3 l min^{-1} and stirring speed of 400 rpm, pH 6.0 with or without constant cyanide feed (Kalnenieks et al. 2000). Under these conditions a steady oxygen concentration, typically in the range between 40 and 50 % of saturation, was established. Vigorously aerated batch cultivations were carried out on a shaker at 160 rpm in 0.75 l unbaffled flasks containing 50 ml culture. Oxygen supply in these cultures was comparable to that in the aerobic chemostat, as judged from the specific rate of acetaldehyde production, which was similar in both cases (not shown). For cultivations or incubations under moderate aeration, shaking (with smaller amplitude) at 100 to 125 rpm in a water bath was applied to 100 ml unbaffled flasks containing 30 to 50 ml culture. Batch cultures referred to as 'anaerobic' were grown under oxygen-limited conditions in 0.5 l flasks containing 0.4 to 0.5 l culture incubated without shaking.

Permeabilization of cells

Cells were permeabilized following a slightly modified procedure of Osman et al. (1987). Bacteria were pelleted by centrifugation and resuspended at 7 g dry mass ml^{-1} in 30 mM potassium phosphate buffer, pH 6.5, containing 2 mM $MgCl_2$. One milliliter of the obtained suspension was centrifuged, and the pellet was resuspended in 0.2 ml of the same buffer, containing 0.2 mg lysozyme. After that, 15 ml of chloroform was added, the sample was vortexed for 45 s, and placed on ice for 10 min. Then, 0.8 ml of ice-cold buffer was added, and the suspension was used for incubations and measurement of ADH activities, methanol solution of chloramphenicol (10 mg ml^{-1}) was added to prevent *de novo* protein synthesis. As shown previously (Kalnenieks et al. 2003), an advantage of this permeabilization procedure (in contrast to ultrasonic breakage of cells) was that it made ADH accessible to external NADH, while totally inactivating NADH oxidase.

Alcohol dehydrogenase assays

Alcohol dehydrogenase activity was estimated in the direction of ethanol oxidation, as described by Neale et al. (1986). The total ADH activity and the activity of ADH I were measured spectrophotometrically at 340 nm after transfer of an aliquot (10 to 20 ml) of the cell suspension into a cuvette with 1.5 ml of 30 mM Tris-HCl buffer, pH 8.5, containing 1 mM NAD^+. The rate of NADH generation was monitored at room temperature. For measurement of the total activity of both isoenzymes, 1 M ethanol was added to the buffer. Discrimination between the two isoenzyme activities was based on the fact that only ADH I, but not ADH II, could oxidize butanol. For measurement of the ADH I butanol-oxidizing activity, which was taken to be half of the ADH I ethanol-oxidising activity (Kinoshita et al. 1985), 200 mM butanol was added in place of ethanol. ADH II activity was found by subtraction of the estimated ADH I ethanol-oxidizing activity from the total ethanol-oxidizing activity. Control assays showed that without ethanol or butanol in the reaction buffer NADH generation did not take place. Furthermore, permeabilised cells did not oxidise externally added NADH.

Analytical methods

Intracellular NAD(P)H concentrations were determined luminometrically with a LKB 'Wallac 1251' luminometer, using the 'Roche' bacterial luciferase assay, as described previously (Karp et al. 1983; Kalnenieks et al. 2002). Calculation of the intracellular NAD(P)H concentration was based on the published value (3.3 ml mg^{-1} dry mass) of *Z. mobilis* intracellular volume (DiMarco, Romano 1985). The time-course of the intracellular NAD(P)H concentration was monitored fluorimetrically (Chance et al. 1979), using an excitation filter with the spectral maximum around 366 nm, and an emission filter with the maximum around 515 nm. Cell concentration was determined as optical density at 550 nm, and dry cell mass of the suspensions was calculated by reference to a calibration curve. Microsoft Excel software was used for calculations, statistical treatment and plotting of data. If not stated otherwise, all chemicals were purchased from 'Sigma'.

Results

Competitive binding of cyanide at the active site of ADH II

Permeabilized cell suspension, obtained from an aerobically cultivated overnight batch

culture, was incubated at 0 °C for 1 h with added NAD^+ (final concentration 100 or 500 mM) and various potassium cyanide concentrations (0 to 500 mM), and both ADH activities were then measured. The dependence of the calculated initial rates of ADH II-catalyzed ethanol oxidation on the inhibitor concentration at both NAD^+ concentrations was plotted on Dixon coordinates (Fig. 1). According to this plot, cyanide acted as a competitive inhibitor of ethanol oxidation, competing with the oxidized cofactor. The apparent inhibition constant K_i was close to 200 mM.

It was not possible to apply the same straightforward approach to the opposite reaction (acetaldehyde reduction), because under the given assay conditions acetaldehyde rapidly reacted with cyanide (unpublished observation). Therefore, to estimate the competition betwen NADH and cyanide, we used an indirect assay. The permeabilized cell suspension was incubated at 0 °C with various NADH or NAD^+ concentrations (0, 20, 200 and 2000 mM) for 20 minutes. Then cyanide was added to a final concentration of 200 mM, and incubation was continued for an additional 45 min. Control samples were incubated under similar conditions without cyanide addition, and their activity remaining after the incubation period was taken as the reference (100 %) for the data shown in the Fig. 2A. After incubation, ADH activity was monitored exactly as in the previous experiment, but using assay buffer without cyanide, which contained standard amounts of NAD^+ and ethanol (or butanol). It was assumed that the initial rate of ADH II reaction in each sample would depend on cyanide previously bound to the enzyme, and, hence, on the competition between cyanide and the cofactors during the incubation phase.

The results showed that the percentage of ADH II activity that remained after a 45-min incubation of permeabilized cells with 200 mM cyanide, indeed strongly depended on the cofactor concentration during the incubation phase (Fig. 2A). NAD^+ caused a small but significant increase of cyanide resistance of ADH II at 200 and 2000 mM concentrations. NADH had a much more pronounced effect, at higher concentrations turning ADH

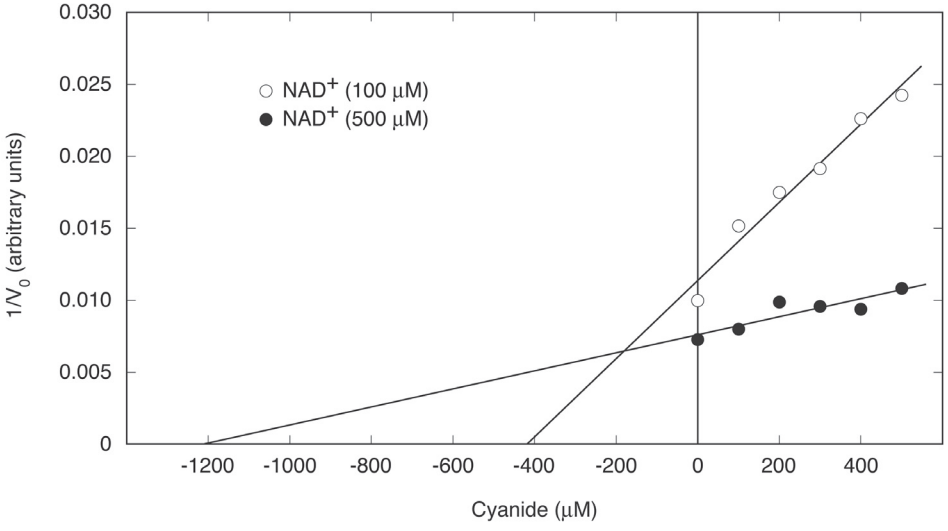

Fig. 1. Dixon plot of ADH II inhibition by cyanide at different NAD^+ concentrations. The plotted values are means of three independent experiments (see comments in the text).

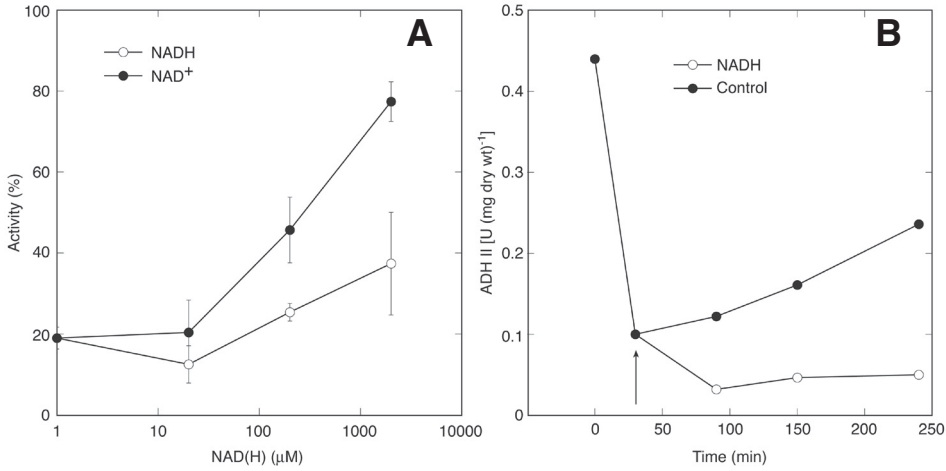

Fig. 2. Elevation of cyanide-resistance of ADH II in the presence of NADH and NAD^+. A, ethanol-oxidising activity of ADH II remaining after a 45-min incubation of aerobically grown permeabilized cell suspension at 0 °C with 200 mM cyanide in the presence of various NADH (open symbols) or NAD^+ (closed symbols) concentrations. In the control samples, incubated without cyanide, the activity of ADH (taken as 100 %) was 0.22 (± 0.06) U mg^{-1} dry mass for ADH I and 0.36 (± 0.20) U mg^{-1} dry mass for ADH II. Mean values of three to five (± SEM) experiments are presented. B, time course of ADH II ethanol-oxidising activity in a permeabilised cell suspension, incubated on a shaker at 15 °C with 200 mM cyanide; 2000 mM NADH added (open symbols), where indicated by an arrow; control (closed symbols) incubated without NADH addition.

II almost insensitive to cyanide. Notably, addition of NADH partially restored ADH II activity. Fig. 2B shows the time-course of gradual restoration of the ADH II activity after NADH addition to permeabilized cells previously treated with cyanide. We speculate that the elevation of cyanide resistance and restoration of ADH II activity can be explained by competition between NADH and cyanide for binding at the active centre of ADH II. The fact that NADH more efficiently competed with cyanide than did NAD^+ was in good agreement with the reported K_M values for both ADH II cofactors (Kinoshita et al. 1985): 12 mM for NADH and 110 mM for NAD^+. In general, this finding means that elevated intracellular NADH concentrations (increased NADH/NAD^+ ratio) can, in principle, increase the apparent cyanide-resistance of ADH II.

Effect of the redox state of culture on the cyanide-sensitivity of ADH II

In order to verify the putative importance of intracellular NADH concentration for ADH II cyanide-resistance in growing cells, bacteria were cultivated under different conditions of aeration and cyanide, and then: (i) cyanide sensitivity of both alcohol dehydrogenases was assayed in permeabilized cell suspensions, and (ii) intracellular NAD(P)H concentrations were determined. The percentage of enzymatic activity remaining after 45 min of incubation of the permeabilized cells with 200 mM cyanide at 0 °C (relative to that of the control sample, incubated for the same period of time without cyanide addition), is presented in Fig. 3A. In agreement with our previous observations (Kalnenieks et al. 2003), ADH I was almost insensitive to cyanide under all experimental conditions. However,

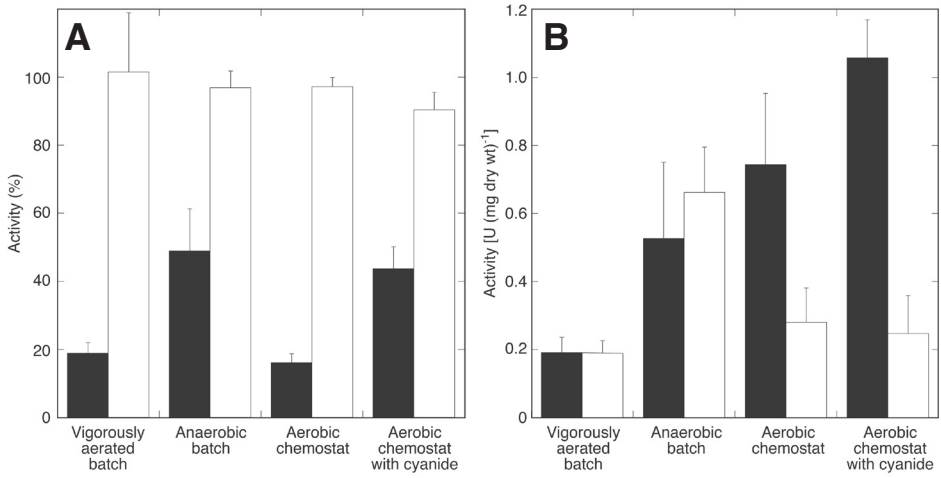

Fig. 3. Dependence of ADH cyanide-sensitivity on culture conditions. Samples for cell permeabilisation were taken from batch cultures in their early stationary phase (after 18 to 20 h of growth), or from steady-state chemostat cultures. Permeabilised cell suspensions were incubated with 200 mM cyanide for 45 min at 0 °C, and the remaining activities (relative to the respective controls without cyanide) of ADH I (empty bars) and ADH II (filled bars) were determined. A, ADH relative activities remaining after incubation with cyanide; B, ADH activities in control samples.

culture aeration strongly affected the cyanide sensitivity of ADH II. It was particularly sensitive to cyanide in aerobically cultivated samples. In permeabilized cells taken from a vigorously aerated chemostat or batch culture, the activity of ADH II after 45 min had decreased to below 20 % of the corresponding control value. At the same time, ADH II in the cells from an anaerobic batch, or from an aerobic cyanide-fed chemostat, retained 40 to 45 % of its activity. Notably, the cyanide resistance of ADH II was not correlated with its absolute activity (Fig. 3B). Elevation of ADH II cyanide resistance in cells grown aerobically with cyanide was demonstrated in our previous paper (Kalnenieks et al. 2003). The present results show that cyanide *per se* is not essential for the increase of ADH II cyanide-resistance. Anaerobic growth conditions led to an equally cyanide-resistant ADH II (Fig. 3), for which the presence of cyanide in the culture medium had no extra effect (not shown).

Also, the intracellular concentrations of reduced nicotinamide nucleotides in *Z. mobilis* depended very much on the culture redox conditions. In Fig. 4, intracellular NAD(P)H concentrations under three different modes of cultivation are presented. The intracellular NADH concentration varied over a range of almost two orders of magnitude, while the NADPH concentration varied to a somewhat lesser extent. A large difference in NADH concentrations (more than an order of magnitude) was seen between a vigorously aerated culture (in a fermenter with 1 l working volume, 3 l min^{-1} air flow, stirring at 400 rpm) and a moderately aerated culture (on a water bath shaker). Doubling of the intracellular NADH concentration (from approx. 300 to 600 mM) took place in 1 h after cyanide addition at a 200-μM final concentration to the moderately aerated batch culture. In good accordance with our data, an intracellular NADH concentration close to 400 mM was reported previously for an anaerobic batch culture during exponential and early stationary

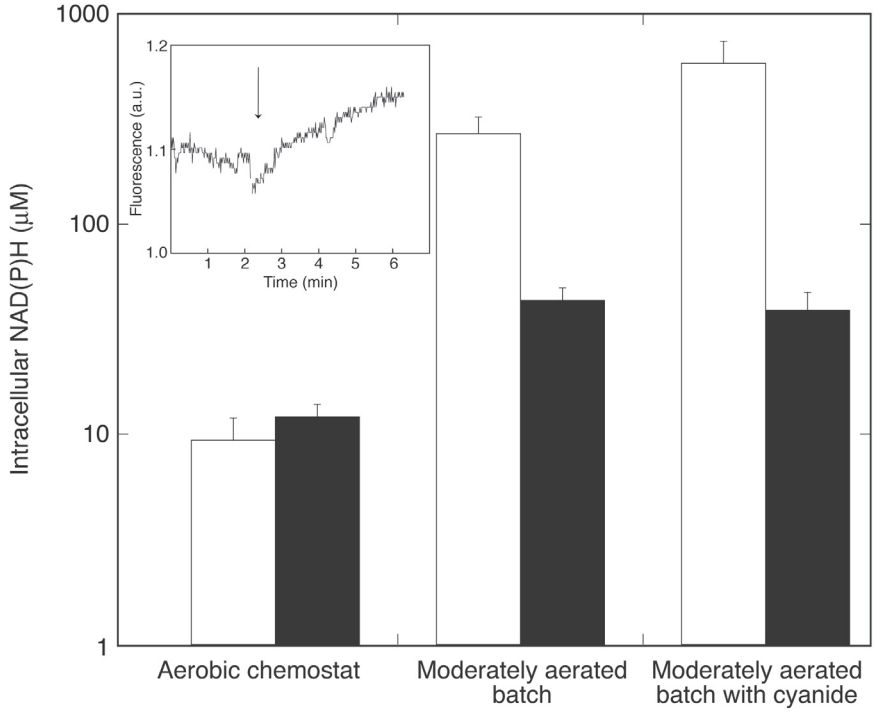

Fig. 4. Intracellular NADH (empty bars) and NADPH (filled bars) concentrations at various cultivation conditions. Mean results of three experiments are presented (± SEM). Inset: the time-course of intracellular NAD(P)H concentration in a suspension of aerobically grown cells after addition of glucose (indicated by the arrow, 1 % final concentration), as monitored fluorimetrically (arbitrary units given).

growth phase (Osman et al. 1987). The characteristic transition times between different NAD(P)H concentration steady-states were in the range of several minutes, as illustrated by the fluorescence data (Fig. 4, inset).

We noted that the range of variation of the ADH II cyanide resistance (Fig. 3) and the corresponding variation of intracellular NADH levels (Fig. 4), fitted reasonably well into the relationship obtained for permeabilized cells with external NADH (Fig. 2A). This finding points to the quantitative relevance of intracellular NADH variation for explaining the observed changes of ADH II cyanide resistance under different growth conditions.

Given that the oxidative damage of ADH II is due to exposure of the active site iron to oxygen (Tamarit et al. 1997), one might speculate that binding of NADH protects the enzyme also against oxidative damage under aerobic conditions. Indeed, data presented in Fig. 5 show a dramatic increase of ADH II resistance to oxygen in permeabilized cells with added external NADH. A suspension of permeabilized cells was incubated at 125 rpm in a water bath shaker at 15 °C. Without addition of external NADH, the activity of ADH II after 4 h of incubation had decreased more than five times, in good agreement with the previously reported results (Tamarit et al. 1997). Yet, even at the lowest concentration of externally added NADH (20 mM), the inactivation of ADH II proceeded much slower:

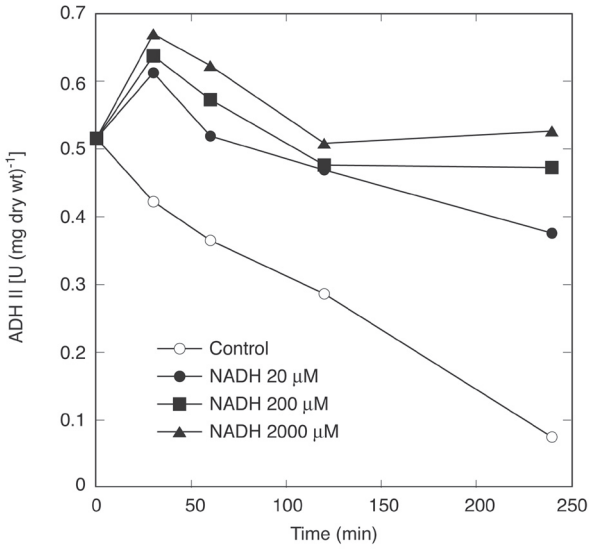

Fig. 5. Time-course of the ethanol-oxidising activity of ADH II in permeabilized cells, incubated on a shaker at 15 °C in the presence of various NADH concentrations. .

only about 20 % of activity was lost after 4 h of incubation. At higher NADH concentrations the activity remained fairly stable.

Discussion

Cyanide is an important ecological factor for microorganisms. It appears in industrial wastewaters, from which various cyanide-degrading species have been isolated (Dubey, Holmes 1995). Many plant species are cyanogenic, particularly in equatorial areas (Francisco, Pinotti 2000): they produce cyanogenic glycosides, which serve to protect the plant against herbivores. Cyanide can be expected to influence also the cyanogenic plant interactions with microorganisms. *Zymomonas mobilis* was first isolated in Mexico from fermenting *Agave americana* juice (Swings, deLey 1977). Although *A. americana* does not belong to the group of cyanogenic plants (Francisco, Pinotti 2000), *Z. mobilis* might well encounter other species in its natural habitat, which are cyanogenic. Therefore, the unusual physiological response of *Z. mobilis* to cyanide might have implications for its natural life cycle, which is poorly investigated.

Z. mobilis responds to cyanide addition to the growth medium in a specific way. Growth in the presence of cyanide does not induce any spectroscopically detectable changes in the *Z. mobilis* respiratory chain (Kalnenieks et al. 2000), nor does it alter the cyanide sensitivity of membrane respiration (Kalnenieks et al. 2003). Instead of expression of a cyanide resistant respiratory pathway, typical for bacteria (Ashcroft, Haddock 1975; Knowles 1976; Kita et al. 1984), *Z. mobilis* elevates the cyanide-resistance of its alcohol dehydrogenase isoenzyme, the iron-dependent cyanide-sensitive ADH II (Kalnenieks et al. 2003). Here we show that the cyanide-sensitivity of ADH II depends on the intracellular redox state, namely, on the concentration of NADH. We hypothesize a simple mechanism

for the variable cyanide-sensitivity of ADH II: the cyanide-resistant (and oxygen-resistant) form of this enzyme is the one with NADH bound to the active site, while the cyanide-sensitive form is the one without a cofactor or, most probably, with a bound NAD^+ (as NAD^+ is more easily replaced by cyanide than is NADH). Under vigorous aeration, ADH II apparently operates in the direction of ethanol oxidation (Kalnenieks et al. 2002) in a microenvironment of high NAD^+ and low NADH concentrations; hence its elevated sensitivity to cyanide in aerobically growing culture is easily explained.

We can summarize the sequence of events after cyanide addition to an aerobic culture and put forward a working hypothesis, explaining the gradual emergence of the cyanide-resistant form of ADH II: (i) inhibition of respiration is the immediate effect of cyanide, (ii) which causes a rise of intracellular NADH level, (iii) the excess NADH occupies the active sites of ADH II, outcompeting cyanide, as well as protecting the enzyme molecule against oxidative damage. The result of these events is a gradual emergence of the "cyanide-resistant" (NADH-bound) form of ADH II, which replaces the inhibited (cyanide-bound) form of ADH II, and, at the same time, is protected against oxygen. Thus, the aerobic culture with cyanide grows in a largely anaerobic manner: it respires slower (Kalnenieks et al. 2000; 2003), yet maintains a larger fraction of active ADH II molecules with bound NADH, obviously participating in ethanol synthesis under oxic conditions.

As far as we know, ADH II is a unique example of an enzyme with an active center, in which nicotinamide cofactors compete for binding with cyanide. Such a dependence of ADH II ligand-binding properties on the intracellular redox cofactors places it in the position of a putative key regulator of respiratory metabolism and ethanologenesis. Interestingly, the NADH/NAD^+ ratio (Leonardo et al. 1996) and, under certain conditions, possibly, *Fnr* (Membrillo-Hernandez, Lin 1999) were demonstrated to act as transcriptional regulators for another iron-containing ADH, the product of the *adhE* gene in *E. coli*. Taking into account the present results, we may speculate that in general the intracellular redox conditions affect the activity of bacterial iron-containing alcohol dehydrogenases at several regulatory levels. The details of transcriptional regulation of *Z. mobilis adhB*, however, still need to be elucidated. Another intriguing question for further study remains, whether ADH II can bind other ligands of physiological significance (e. g. nitric oxide) in a redox cofactor-dependent manner.

Acknowledgements

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Iekššūnas redoks-statuss regulē *Zymomonas mobilis* alkoholdehidrogenāzes II rezistenci pret cianīdu un skābekli

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Kopsavilkums

Darba mērķis bija noskaidrot mehānismu, kas nosaka baktērijas *Zymomonas mobilis* dzelzi saturošās alkoholdehidrogenēzes izoenzīma (ADH II) mainīgo cianīda jutīgumu un jutību pret skābekli. Visaugstāko ADH II cianīda jutīgumu novēroja šūnās, kuras kultivēja intensīvas aerācijas režīmā, kad iekššūnas NADH koncentrācija ir zema. Anaerobi augušajās baktērijās, kā arī tajās, kuras kultivēja aerobi cianīda klātbūtnē, gan ADH II cianīda izturība, gan arī iekššūnas NADH koncentrācija bija augstāka. Aerobi audzētās permeabilizētās šūnās cianīds izraisīja pakāpenisku ADH II inhibēšanu, kuru mazināja NADH un, mazākā mērā, NAD pievienošana. Parādīts, ka cianīds darbojas kā ADH II konkurentais inhibitors, konkurējot ar nikotīnamīda kofaktoriem. NADH palielina kā ADH II izturību pret cianīdu, tā arī tās noturību pret gaisa skābekļa iedarbību.

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The effect of stringent control on valine biosynthesis by *Corynebacterium glutamicum*

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Abstract

The present study focused on methods of stringent response induction and the investigations of its effect on valine synthesis by the isoleucine auxotroph *Corynebacterium glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*. The intracellular concentration of guanosine tetraphosphate increased and bacterial growth rate decreased, i.e. intracellular stringent response, was induced under conditions of (i) cell culture growth in medium lacking isoleucine and (ii) reduced isoleucine uptake by cells. The induction of the cellular stringent response was followed by an increase in the activity of acetoxydroxy acid synthase, e.g. *ilvBN*-encoded key enzyme of valine synthesis, and by a drastic increase in the cell specific rate of amino acid synthesis. It was demonstrated that induction of the stringent response can be used as method to increase valine overproduction by *C. glutamicum* cells.

Key words: *Corynebacterium glutamicum*, ppGpp, stringent control, valine biosynthesis.

Introduction

The stringent response is a pleiotropic physiological response of bacterial cells to amino acid or energy source limitation (Cashel et al. 1996). Many features of the stringent response are mediated by the accumulation of hyperphosphorylated guanosine nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp). Usually two related proteins RelA and SpoT are involved in the metabolism of ppGpp in bacterial cells. The RelA protein encoded by *relA* gene has synthase activity and is responsible for ppGpp biosynthesis during amino acid limitation. *RelA* is activated when uncharged tRNA binds to the acceptor site of a translating ribosome. The SpoT protein encoded by the *spoT* gene is primarily a ppGpp hydrolase, but it also catalyzes ppGpp synthesis in response to glucose starvation (Cashel et al. 1996).

The *relA*- and *spoT*-regulated stringent mechanism has been described in gram negative bacteria *Escherichia coli* and *Salmonella typhimurium* (Cashel et al. 1996). In contrast, a *relA*/*spoT* homologous gene (*rel*) that encodes a bifunctional enzyme with ppGpp synthase and ppGpp-degrading activities has been described in the gram positive bacterium *Corynebacterium glutamicum* (Wehmeier et al. 1998), also in *Streptococcus equisimilis* (Mechold, Malke 1997), *Bacillus subtilis* (Wendrich, Marahiel 1997) and *Mycobacterium tuberculosis* (Tauch et al. 2001).

Intracellular ppGpp in bacterial cells functions as an organiser of the adaptive cellular response to starvation. ppGpp co-ordinates the global transcriptional pattern with the

current growth conditions: it mediates feedback repression (negative control) or even induction of the expression (positive control) of many genes in bacterial cells. Since ppGpp serves as a specific alarmone of the stringent response, it may be used as an indicator of starvation for amino acids and an energy source in bacterial cells (Cashel et al. 1996).

The negative stringent control usually concerns reactions typical of growth and reproduction: an immediate reduction of the ribosomal RNA (*rrn*) operon transcription and a massive reduction of the synthesis of stable RNA (rRNA and tRNA; Cashel et al. 1996; Zhang et al. 2002), also a reduction of the synthesis of a certain mRNAs and an increase in the rate of protein degradation in bacterial cells (Lewin 2000). The accumulation of ppGpp in bacterial cells can also cause negative effects on the biosynthesis of lipids, polyamines and peptidoglycan (Cashel et al. 1996). All of these negative effects lead to changes in bacterial physiology, predominantly to the reduction of bacterial growth rate.

In contrast, in some cases ppGpp appears to have a positive regulatory effect on the expression of genes and on the translation level of enzymes involved in the biosynthesis of some amino acid. Induction of the ppGpp-mediated *relA*-dependent stringent control was found to be required to increase the expression of the genes encoding branched-chain amino acid synthesis in *E. coli*, *S. enterica* serovar typhimurium (Tedin, Norel 2001) and *B. subtilis* (Eymann et al. 2002). It has also been shown that induction of this control is required to elevate the translation level of branched chain amino acid synthesis enzymes in *B. subtilis* (Eymann et al. 2002). However, further research is required to establish whether the synthesis of branched-chain amino acids in other bacteria is under the strong control by stringent response mechanism as well.

The gram-positive soil micro-organism *Corynebacterium glutamicum* is of a special interest for the industrial production of amino acids. During the recent 40 years various mutants of *C. glutamicum* have been isolated with the capacity to produce significant amounts of different L-amino acids (glutamic acid, lysine, threonine and others). In spite of the great demand on the world-wide market, industrial production of L-valine with this or another bacteria is still not developed. The main reason is a complicated control of valine synthesis in bacterial cells. For example, acetohydroxy acid synthase (AHAS), the key enzyme of valine biosynthesis in *C. glutamicum* is common for the synthesis of all branched chain amino acids and is feedback inhibited by all of them (Morbach et al. 2000). Hence, it is very important to construct strains that will aid to increase our knowledge about the cellular metabolism control and to find methods to increase valine overproduction in bacterial cells.

It has been reported that lysine synthesis by *C. glutamicum* RC 115 can be significantly enhanced by the induction of the ppGpp-mediated stringent response (Ruklisha et al. 1995; Ruklisha et al. 2001). This effect was explained as an increase in lysine synthesis activity in cells as a consequence of an increased intracellular precursor availability.

The goal of this study was to identify methods of the stringent response induction in *C. glutamicum* cells and to investigate the effect of this response on valine synthesis by its producing bacteria.

Materials and methods

Bacterial strains and culture conditions

The strain used in this study was *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*. This strain,

auxotrophic for isoleucine, was shown to be able to excrete L-valine and D-pantothenic acid (Sahm, Eggeling 1999).

Brain heart infusion (BHI) medium was used for pre-culture growth. CG XII minimal medium (Keilhauer et al. 1993) with some modifications (isoleucine concentration 0.57 mM or 1.14 mM, 3 mg l^{-1} deferoxamine instead of 30 mg l^{-1} protocatechuic acid, 2 mg l^{-1} biotine instead of 0.2 mg l^{-1} and 50 mg ml^{-1} kanamycin) was used for main culture growth. The pH was maintained at 7.0 due to the addition of 42 g l^{-1} MOPS in the culture medium. The cultivations were performed at 30 °C in buffled Erlenmeyer flasks on a rotary shaker (220 rpm). At least three runs of experiments were performed in order to estimate kinetic parameters.

Physiological parameters

Bacterial growth was followed by measuring the optical density of the cell culture at 600 nm in a Helios UV-Visible spectrophotometer (Thermo Spectronic, UK). Concentrations of amino acids in the cell culture were quantified by HPLC method as described previously (Ruklisha, Paegle 2001). Using the kinetic experimental data for measured biomass and valine concentrations, the specific rates of bacterial growth (μ) and valine synthesis (q_p) were calculated (Ruklisha et al. 1995).

Determination of acetoxydroxy acid synthase activity

The cells were harvested by centrifugation for 15 min at 10 000 g_n at 4 °C, washed twice with 2 % KCl and resuspended in 100 mM potassium phosphate buffer (pH 7.3) containing 0.5 mM ditiothreitol and 20 % (v/v) glycerol. The cells were disrupted by pulsed sonication ('Dr. Hielscher' ultrasonic processor, Germany) of 8 min total duration, with 0.5-s pulses and 0.5-s intervals between them. Cell debris and intact cells were separated from the cell extract by centrifugation (30 min, 4 °C, 12 000 g_n). The activity of acetoxydroxy acid synthase (AHAS, EC 4.1.3.18) was assayed in the cell-free extracts as described by Leyval et al. (2003). The method is based on the conversion of pyruvate to α-acetolactate, which was subsequently decarboxylated to acetoin and detected by the colorimetric method of Westerfeld (1945) using a Helios UV-Visible spectrophotometer (Thermo Spectronic, UK). The AHAS specific activity was expressed in nmol of α-acetolactate formed per mg protein per min. The protein concentration in cell free extracts was determined by Lowry's method (Lowry et al. 1951).

Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) assays

ppGpp was extracted from the cells with 0.2 M KOH as described by Zhang et al. (2002). Nucleotides in cell extracts were separated by isocratic ion exchange HPLC (Waters 501, USA) with a 4.6 × 250 mm Hypersil Sax 5 μm column (Alltech, Belgium), using a 0.03 M potassium phosphate buffer, pH 3.4, supplemented with 14 % acetonitrile and 0.01 M tetrabutylammonium phosphate as the mobile phase. ppGpp was quantified by measuring absorbance at 254 nm using a Tunable Absorbance Detector (Waters 486, USA).

Chemicals

ppGpp was obtained from TrilinkBiotech (USA). BHI was purchased from Liofilchem (Italy). All other biochemicals were of analytical grade and purchased from Sigma-Aldrich.

Results

In order to induce the stringent response in bacterial cells and to estimate the consequences of this response on valine synthesis, short-term experiments of *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* growth under isoleucine-unlimited or limited conditions (control and experimental version, respectively) were carried out.

Cells were precultured in a modified CGXII minimal medium with isoleucine concentration 0.57 mM. Cell cultures in the exponential growth phase ($\mu = 0.20 \pm 0.01$ h^{-1}) were harvested by centrifugation, then washed with CGXII minimal medium lacking isoleucine and resuspended in a medium either with or without 1.14 mM isoleucine. Cells were re-cultivated for 7 h.

The experimental results showed that bacterial growth was significantly influenced by isoleucine limitation (Fig. 1): the biomass concentration in the cell culture subjected to isoleucine starvation for 3.5 h was 1.9 times lower in comparison with the concentration achieved in a standard medium. Also, the calculated value of the specific growth rate of cells cultured under isoleucine limited conditions was low ($\mu = 0.06 \pm 0.00$ h^{-1}), compared to the value achieved by cells cultured in a standard medium ($\mu = 0.28 \pm 0.01$ h^{-1}). The intracellular concentration of ppGpp showed a slight increase after 1 to 2 h and a drastic increase after 3.5 h of cell culture growth under isoleucine limited conditions. The final intracellular concentration of ppGpp under the latter conditions reached 0.27 nmol mg^{-1} DM (Fig. 1). In contrast, intracellular ppGpp was not detected in cells grown under isoleucine-unlimited conditions.

To verify the effect of isoleucine limitation on ppGpp synthesis and the stringent response induction in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells, additional experiments were carried out: isoleucine uptake by cells was reduced by supplementation of the cell culture with valine. As competition occurs between isoleucine and valine for uptake by BrnQ, a carrier that transports all branched-chain amino acids into *C. glutamicum* cells (Lange et al. 2003), the inhibition of isoleucine uptake can be achieved by increased concentrations of valine in the medium. High concentrations of valine in the medium can eventually evoke the same isoleucine starvation effect in bacterial cells.

The scheme of experiments for investigating the effect of isoleucine uptake reduction on bacterial growth and ppGpp accumulation in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*

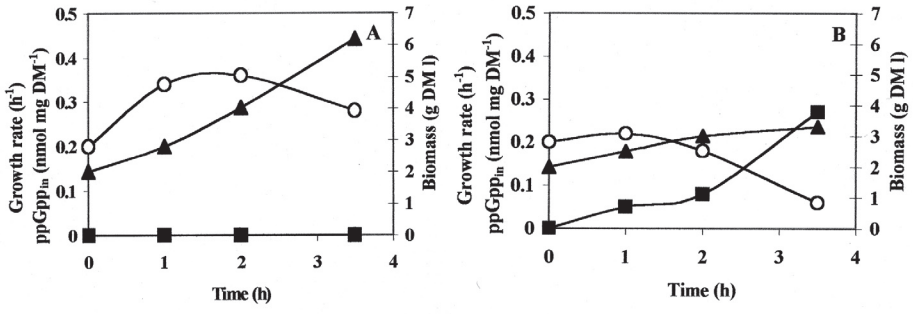

Fig. 1. Changes in biomass concentration (▲), bacterial growth rate (❍) and ppGpp concentration (■) in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under isoleucine unlimited (A) or isoleucine limited (B) conditions achieved by its extracellular limitation.

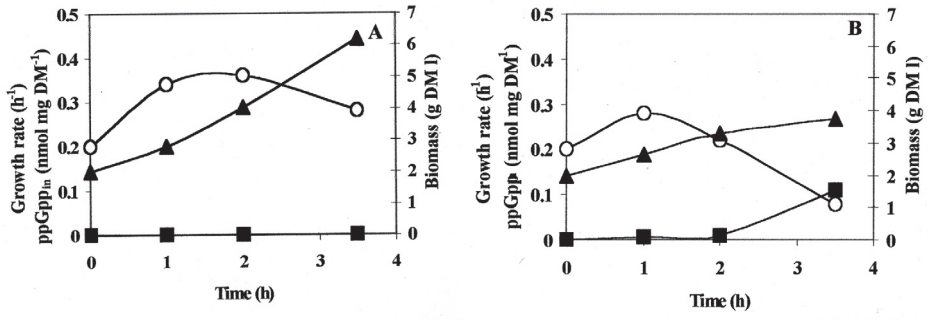

Fig. 2. Changes in biomass concentration (▲), bacterial growth rate (❍) and ppGpp (■) concentration in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under isoleucine unlimited (A) or isoleucine uptake limited (B) conditions achieved by cell culture supplementation with 175 mM valine.

cells was similar to that used to estimate the effect of isoleucine limitation in the medium. Cells were precultured in CGXII minimal medium with an isoleucine concentration of 1.14 mM for 4 h. Then the cell culture was collected and re-cultivated in CGXII medium without or with 175 mM valine for 7 h.

The re-cultivations showed a significant decrease in biomass formation and bacterial growth rate, as well as an increase in the intracellular concentration of ppGpp under conditions when cell culture was exposed with 175 mM valine (Fig. 2).

There was an inverse relation between biomass formation and intracellular ppGpp accumulation in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under conditions with extracellularly limited isoleucine or those with restricted isoleucine uptake, and ppGpp accumulation was absent in cells grown under isoleucine unlimited conditions. This indicated that *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* metabolism under both of these conditions might be regulated in a stringent response manner using ppGpp as an alarmone.

Further, the effect of the stringent response on valine biosynthesis by *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* was investigated. It was observed that an increase in ppGpp accumulation was followed by a significant increase in AHAS activity and an increase in the specific rate of valine synthesis of cells subjected to isoleucine starvation (Table 1). This suggested that the stringent response might lead to an increase in valine overproduction.

It should be noted that a more significant increase in the cell specific rate of valine synthesis observed under stringent response conditions induced in *C. glutamicum* cells by the restriction of isoleucine uptake. Thus, besides AHAS activity, other intracellular conditions (intracellular precursor concentrations or others) might also be important enhancing the valine synthesis activity of bacterial cells under the stringent response conditions.

Discussion

The intracellular concentration of ppGpp increased and the specific growth rate of *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells decreased under conditions of isoleucine limitation e.g. bacterial growth in a medium lacking isoleucine or those with reduced

Table 1. The effect of isoleucine limitation on 5'-diphosphate 3'-diphosphate ($ppGpp_{in}$) concentration, acetohydroxy acid synthase (AHAS) activity and cell-specific rate of valine synthesis of *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD*. Parameters were estimated after 3.5 h ([a]) or 7.0 h ([b]) of cell culture growth under the specified conditions. The data shown are means and standard deviations from three runs of experiments with five replicates for each estimation

Culture conditions	Medium modification	ppGppin (nmol mg^{-1} DM)[a]	AHAS activity (nmol min^{-1} mg^{-1} protein)[a]	Valine synthesis (g h^{-1} g^{-1} DM)[b]
Isoleucine unlimited	–	0	362 ± 10	0.043 ± 0.002
Isoleucine limited	Isoleucine omittion	0.270 ± 0.008	590 ± 22	0.073 ± 0.003
Isoleucine limited	Supplementation with 175 mM valine	0.110 ± 0.005	692 ± 24	0.113 ± 0.004

isoleucine uptake by valine. In contrast, intracellular ppGpp was not detected in cells grown under isoleucine-unlimited conditions. Consequently, the $ppGpp_{in}$ concentration was increased and the stringent response was induced in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* cells under conditions of isoleucine limitation.

Further, the effect of the stringent response on valine synthesis by *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* was investigated. An increase in the activity of AHAS e.g. a key enzyme of valine synthesis, and the cell specific rate of valine synthesis was enhanced under conditions with extracellular or intracellular limitation of cell culture growth by isoleucine. Therefore, an increase in AHAS activity in cells was directly related with an increase in ppGppin concentration and inverse related with the specific bacterial growth rate and biomass concentration achieved in the cell culture. Consequently an increase in valine synthesis might be a result of the stringent response induced in bacterial cells by isoleucine limitation.

It has been reported that transcription of *ilvBNC* operon encoding acetoxydroxy acid synthase and isomeroreductase, e.g. key enzymes of L-isoleucine, L-valine and L-leucine synthesis in *C. glutamicum*, is repressed by an excess of branched chain amino acids (5 mM each) and that this transcription may be increased under limiting conditions for any of these amino acids (0.5 mM each) (Morbach et al. 2000). It has also been reported that exposure of *C. glutamicum* cells with 40 mM valine, demonstrating the effect of isoleucine uptake restriction, causes an increase in *ilvBN* mRNA levels, and also an increase in the intracellular level of the *ilvB* protein product i.e. the large subunit of AHAS (Lange et al. 2003). Therefore, isoleucine limitation may be a pre-condition for the derepression of transcription of genes encoding enzymes of branched chain amino acid synthesis, also a pre-condition for the increase of enzyme translational level in *C. glutamicum*. However, transcriptome analysis of *B. subtilis* wild type strain (*relA*$^+$) and *relA*$^-$ mutant proved that functioning of the *relA* gene was absolutely required for the derepression of transcription of *ilvBNC* operon under branched-chain amino acid limited conditions (Eymann et al. 2002). Moreover, a *relA*$^-$ mutation in *B. subtilis* resulted in a strain auxotrophy for valine and a weaker one for isoleucine, leucine and methionine (Wendrich, Marahiel 1997). It

was also shown that *relA*⁻ mutants of *E. coli* K-12 strains failed in derepression of *ilvBN* (Freundlich 1977). These investigations clearly demonstrated that transcription of operons encoding synthesis of branched chain amino acids in *B. subtilis* and *E. coli* K-12 was strongly dependent on *relA* and the stringent response induction. However it is not clear if synthesis of branched chain amino acids in other bacteria is under strong dependence on *relA* induction as well.

Our results suggest that an increase in AHAS activity in *C. glutamicum* under isoleucine limited conditions might be a consequence of the derepression of *ilvBN* operon. Correlation between the intracellular concentration of ppGpp, the activity of AHAS and the cell specific rate of valine synthesis, may indicate that the stringent response mechanism might directly control valine synthesis by this bacterium. However, further research is required to estimate whether *rel*-dependent, ppGpp mediated stringent response is required for induction of the transcription of operons encoding branched chain amino acid synthesis in *C. glutamicum*.

The effect of the stringent response on synthesis of branched chain amino acids and other metabolic processes in bacterial cells might also be indirect. Moreover, transcriptome and proteome analysis of *B. subtilis* wild type and *relA*⁻ mutant strain showed that some genes may be induced or downregulated independently of *relA* even under the stringent response conditions.

The positive effect of isoleucine limited conditions on valine synthesis by *C. glutamicum*, shown in this study, might be a result of the derepression of *ilvBN* transcription in response to isoleucine limitation, also a result of *rel*-dependent induction of *ilvBN* transcription. This means that valine synthesis in this bacterium might be indirectly or directly controlled by the stringent control mechanism.

It can not be excluded that valine synthesis in *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* might also be an indirect effect of the intracellular stringent response situation e.g. effect of an increased availability of precursors leading to increased activity of valine synthesis enzymes. AHAS in *C. glutamicum* exhibits a weak affinity for pyruvate (K_M = 8.3 mM) (Leyval et al. 2003). Consequently, the increase in intracellular pyruvate concentration might have a significant impact on the activity of this enzyme in bacterial cells.

Therefore, stringent response induction in *C. glutamicum* cells might be used as a method to enhance valine synthesis by this bacterium. However further research should be done to estimate whether an increase in valine overproduction by this bacterium is a direct consequence of the *rel*-dependent induction of valine synthesis encoding genes.

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***Stringent* mehānisma loma valīna biosintēzes regulācijā *Corynebacterium glutamicum* šūnās**

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Kopsavilkums

Pētījumu mērķis bija noskaidrot *stringent* kontroles nozīmi valīna biosintēzes regulācijā *Corynebacterium glutamicum* šūnās. *Stringent* kontroli izoleicīna auksotrofa *C. glutamicum* 13032 Δ*ilvA* pJC1*ilvBNCD* celma šūnās inducēja ārpussūnas izoleicīna ierobežojuma apstākļos, kā arī samazinot šīs aminoskābes transportu šūnās: abos gadījumos palielinājās iekššūnas guanozīna tetrafosfāta koncentrācija un samazinājās šūnu augšanas ātrums. Vienlaicīgi šīs kontroles inducēšana izraisīja krasu valīna sintēzes regulatorā fermenta acetohidroksiskābes sintēzes aktivitātes, kā arī šūnu valīna sintēzes specifiskā ātruma palielināšanos. Pētījumi liecina, ka *stringent* kontroles inducēšanu var pielietot, lai palielinātu valīna virssintēzi *C. glutamicum* šūnās.

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Impact of changes in agricultural land use on the Corncrake *Crex crex* population in Latvia

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Abstract

Data on Corncrake numbers and agricultural land use were collected in 68 freely chosen sample plots (0.67 - 44.38 km2; mean = 8.42; SD = 7.458) in Latvia 1984 - 2004. The annual TRIM index of Corncrake numbers in Latvia increased significantly during the study period ($p < 0.003$). The total area of all abandoned agricultural lands have increased significantly ($p < 0.0006$) at the expense of cultivated pastures ($p < 0.015$) in the survey plots. The area of intertilled crops also decreased ($p < 0.07$). The area of both cultivated and uncultivated meadows increased significantly during the period of 1989 - 1998 ($p < 0.005$), and decreased in 1999 - 2004 ($p < 0.025$). The habitat-specific annual TRIM index of Corncrake numbers was positively correlated with the TRIM index of area of uncultivated meadows ($p < 0.001$), all meadows combined ($p < 0.001$), uncultivated pastures ($p < 0.05$) and abandoned agricultural lands ($p < 0.0025$), but negatively with the TRIM index of area of intertilled crops ($p < 0.05$). The total Corncrake TRIM index was positively correlated ($p < 0.002$) with the total amount of precipitation during the Corncrake breeding season (May - July). The highest breeding density (on average – 3.05 males km^2) was observed in abandoned grasslands, followed by uncultivated meadows > abandoned arable lands > cultivated meadows > other (miscellaneous) habitats > uncultivated pastures > shrubland > winter crops > cultivated pastures > spring crops > intertilled crops. More Corncrakes than expected were observed in abandoned grasslands, uncultivated meadows and abandoned arable lands ($p < 0.001$), but Corncrake numbers were smaller in winter crops, cultivated pastures, spring crops and intertilled crops ($p < 0.001$). Despite the recent increase of the Corncrake numbers in Latvia, the projected long-term dynamics since 1940 show a significant decrease in numbers ($p < 0.0001$) due to decrease of area of suitable habitats (e.g. meadows) in Latvia.

Key words: agricultural land use, Corncrake, *Crex crex*, habitat preference, population trends.

Introduction

Agriculture has shaped various ecosystems worldwide. It has been recently recognized that production of food for the still growing human population in environmentally and ecologically sustainable way might be the greatest challenge for agriculture (Robertson, Swinton 2005). Agricultural habitats are used by many organisms, even rare and endangered species, and thus it is very important to achieve better integration of

agriculture and conservation biology (Banks 2004). In Europe, semi-natural grasslands i.e. hay meadows and pastures, especially those managed using traditional method, are the main breeding habitat for Corncrake *Crex crex*, a bird species recognized as *near-threatened* globally by the IUCN (Hilton-Taylor 2000). In Western Europe, Corncrakes have been declining in numbers and their distribution range has been shrinking since the 19th century, when mechanical grass mowing was introduced and earlier mowing became possible due to accelerated grass growth stimulated by intensive fertilization of fields (Glutz von Blotzheim et al. 1973). Although the species has declined also in Latvia since the First World War (von Transehe 1965), today the Baltic States support a considerable part of the European Corncrake population (Green et al. 1997). The recent increase of the Corncrake population in Europe (Schäffer, Koffijberg 2004) is predicted to be short term since both intensive agriculture and cession of agriculture is detrimental for the species (Keišs 2003) and its conservation status in European Union therefore has been evaluated as *unfavourable* (Papazoglou et al. 2004).

Despite the fact that historic declines of Corncrake numbers are often associated with changes in agricultural practices, reliable data are rarely available (Glutz von Blotzheim et al. 1973). Green and Stowe (1993) analyzed Corncrake declines in Britain and Ireland associated with changes in vegetation of the Corncrake habitats and changes in land use (Stowe et al. 1993). Vegetation impact on habitat selection in Corncrakes has been described also by Schäffer and Münch (1993) in Murnauer Moss, Germany and by Schäffer (1999) in valleys of the rivers Biebrza and Narew in Poland. Despite these indepth studies on vegetation, long-term data connecting agricultural land use (e.g. availability of various Corncrake habitats in a scale of a country) and respective Corncrake numbers are still lacking [but see "snapshots" provided by Elts (1997) for Estonia and Keišs (1997) for Latvia].

In the present study, the dynamics of Corncrake population numbers is related to changes in agricultural land observed in sample plots. Historical dynamics of Corncrake population in Latvia is projected by past land-use data for the territory of Latvia.

Materials and methods

Data were collected in 68 freely chosen sample plots in 1989 - 2004, the Snēpele sample plot has been surveyed since 1984 (Fig. 1, Table 1). On average, 19 sample plots were surveyed each year, but only four plots were surveyed for more than ten years. The area of the sample plots were determined by using 1:50 000 and 1:10 000 topographic maps. Total area of open landscape (excluding forests, open waters and towns) in sample plots varied between 0.67 and 44.38 km^2 (mean = 8.42; SD = 7.458). Habitats were mapped and the area of each habitat was calculated according to following categories: (i) cultivated meadows and perennial grasslands – sown or fertilized and managed grasslands, used for mowing; (ii) uncultivated meadows – semi-natural grasslands which are not fertilized and are mowed once per year; (iii) cultivated pastures – see (i), but used for grazing; (iv) uncultivated pastures – see (2), but used for grazing; (v) winter crops – fields of winter rye, winter wheat, winter barley and triticale; (vi) spring crops – fields of spring barley, oats, spring wheat, spring rye, buckwheat and mixed cereals; (vii) intertilled crops – fields of various kinds of intertilled crops (potatoes, beets etc.), this category was called "other arable land" in Keišs (1997); (viii) abandoned grasslands; (ix) abandoned arable lands; (x)

abandoned lands with unknown last usage; (xi) shrubland; (xii) other (miscellaneous) habitats.

Preferably, habitat mapping were repeated every year before the Corncrake surveys. At least once, the habitats were mapped in 63 sample plots. Corncrakes were surveyed at night by counting all calling males. Each calling male was attributed to one of the given habitat categories. In 205 (67.7 %) cases of all 303 cases Corncrakes were surveyed twice per season, in 129 (62.9 %) of all these cases (n = 205) calling males were recorded on the map and if males were observed >250 m in first and second count, they were considered as two different individuals (Peake, McGregor 2001; Schäffer, Mammen 2003). For the rest of repeated surveys, survey with greatest observed number was used for analyses.

The *TRends and Indices for Monitoring data* (TRIM) version 3 software (Pannekoek, van Strien 2001) was used for analyses of Corncrake count and habitat data. To meet the requirements of the mathematical model, data used for calculation of the annual TRIM index for Corncrakes were taken only from those sample plots (n = 61) where surveys were conducted two or more years (Fig. 1). Similarly, the annual TRIM index for the area of each habitat category could only be calculated for those plots where habitat data were available for two or more years and only for the given habitat was present at least in one year (i.e. value is not 0). Therefore, the number of sample plots used for calculating annual TRIM index of each habitat category differed. For analytical purposes, habitat- specific annual TRIM indices for Corncrake numbers were calculated using the same subset of sample plots as for respective habitat category index (Table 2). The Spearman's rank correlation coefficient (ZAR 1996) was calculated for the obtained habitat-specific Corncrake indices. The φ-test (Plohinski 1972) was used to determine if Corncrakes prefer a specific habitat category.

Latvian State Meteorological Agency data on precipitation in six observational stations in Latvia (Ainaži, Daugavpils, Rūjiena, Stende, Zīlāni and Zosēni) during the Corncrake breeding season (May - July) were obtained and the relationship with the annual Corncrake

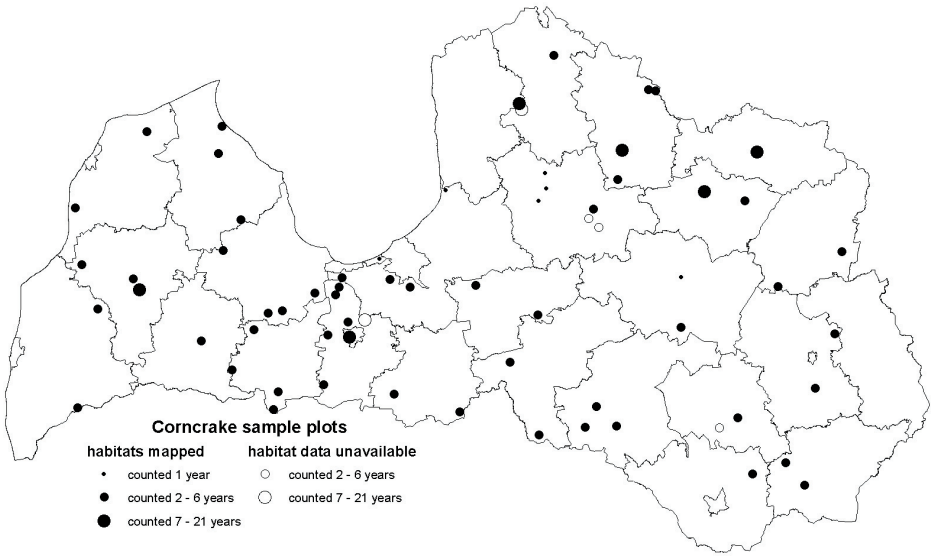

Fig. 1. Locations of Corncrake survey sample plots in Latvia 1984 - 2004.

Table 1. Corncrake survey plots and annual breeding density in Latvia (1984 - 2004)

Year	Surveyed plots with habitat data	All surveyed plots (plots, started in the given year)	Annual density (males km^{-2})
1984	1	1 (1)	0.65
1985	1	1 (0)	0.48
1986	1	1 (0)	1.31
1987	1	1 (0)	0.54
1988	1	1 (0)	0.48
1989	4	5 (4)	1.02
1990	7	10 (7)	1.38
1991	7	12 (4)	1.10
1992	4	7 (2)	0.78
1993	2	8 (0)	0.86
1994	8	14 (6)	0.73
1995	11	18 (1)	1.92
1996	22	25 (17)	1.76
1997	7	10 (4)	1.55
1998	3	11 (3)	1.99
1999	6	14 (8)	2.26
2000	6	12 (1)	1.38
2001	10	19 (1)	2.10
2002	35	44 (5)	2.14
2003	42	50 (4)	1.73
2004	42	45 (0)	1.97

TRIM index was determined.

The historical population dynamics of Corncrakes were projected by using statistical information on land use in Latvia in the past (Appendix 1; Anonymous 1959; 1967; 1976; 1986; 1991; 1993; 1994; 1996; 1997a; 1997b; 1999a; 1999b; 2000a; 2000b; 2000c; 2001; 2002; 2003a; 2003b; 2004a; 2004b). Habitat categories used in this study are consistent with statistical data collected on agricultural land use in Latvia and therefore allow to project the total number of calling Corncrakes in Latvia. Since the categories of statistical data collected in various decades since 1940 has slightly changed, for projection of Corncrake numbers in the past, it was necessary to combine some categories (e.g. cultivated and uncultivated meadows into "meadows"; cultivated and uncultivated pastures into "pastures"; meadows and pastures together and all types of abandoned lands together, Appendix 1).

Results

The annual TRIM index of Corncrake numbers in Latvia increased significantly during the period of observation (1989 - 2004: $r = 0.79$; $p < 0.0003$; adding the data of the single

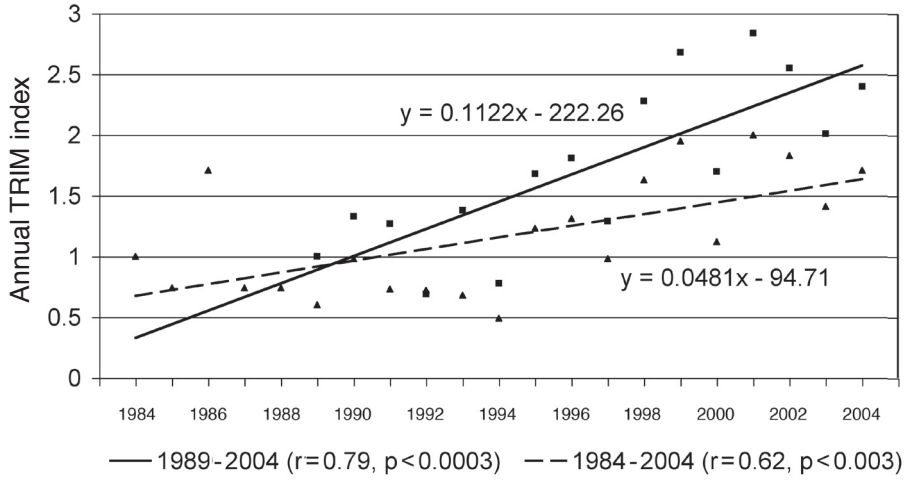

Fig. 2. Changes in the annual TRIM index of Corncrakes in Latvia (in the period 1984 – 1988 only one sample plot was surveyed).

plot available for period 1984–1988, r = 0.62; p < 0.003; Fig. 2).

The area TRIM indices of agricultural land use in Corncrake survey sample plots during the period of 1989 - 2004 changed as follows: area of abandoned agricultural lands increased very significantly (r = 0.76; p < 0.0006), while the area of cultivated pastures decreased (r = –0.60; p < 0.015), along with the area of intertilled crops (r = –0.47; p < 0.07). The area of both cultivated and uncultivated meadows increased significantly

Table 2. Number of sample plots surveyed at least two years and used to calculate TRIM indices for area of specific habitat categories (habitat present at least one year) and indices of total Corncrake number

Habitat category	Survey plots used in analyses (number)
Cultivated meadows	43
Uncultivated meadows	44
All meadows	47
Cultivated pastures	33
Uncultivated pastures	35
All pastures	41
Winter crops	41
Spring crops	43
All crops	45
Other arable land	42
Abandoned neadows	40
Abandoned arable land	35
All abandoned agricultural land	45

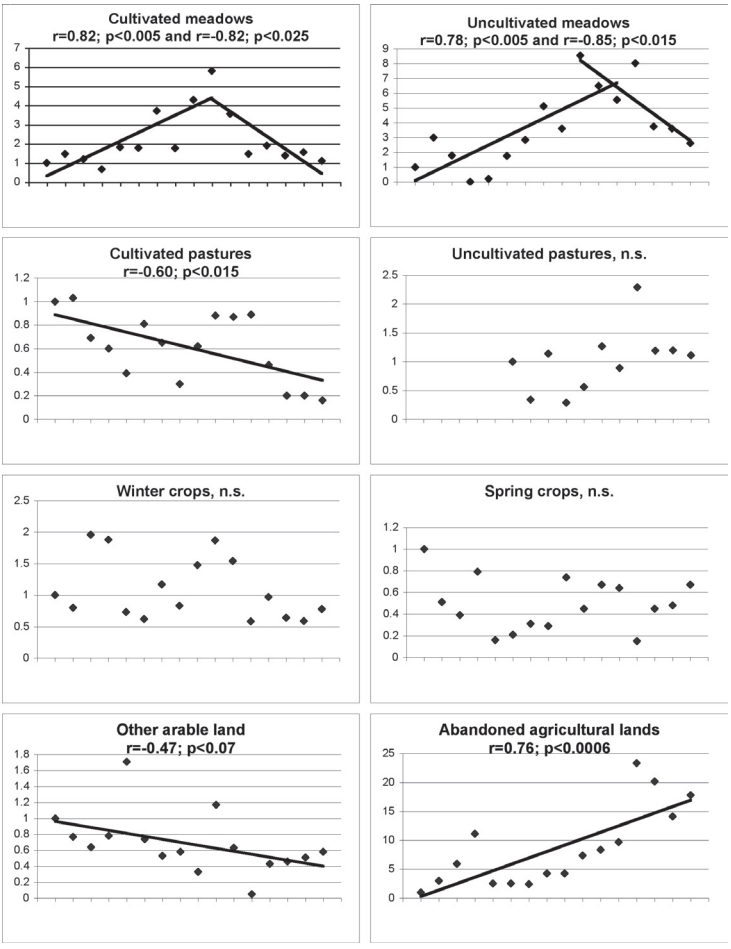

Fig. 3. Changes in the annual TRIM indices of various agricultural habitat categories in Corncrake sample plots in Latvia 1989 - 2004.

until 1998 ($r = 0.82$ for cultivated and $r = 0.78$ for uncultivated meadows; $p < 0.005$), but decreased in 1999 - 2004 ($r = –0.82$; $p < 0.025$ for cultivated and $r = –0.85$; $p < 0.015$ for uncultivated meadows). The area indices of uncultivated pastures, winter crops and spring crops did not change significantly in sample plots during the study period (Fig. 3).

The Corncrake index calculated for habitat-specific subsets of sample plots (see *Materials and Methods*, and Table 2) was positively correlated (Fig. 4) with the area index of uncultivated meadows in respective subset of sample plots (Spearman's rank test, $p < 0.001$), all meadows combined ($p < 0.001$), uncultivated pastures ($p < 0.05$) and abandoned agricultural lands combined ($p < 0.0025$). The area index of intertilled crops and index of Corncrake number were negatively correlated ($p < 0.05$), but the correlation coefficients for area indices of cultivated pastures, spring crops and winter crops related to the respective indices of Corncrake numbers were close to zero (Fig. 4).

The total Corncrake TRIM index and the total amount of precipitation during the

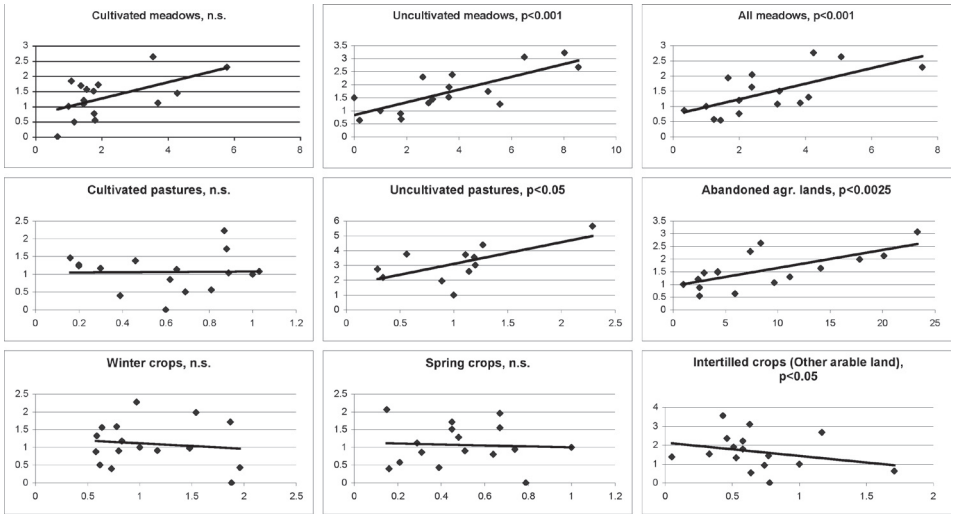

Fig. 4. Annual TRIM index of Corncrake numbers (Y-axis) explained by the annual TRIM index of the respective habitat (X-axis), p values for Spearman's rank correlation coefficient are given (see also Table 2).

Corncrake breeding season (May - July) was positively correlated (Fig. 5; $r = 0.86$; $p < 0.002$).

The habitat area data were available for 3300 Corncrake registrations during the period of 1989 - 2004. Calling male density was calculated and observed vs. expected Corncrake proportion in each habitat type was compared (Table 3). The highest breeding density (3.05 calling males per km^2) was observed in abandoned grasslands, followed by uncultivated meadows and abandoned arable lands. More Corncrakes than expected by area covered of the respective habitat category were observed in all of these three habitat categories (φ-test, $p < 0.001$). Cultivated meadows, other habitats, uncultivated pastures

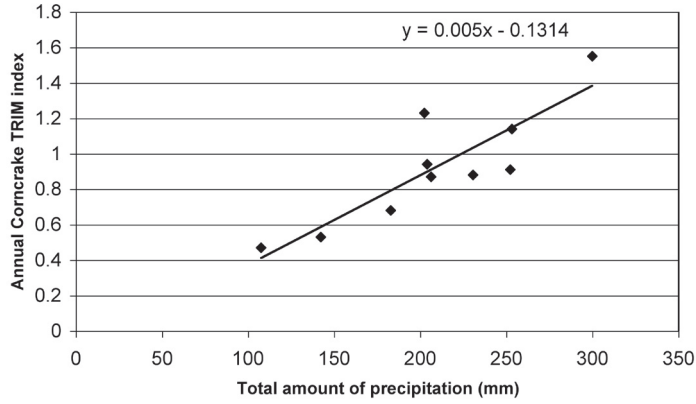

Fig. 5. Annual TRIM index of Corncrake numbers explained by the total amount of rainfall in May-July in six meteorological stations in Latvia in the respective year..

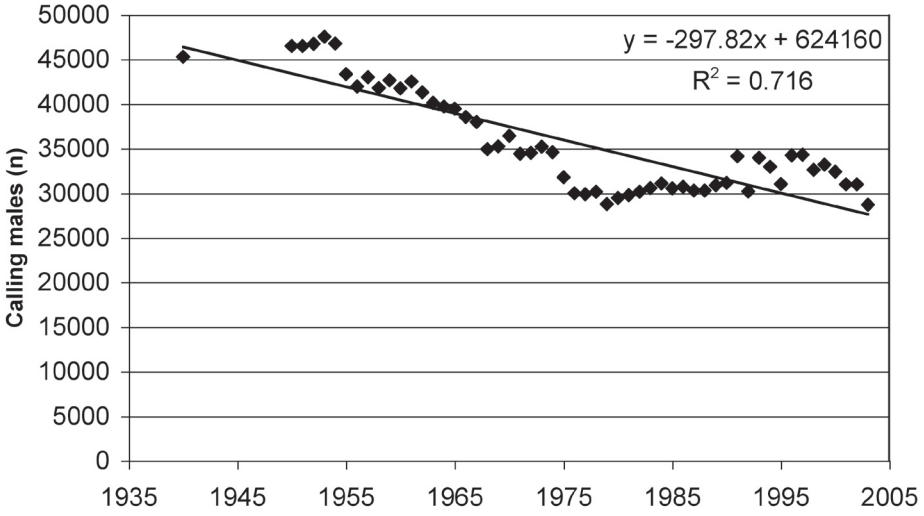

Fig. 6. Expected population of Corncrake in Latvia according to land-use data (for sources see *Materials and methods* and Appendix 1).

and shrubland had insignificantly less Corncrakes than expected, but in some years there were more Corncrakes than expected in a respective habitat (Table 3). Observed Corncrake numbers were smaller than expected in winter crops, cultivated pastures, spring crops, and intertilled crops (φ-test, $p < 0.001$ for all respective habitats).

The dynamics of Corncrake population numbers since 1940 (Fig. 6), obtained by habitat specific breeding densities (Table 3) and respective agricultural statistics (see *Materials and methods*), show a significant decrease in numbers ($r = 0.80$; $p < 0.0001$).

Discussion

Precision of the survey

This study used registrations of calling males as a basis for all calculations and conclusions on Corncrake population dynamics. This method has been used in many other Corncrake studies across Europe (Schäffer, Koffijberg 2004). However, a calling Corncrake male does not always mean that breeding on the site occurs (Schäffer 1994). Further, even if breeding occurs, the population density alone might not always show the quality of the site adequately (van Horne 1983), because breeding success is not known (e.g. Schäffer 1994) and might be lower in high density sites due to so called "ecological traps" caused by anthropological impact, predators etc. (Bock, Jones 2004). Despite all of the given uncertainties, no better method for surveying Corncrakes is available.

Two counts per season is a widely recognized method for surveying Corncrakes (Schäffer, Mammen 2003) as well as "the 250 m rule" for combining of the results of both counts (Peake, McGregor 2001). If Corncrakes are moving more than 250 m, this method might lead to an overestimation of the number of Corncrakes present. Nevertheless, Peake and McGregor (2001) showed that only 66 % of resident males in a given territory

Table 3. Number of sample plots surveyed at least two years used to calculate TRIM indices for area of specific habitat categories (habitat present at least one year) and indices of total Corncrake number

Habitat category	Density (males km^{-2})	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	All years together
Abandoned neadows	3.05	n.a.	+	+*	+**	+	+	+*	+*	+*	+	+	+	+*	+*	+*	+***	+***
Uncultivated meadows	2.85	+	+	+	+	0	+*	+*	+***	+**	+*	+*	–	–	+***	+***	+***	+***
Abandoned arable land	2.70	+	+	0	n.a.	0	–	+	+	+	n.a.	+	+	–	+	–	+**	+***
Cultivated meadows	1.68	+	+	0	–*	+	+	–*	+	–*	–	–*	0	+	–*	+	+	–
Other habitats	1.60	+	n.a.	n.a.	n.a.	n.a.	0	+	n.a.	n.a.	+	n.a.	n.a.	–	–	–	–	–
Uncultivated pastures	1.56	n.a.	n.a.	n.a.	n.a.	n.a.	0	+	–	0	0	+	+	–*	–	–	–*	–
Bushes	1.27	n.a.	n.a.	n.a.	n.a.	n.a.	+	0	n.a.	n.a.	n.a.	n.a.	n.a.	–*	–	–***	–	–
Winter crops	1.25	–	–	–	–***	+	–**	–	–	0	–	+	+	+	+	–***	–***	–***
Cultivated pastures	0.81	–*	–*	–*	–**	–	–***	–	–	–**	–*	–	–	–	–*	–	–	–***
Spring crops	0.69	–	–	+	–***	–	–**	–	–***	–	–**	–*	–	–	–***	–***	–***	–***
Other arable land	0.12	–*	–**	–	–	–*	–***	–	–***	–*	–*	–**	n.a.	–	–***	–***	–***	–***
Corncrakes registered		47	65	34	29	10	57	113	305	83	64	164	48	223	646	643	769	3300

are detected in two counts when "the 250 m rule" is applied. Reduced calling activity of males during pairing (Schäffer 1995; Tyler, Green 1996) was mostly responsible for the undetected 34 %. Therefore, the results obtained by this method are underestimates rather than overestimates of population size and calling male density.

Volunteers were allowed to choose sample plots freely, which might lead to overestimates, because observers will always tend to count birds where they are, but not in places, where birds are absent. In the last three years (2002 - 2004) the number of surveyed plots increased substantially (Table 1), which might be another source of error. However, the majority of these plots were been initialy surveyed already in previous years. Most of the sample plots were established in 1996, when plots were chosen randomly (Keišs 1997) and survey in 17 of them was repeated in following years. Corncrake is still evenly distributed in the whole territory of Latvia (Repeated Latvian Breeding Bird Atlas 2000 - 2004, Ornithological Society of Latvia unpublished data) and observers were specially asked to include all available potential Corncrake habitats (all open agricultural lands) at the site in the survey plot. Therefore, it is doubtful that the choice of the study plots had a major bias on the obtained results.

Impact of agricultural land use on Corncrakes

Differences in the intensity of agriculture and recent changes in the political systems in Eastern Europe are having an effect on bird populations (Green, Rayment 1996; Donald et al. 2001; Auniņš et al. 2001). The rapid increase of agriculturally abandoned lands in Latvia in the 1990s seems to be the main reason for the increase of Corncrake numbers (Fig. 2; Fig. 3; Fig. 4). Pesticide use in Latvian farmlands has dropped considerably after 1990 (Anonymous 1999c) suggesting that generally the management of those agricultural lands still in use (meadows, pastures and even winter crops) has been under low management intensity during the 1990s, which allowed Corncrake numbers to increase. Müller and Illner (2001) showed that in Germany, Corncrakes can successfully reproduce in managed arable lands such as crop fields,which is probasbly true also in Latvia.

Auniņš and Priednieks (2005) observed that Corncrake has shown a tendency to decline in Latvia since 2000, but the methods (morning 5 min counts) applied by Auniņš and Priednieks (2005) were not designed to survey Corncrakes and their results might well be only artifacts. Although a decline of Corncrake population is expected in the future (Keišs 2003), the annual TRIM index (Fig. 2) shows that Corncrake numbers fluctuate, but have not decreased since 2000.

The observed increase of meadows until the end of the 1990s in the sample plots (Fig. 3) are in conflict with the decline tendency observed in Latvia in general (Appendix 1). This might be explained by a methodological error, caused by additional arable lands with grass vegetation and abandoned grasslands without any shrubs to the category "meadows". When shrubs become clearly visible, these habitats become "abandoned agricultural lands". Unfortunately, observers were not given specific instruction on when "abandoned agricultural land" should be counted as "shrubland". Therefore observers might have interpreted this category differently. It was not possible to find also any published definition on the term "shrubland" used in official land-use statistics. However, the total area of shrublands in the sample plots was small. Since Latvia's accession to European Union, many abandoned areas have been moved or even ploughed again in 2005, since EU funds became available for farmers. Consequently, the land use in

Latvia is again experiencing changes. It is most likely that these changes will not favour Corncrakes, since intensive farming methods are been introduced.

Due to many uncertainties associated with the calculation of Corncrake numbers in the past, the results shown (Fig. 6; Appendix 1) can be classified as a crude estimation. Although the calculations are very simple and speculative, since Corncrake surveys in the past in Latvia were not performed, the obtained results indicate that the recent population increase in Latvia has not compensated the losses experienced earlier (Fig. 6). The estimation illustrates only losses due to land-use changes, mainly due to decrease of the area of meadows (Appendix 1). Quality of habitats (e.g. meadows) might have also decreased since 1940 due to changes in mowing methods from hand-scythe and horse-drawn mower, which ensured slow and prolonged mowing late in the season. Therefore, the decline of the Corncrake population in Latvia in the past century might well have been larger than shown in Fig. 6.

This study showed a close positive correlation between amount of precipitation during the Corncrake breeding season (May - July) and the annual Corncrake index. The tendency to have higher Corncrake numbers in years with more rainfall has been observed also by Kiss (2004) in southern Hungary. Better food availability in wet years might be one of possible explanation for the observed tendency. Koffijberg and van Dijk (2001) hypothesized that the influx of Corncrakes in The Netherlands in 1998 was due to immigration from populations in Belarus and Russia, where large amounts of rainfall in 1998 resulted in high water tables, which prevented breeding in floodplain meadows. Such as immigration cannot be excluded also in Latvia, further investigation in Corncrake habitats during the breeding season is needed to confirm this.

International and standardized Corncrake monitoring has been started only recently (Schäffer, Mammen 2003), and therefore precise information on the population dynamics even in the past decade is scarce in most European countries. This case study provides useful insights into Corncrake population dynamics on the East-European scale.

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Lauksaimniecības zemes lietošanas izmaiņu ietekme uz griezes *Crex crex* populāciju Latvijā

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Kopsavilkums

Griežu un lauksaimniecības zemes izmantošanas monitoringu veica 68 brīvi izvēlētos parauglaukumos (0,67 - 44,38 km^2; vid. = 8,42; SD = 7,458) Latvijā no 1984. līdz 2004. gadam. Griežu kopskaita indekss novērojumu periodā būtiski pieauga ($p < 0,003$). Parauglaukumos novērojumu laikā pieauga atmatu platības indekss ($p < 0,0006$), bet kultivētu ganību ($p < 0,015$) un rušināmkultūru indekss samazinājās ($p < 0,07$). Gan kultivētu, gan nekultivētu pļavu platības indekss parauglaukumos pieauga līdz 1998. gadam ($p < 0,005$), bet pēc tam krasi samazinājās ($p < 0,025$). Griežu skaita indekss pozitīvi korelēja ar nekultivētu pļavu platības indeksu ($p < 0,001$), visu pļavu indeksu ($p < 0,001$), atmatu platības indeksu ($p < 0,0025$) un nekultivētu ganību platības indeksu ($p < 0,05$). Negatīvi griežu indeksu ietekmēja rušināmkultūru indekss ($p < 0,05$). Griežu indekss korelēja ar kopējo nokrišņu daudzumu griežu ligzdošanas sezonā: maijā, jūnijā un jūlijā ($p < 0,002$), kas norāda uz citu populāciju īpatņu iespējamu ieceļošanu Latvijā slapjās vasarās. Vislielākais griežu ligzdošanas blīvums (3,05 tēviņi uz km^2) bija neapsaimniekotās pļavās, tām dilstošā secībā seko nekultivētas pļavas (2,85) > aramzeme atmatā (2,70) > kultivētas pļavas (1,68) > citi biotopi (1,60) > nekultivētas ganības (1,56) > krūmāji (1,27) > ziemāji (1,25) > kultivētas ganības (0,81) > vasarāji (0,69) > rušināmkultūras (0,12). Analizējot 3300 griežu reģistrācijas dažādos biotopos, neapsaimniekotās pļavās, nekultivētās pļavās un atmatā atstātās aramzemēs tika novērots ievērojami vairāk griežu, nekā sagaidāms ($p < 0,001$), bet griežu bija ievērojami mazāk ziemājos, kultivētās pļavās, vasarājos un rušināmkultūrās ($p < 0,001$). Neskatoties uz pašreizējo griežu skaita pieaugumu Latvijā, ilgtermņa populācijas dinamika kopš 1940. gada parāda būtisku skaita samazināšanos ($p < 0,0001$) piemēroto biotopu (pļavu) platības samazināšanās dēļ.

Appendix 1. Corncrake population density and statistical data on agricultural land use (thousands of hectares) used for calculations of Corncrake numbers 1940–2004 (statistical data compiled from Anonymous 1959; 1967; 1976; 1986; 1991; 1993; 1994; 1996; 1997a; 1997b; 1999a; 1999b; 2000a; 2000b; 2000c; 2001; 2002; 2003a; 2003b; 2004a; 2004b); n.a. – data were not available; * – the value is most likely 0; values in bold are used for calculations; values in parentheses are calculated assuming gradual change between two closest available values.

Appendix 1. *Continued*

Land use		Years														
		CD	**1940**	**1950**	**1951**	**1952**	**1953**	**1954**	**1955**	**1956**	**1957**	**1958**	**1959**	**1960**	**1961**	**1962**
1	Sown perennial grasslands	1.68	520.2	282.2	318.5	333.1	410.8	380.8	376.6	364.1	479.9	491.3	521.5	504.7	532.4	494.4
2	Meadows	1.88	915.8	905.9	904.2	897.8	884.9	865.5	694.8	684.6	691.1	691.1	697.6	706.5	723.4	742.8
3	Pastures	0.89	600.5	423.8	419.8	419.8	394.0	394.0	437.1	394.0	387.5	381.1	374.6	362.4	342.3	297.1
4	Meadows and pastures	1.38	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	Winter crops in total	1.25	362.2	257.0	235.0	269.3	234.7	264.0	221.4	281.8	265.7	213.6	270.7	249.0	276.0	280.0
5.1	*Winter rye*	n.a.	291.9	227.8	202.0	221.0	189.0	203.3	176.8	216.8	212.1	178.1	220.1	220.0	240.0	227.0
5.2	*Winter wheat*	n.a.	70.3	29.2	33.0	48.3	45.7	60.7	44.6	65.0	53.6	35.3	50.6	29.0	36.0	53.0
5.3	*Winter barley*	n.a.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	n.a.	n.a.	n.a.	n.a.
5.4	*Triticale*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6	Spring crops in total	0.69	730.2	518.9	525.0	556.2	588.2	570.0	370.6	322.6	284.3	295.6	275.8	301.0	329.0	265.0
6.1	*Spring wheat*	n.a.	87.6	105.1	127.4	131.7	179.0	145.0	99.2	98.3	54.8	56.2	38.2	34.0	34.0	28.0
6.2	*Spring rye*	n.a.	3.0	1.0	0.8	0.9	1.2	1.0	0.4	0.2	0.2	0.1	n.a.*	n.a.*	n.a.*	n.a.*
6.3	*Spring barley*	n.a.	169.9	110.8	121.1	138.8	199.0	164.5	93.0	62.8	63.4	78.5	72.7	84.0	102.0	114.0
6.4	*Oats*	n.a.	387.1	213.5	203.0	212.6	156.8	207.2	129.6	111.0	114.6	102.3	103.9	102.0	54.0	31.0
6.5	*Mixed crops*	n.a.	n.a.	42.3	35.7	40.0	38.4	39.0	37.1	40.4	39.7	45.3	47.0	59.0	96.0	71.0
6.6	*Mixed crops and legumes*	n.a.	82.6	46.2	37.0	32.2	13.8	13.3	11.3	9.9	11.6	13.2	14.0	22.0	43.0	21.0
6.7	*Buckwheat*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	Intertilled crops (other arable land) in total	0.12	303.9	277.6	295.5	316.3	279.2	297.8	380.9	363.4	332.9	327.0	329.7	372.9	372.0	425.2
7.1	*Legumes*	n.a.	35.5	24.4	20.1	20.2	8.5	16.4	15.9	15.0	10.1	8.8	9.0	14.0	28.0	14.0
7.2	*Oil flax*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.3	*Long-fibred flax*	n.a.	58.6	42.6	50.3	55.0	31.6	35.3	45.6	49.9	42.7	32.8	28.9	30.2	29.5	29.1
7.4	*Sugar beets*	n.a.	14.9	17.2	17.5	18.3	20.2	20.9	18.9	18.0	17.5	18.9	19.2	19.8	20.5	19.0
7.5	*Potatoes*	n.a.	138.7	149.1	150.6	155.4	149.8	149.0	142.2	142.6	151.7	152.6	159.8	159.8	154.2	142.8
7.6	*Vegetables*	n.a.	9.1	15.8	20.9	21.4	20.9	21.9	20.5	22.7	23.5	20.1	18.8	16.1	14.8	15.3
7.7	*Fodder roots*	n.a.	47.1	21.4	24.0	25.3	25.5	25.5	21.6	24.7	19.2	24.3	24.0	22.0	24.0	44.0
7.8	*Fodder sugar beets*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.9	*Fodder cabbage*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.10	*Green crops and ensilage excl. maize*	n.a.	n.a.	7.1	12.1	20.7	22.7	28.8	6.7	24.5	39.4	49.7	43.0	50.0	12.0	50.0
7.11	*Maize*	n.a.	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	48.5	35.8	18.8	13.9	27.0	62.0	89.0	111.0
7.12	*Green manure cultures*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	61.0	30.2	10.0	5.9	n.a.	n.a.	n.a.	n.a.
7.13	*Nectar cultures*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	All abandoned agriculture lands	2.87	n.a.*	251.2	239.0	213.1	219.6	213.1	315.4	290.6	284.2	258.4	251.9	230.8	219.6	226.1
9	Shrubland	1.27	n.a.*	294.3	297.1	297.1	290.7	297.1	248.1	235.6	229.2	229.2	222.7	212.7	196.9	190.4

Appendix 1. *Continued*

Land use	Years																				
	1963	**1964**	**1965**	**1966**	**1967**	**1968**	**1969**	**1970**	**1971**	**1972**	**1973**	**1974**	**1975**	**1976**	**1977**	**1978**	**1979**	**1980**	**1981**	**1982**	**1983**
1	475.0	435.3	511.2	469.7	467.0	482.0	502.9	597.4	602.8	633.9	662.6	657.2	664.1	524.0	525.0	531.0	527.0	523.5	555.3	569.7	582.5
2	788.0	794.4	745.2	737.3	704.0	484.4	484.4	469.9	258.4	258.4	264.8	258.4	229.8	229.4	228.0	228.2	214.3	231.8	235.4	238.1	238.9
3	322.9	316.5	278.3	428.0	490.9	755.7	762.2	752.8	943.0	943.0	936.5	955.9	628.0	610.2	599.2	596.8	614.3	599.6	598.5	595.6	596.2
4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	199.0	346.0	360.0	231.0	256.0	257.0	216.0	183.6	193.0	196.0	208.0	186.0	189.2	229.0	174.0	213.0	77.0	193.9	158.0	181.0	240.0
5.1	161.0	278.0	277.3	183.0	196.0	189.0	141.0	108.8	111.0	120.0	129.0	99.0	105.2	123.0	80.0	111.0	53.0	111.1	90.0	n.a.	n.a.
5.2	38.0	68.0	80.6	48.0	60.0	68.0	75.0	74.8	82.0	76.0	79.0	87.0	84.0	106.0	94.0	102.0	24.0	82.8	68.0	n.a.	n.a.
5.3	n.a.	n.a.	2.1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6	409.0	294.0	244.1	301.0	309.0	296.0	357.0	383.0	411.0	375.0	399.0	434.0	449.8	467.0	542.0	522.0	580.0	493.9	523.0	531.0	480.0
6.1	48.0	33.0	25.8	23.0	21.0	13.0	5.0	2.2	1.0	1.0	0.0	0.0	0.3	0.0	0.0	n.a.	n.a.	0.2	n.a.	n.a.	n.a.
6.2	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*
6.3	179.0	128.0	119.6	156.0	166.0	179.0	245.0	278.3	294.0	269.0	291.0	313.0	322.8	356.0	415.0	404.0	490.0	396.7	396.0	n.a.	n.a.
6.4	45.0	48.0	50.7	68.0	84.0	104.0	91.0	88.9	104.0	93.0	95.0	107.0	113.5	97.0	111.0	104.0	78.0	81.7	90.0	n.a.	n.a.
6.5	109.0	66.0	47.5	54.0	38.0	n.a.	16.0	13.6	12.0	12.0	13.0	14.0	13.2	14.0	16.0	14.0	12.0	15.3	28.0	n.a.	n.a.
6.6	28.0	19.0	0.3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6.7	n.a.	n.a.	0.2	n.a.	n.a.	n.a.	n.a.	0.0	n.a.	n.a.	n.a.	n.a.	0.0	n.a.	n.a.	n.a.	n.a.	0.0	n.a.	n.a.	n.a.
7	381.3	362.9	393.2	372.4	351.6	350.9	304.3	329.7	304.3	340.3	317.0	328.9	302.5	292.0	292.0	286.0	334.0	318.1	289.6	144.9	144.8
7.1	19.0	11.0	19.7	17.0	12.0	9.0	6.0	6.2	6.0	8.0	8.0	6.0	6.1	7.0	9.0	5.0	4.0	4.1	9.0	n.a.	n.a.
7.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.3	27.0	26.6	24.7	23.3	23.0	22.9	21.9	18.9	18.9	18.9	18.9	18.9	18.9	19.0	18.0	18.0	18.0	17.8	15.0	14.6	14.4
7.4	26.1	22.5	22.4	22.0	22.3	17.2	9.8	9.8	9.6	9.4	9.5	9.6	9.6	11.0	12.0	11.0	13.0	13.0	12.7	13.1	13.5
7.5	135.6	131.1	140.6	137.6	135.9	135.4	132.0	130.7	119.4	119.4	119.8	120.6	120.2	107.0	108.0	107.0	107.0	105.9	103.7	102.6	102.2
7.6	15.6	14.7	14.5	14.5	14.4	14.4	14.6	15.0	12.4	12.6	12.8	12.8	12.8	11.0	11.0	14.0	15.0	15.3	15.2	14.6	14.7
7.7	34.0	34.0	26.1	34.0	33.0	35.0	32.0	27.9	34.0	36.0	39.0	39.0	39.1	41.0	40.0	38.0	39.0	38.6	36.0	n.a.	n.a.
7.8	n.a.	n.a.	10.9	n.a.	n.a.	n.a.	n.a.	3.9	n.a.	n.a.	n.a.	n.a.	0.1	n.a.	n.a.	n.a.	n.a.	0.6	n.a.	n.a.	n.a.
7.9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.10	22.0	46.0	73.2	79.0	65.0	75.0	54.0	74.7	54.0	85.0	52.0	59.0	35.0	31.0	38.0	44.0	96.0	77.5	47.0	n.a.	n.a.
7.11	102.0	77.0	61.1	45.0	46.0	42.0	34.0	42.6	50.0	51.0	57.0	63.0	60.7	65.0	56.0	49.0	42.0	45.3	51.0	n.a.	n.a.
7.12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	148.6	135.6	129.3	119.7	90.4	45.2	38.8	41.2	25.8	25.8	19.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	171.1	164.6	187.5	185.5	184.0	177.5	177.5	176.4	190.4	184.0	177.5	164.6	190.8	189.9	180.2	173.7	167.4	164.7	157.2	148.8	144.6

Appendix 1. *Continued*

Land use	Years																			
	1984	**1985**	**1986**	**1987**	**1988**	**1989**	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**
1	591.3	595.1	604.1	608.3	624.5	634.7	655.9	861.6	598.6	536.0	540.6	374.7	398.4	389.7	392.7	383.1	347.2	344.3	335.1	282.9
2	241.0	240.0	238.3	239.0	246.3	248.6	245.8	246.3	n.a.	n.a.	n.a.	n.a.	246.0	n.a.	238.6	211.8	n.a.	217.2	n.a.	n.a.
3	597.6	598.6	599.3	596.6	599.7	599.1	598.0	597.0	n.a.	n.a.	n.a.	n.a.	479.1	n.a.	379.1	346.6	n.a.	246.4	n.a.	n.a.
4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1423.7	1339.4	1341.1	1172.8	n.a.	1067.6	n.a.	n.a.	958.5	n.a.	948.2	811.5
5	238.0	195.0	204.4	184.8	206.1	246.6	272.8	142.7	261.2	352.4	141.1	136.0	177.5	180.8	179.7	152.8	182.4	206.4	178.5	194.6
5.1	n.a.	100.7	107.3	82.1	101.2	128.7	130.7	69.2	131.4	187.6	62.7	40.4	56.4	62.5	57.7	47.2	54.8	55.8	42.3	44.2
5.2	n.a.	94.3	97.0	102.4	104.7	117.8	140.7	69.8	123.1	153.0	72.9	85.5	117.4	109.7	109.2	95.2	117.4	131.3	115.9	127.9
5.3	n.a.	n.a.	0.1	0.3	0.2	0.1	1.4	1.1	3.4	5.0	2.4	7.4	2.0	5.8	7.5	4.6	4.3	6.3	4.8	3.4
5.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.6	3.3	6.8	3.1	2.7	1.7	2.8	5.3	5.8	5.9	13.0	15.5	19.1
6	515.0	495.8	495.2	484.6	429.2	419.3	402.6	505.6	435.5	341.2	345.2	272.4	268.7	302.0	282.6	263.0	237.6	237.3	236.5	233.9
6.1	n.a.	0.6	0.5	0.7	0.2	0.2	0.8	1.7	5.5	16.1	21.7	24.1	31.8	42.6	41.7	51.0	40.7	35.5	37.6	39.9
6.2	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*	n.a.*
6.3	n.a.	396.9	408.1	402.0	352.5	331.6	306.6	397.4	347.0	270.3	264.1	195.9	176.4	188.7	165.9	142.7	130.6	124.0	132.1	129.2
6.4	n.a.	91.6	77.1	67.3	65.5	75.6	82.4	92.7	69.4	48.5	54.0	45.6	53.6	59.1	59.7	47.2	45.5	55.2	47.1	49.4
6.5	n.a.	6.7	9.5	14.6	11.0	11.9	12.7	13.7	13.5	6.2	5.3	6.8	6.8	11.0	13.6	7.9	5.3	4.1	3.4	2.9
6.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	11.9	9.3	7.9	5.8	6.0
6.7	n.a.	0.0	0.0	n.a.	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.6	1.7	2.3	6.2	10.6	10.5	6.5
7	144.6	279.3	269.4	270.6	270.0	237.5	221.5	291.1	274.5	192.7	164.5	145.2	139.7	129.1	122.4	106.3	105.3	112.4	107.7	113.4
7.1	n.a.	36.6	36.6	27.2	20.4	14.6	10.5	9.0	6.7	2.8	2.8	3.0	3.6	4.7	6.8	2.5	2.1	3.2	2.5	2.9
7.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.3	0.4	0.1	0.1
7.3	14.5	14.3	13.7	13.2	12.9	12.7	11.9	8.8	7.6	0.6	1.5	1.4	1.3	1.6	2.2	2.0	1.6	1.4	2.1	2.1
7.4	13.7	13.5	13.5	13.6	13.6	13.5	14.7	14.6	24.8	12.1	12.0	9.5	10.0	10.9	16.3	15.5	12.7	14.1	15.9	14.4
7.5	101.6	94.3	91.9	90.3	87.6	84.7	80.3	82.2	96.9	87.7	80.4	75.3	78.7	69.6	58.8	50.1	51.3	55.1	53.6	54.6
7.6	14.8	12.1	12.1	12.2	12.2	11.0	10.8	12.7	19.1	18.6	17.5	17.5	15.7	13.5	11.6	9.8	9.7	13.3	12.5	14.3
7.7	n.a.	33.4	34.1	35.3	35.1	35.6	36.2	39.1	36.5	29.6	26.2	19.8	17.3	14.9	13.1	9.1	9.0	9.6	7.5	7.1
7.8	n.a.	0.5	0.4	0.6	0.6	0.5	0.8	0.3	0.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7.9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.4	0.7	0.5	0.3	0.3	0.2	0.3	0.1	n.a.	n.a.	0.0	0.1
7.10	n.a.	26.4	19.3	29.3	40.8	18.7	11.5	84.9	56.7	31.4	20.9	17.8	11.6	13.2	12.8	12.0	11.4	8.4	7.2	9.9
7.11	n.a.	48.2	47.8	48.9	46.8	46.2	44.8	39.5	24.8	9.2	2.7	0.6	1.2	0.5	0.5	0.7	1.2	1.0	1.2	1.7
7.12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	4.5	6.0	4.7	4.1	4.8
7.13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.2	1.0	1.4
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	(169.2)	(233.8)	279.0	390.0	402.0	361.8	434.0	(410.6)	(387.2)	(363.8)	340.4
9	140.6	143.7	(137.7)	(131.8)	125.8	124.5	112.1	111.7	(114.9)	(118.0)	(121.2)	124.3	131.9	144.3	144.2	149.2	(154.2)	(159.2)	(164.2)	(169.2)

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Effect and after-effect of barley seed coating with phosphorus on germination, photosynthetic pigments and grain yield

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Abstract

Phosphorus is an important macronutrient for barley, but commonly it has low availability in soils. Therefore, seed coating with readily available phosphorus is used in agricultural practice. The aim of this work was to investigate the influence of barley seed coating with phosphorus on germination, the content of pigments of green plastids in shoots, the seed yield and the presence of an after-effect on the second generation. The coating of barley seeds with phosphorus depressed germination in the beginning of the development of plants, but there was a positive influence of the coating on the amount of pigments of green plastids in the shoots, and the yield of barley seeds increased by 3 to 91 %. The treatment of barley seeds with the phosphorus influenced positively the physiological activity of next generation seeds. The usage of phosphorus-treated barley seeds is effective: physiological activity of the seeds increases as well as the yield of the seeds. Also, phosphorus uptake is improved, which contributes to better environmental protection.

Key words: mineral nutrition, phosphorus seed coating, photosynthetic pigments, seed germination, spring barley, yield.

Introduction

Phosphorus is the second limiting element after nitrogen for plant growth – plants can not grow without a sufficient supply of this nutrient. Phosphorus plays an essential role in all physiological and biochemical processes in plants (Hartmann 1997; Schachtman et al. 1998; Marschner 1999; Lott et al. 2000). The amount of phosphorus in plants ranges from 0.05 % to 0.50 % of total dry weight (Bieleski 1973; Theodorou, Plaxton 1993; Marschner 1999). It is a component of key molecules such as nucleic acids, phospholipids, and ATP. Phosphorus is also involved in controlling key enzyme reactions and in the regulation of metabolic pathways (Theodorou, Plaxton 1993; Marschner 1999; Abel et al. 2002). Although bound phosphorus is quite abundant in many natural soils, it is largely unavailable for uptake. Phosphorus availability in soils declines with rapid formation of insoluble complexes with cations and by use of soil microorganisms. Therefore, to improve yields, the phosphorus content in soils needs to be supplemented by means of mineral nutrition. However, even under adequate phosphorus fertilisation, only 20 % or less of that applied is removed by the first year's growth (Bieleski 1973; Schachtman et al. 1998;

Vance 2001). This results in phosphorus loading of prime agricultural land. Runoff from phosphorus loaded soils is a primary factor in eutrophication and hypoxia of lakes and marine estuaries in the developed world. As noted by Abelson (1999), a potential phosphate crisis looms for agriculture in the 21st century.

Low availability in bulk soil limits phosphorus uptake by plants. More soluble minerals such as potassium move through the soil via bulk flow and diffusion, but phosphorus is moved mainly by diffusion. Since the rate of diffusion of phosphorus is slow (10^{-12} to 10^{-15} m^2 s^{-1}), high plant uptake rates create a zone around the root that is depleted of phosphorus (Schachtman et al.1998).

Therefore, to achieve more rational use of phosphorus fertilisation, specifically phosphorus treated seeds are used (Stramkale et al. 2004). Usage of phosphorus-treated seeds improves utilisation of mineral nutrient, avoiding contamination of the environment (Gravalos et al. 2000).

A seed coating creates a nutritious environment around germinating seed providing nutritional support in early phase of crop development (Taylor, Herman 1990). The main nutrient supplied in a form of seed coating is phosphorus (Taylor et al. 1998).

Seed coating containing phosphorus has been shown to be an effective way of promoting early seedling growth in phosphorus-deficient soils (Valdes et al. 1987; Refalka et al. 1993; Ros et al. 2000). Even in soils with sufficiently high phosphorus content, a seed coating with phosphorus can be beneficial due to imporved availability of the nutrient (Scott et al. 1991).

Seed coating with phosphorus has been succesfully used in agricultural practice in different regions (Scott 1989). However, there is only limited information available on the exact mechanisms of how phosphorus coating promotes early development of seedlings (Stramkale et al. 2004) and on the possible after-effects of using phosphorus coated seeds on the next generation of crops. The aim of the present experiments was to investigate the effect of phosphorus coating on germination characteristics, photosynthetic pigment content, and the grain yield of barley. A special attention was focused on the effect of phosphorus coating on early seedling development in the next generation using un-coated barley seeds grown from phosphorus coated seed material.

Materials and methods

Spring barley (*Hordeum vulgare* L.) 'Anabell' bred in Germany was used in the laboratory and field tests (in 2002 and 2003). The cultivar provides high yield with excellent quality, it demonstrates good resistance to different plant diseases and has high logging resistance. This cultivar is well suited for brewery and also for food and forage.

In the experiments the seeds treated with phosphorus-powdered nutrition were provided by Finnish company "Kemira Grow How". The nutrition is fixed to the seeds with the help of a binding agent. The patent for this treatment method (iSeed™) belongs to the Finnish company "Kemira Grow How" and the patent for the binding agent belongs to "Kemira Grow How" and "Fortum Oil and Gas".

The addition, barley seeds of the second generation from field grown barley plants established from phosphorus treated seeds were used. This helped to determine whether the phosphorus treatment has an effect also on the next generation.

In the laboratory tests the seeds were germinated in the dark at 20 °C in a Petri dish

lined with two layers of filter paper. The seeds were damped with 5 ml of distilled water. To avoid drying of the seeds, a filter paper bridge connected the Petri dish with a distilled water dish. To prevent evaporation the Petri dish was covered with a lid, and a narrow gap was left to allow air circulation. The percentage of germinated seeds was determined every 5 h. A seed with at least 1 mm of protruded radicle was detected as germinated. The number of repetitions was five. The number of seeds in every repetition was 50. As soon as seed lobes appeared, the Petri dish was placed under light conditions to promote formation of pigments of green plastids.

In the laboratory experiments the following were determined:

(i) Germination, germinating power and germination energy of barley seeds. Germination power is the final percentage of germinated seeds. Germination energy is germination percentage at the time of the most intensive germination (Hartmann et al. 1997; Kutschera 1998).

(ii) Content of pigments of green plastids in 7-day old shoots was measured spectrophotometrically in acetone extract, by determining the optical density (D) at wave-lengths corresponding to absorption maxima of chlorophyll *a*, chlorophyll *b* and carotenoids. Concentration of pigments (C; mg l^{-1}) was calculated according to the following equations (Gavrilenko, Zigalova 2003):

$$C_{Chl\,a} = 9.784\ D_{662} - 0.990\ D_{644};$$
$$C_{Chl\,b} = 21.426\ D_{644} - 4.650\ D_{662};$$
$$C_{car} = 4.695\ D_{440.5} - 0.268\ C_{Chl\,a} + C_{Chl\,b}.$$

Field tests were performed in 2002 and 2003 at the Latgale Scientific Agricultural Centre in Eastern Latvia. In 2002, barley was sown on April 24 and harvested on August 20, and in 2003, it was sown on May 8 and harvested on September 6. The field test design was random blocks with four repetitions. The total area for a block was 2 m × 10 m = 20 m^2. The total space of the test was 1259 m^2. The soil type was humus podzolic gley. The content of organic substances in the soil was 38 - 52 g kg^{-1}, pH_{KCl} – 7.3, P – 63 - 99 mg kg^{-1}, K – 98-147 mg kg^{-1}. The pre-plant was wheat. As a basic fertiliser "Kemira Grow How" complex mineral nutrition 18:9:9 was used, the mineral supplement was ammonium nitrate (the reccomendations of the supplier). In field tests the yield of barley was determined. The barley was cropped in the seed ripening phase using a seed combine harvester Sampo-130.

During the field test, May 2002 was warm but dry (there was no rainfall in three succesive decades). The mean temperature in June was 16.2 °C, rain from 3.4 mm (in the 1st decade) up to 16.6 mm (in the 3rd decade). July was warm and sunny. The mean temperature in July was 20.1 °C, which exceeded the mean monthly temperature by 3.6 °C. Precipitation was insufficient – only 38.7 % of the monthly mean. Hot and dry weather favoured grain ripening at the end of a summer of 2002. May 2003 was warm and wet. The sufficient moisture level positively influenced barley seed germination. June was hot and dry. In July, precipitation increased over the mean by 1.4 times. During the harvest (in the 1st decade of August) precipitation exceeded the mean by about 4.2 fold. Unfortunately, this factor affected the yield negatively.

The mathematical data processing (standard error, standart error of difference) and figures were produced using *MS Excel*.

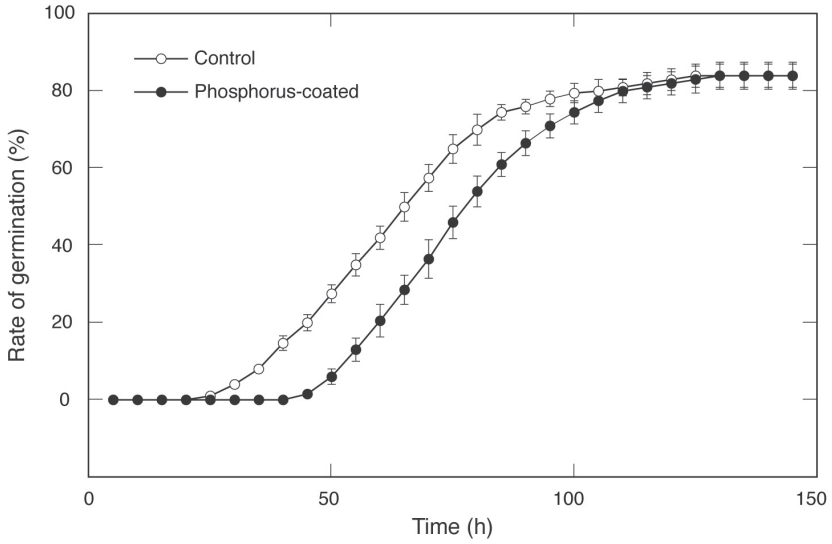

Fig. 1. Effect of phosphorus coating on germination rate of barley (*Hordeum vulgare* L.) seeds.

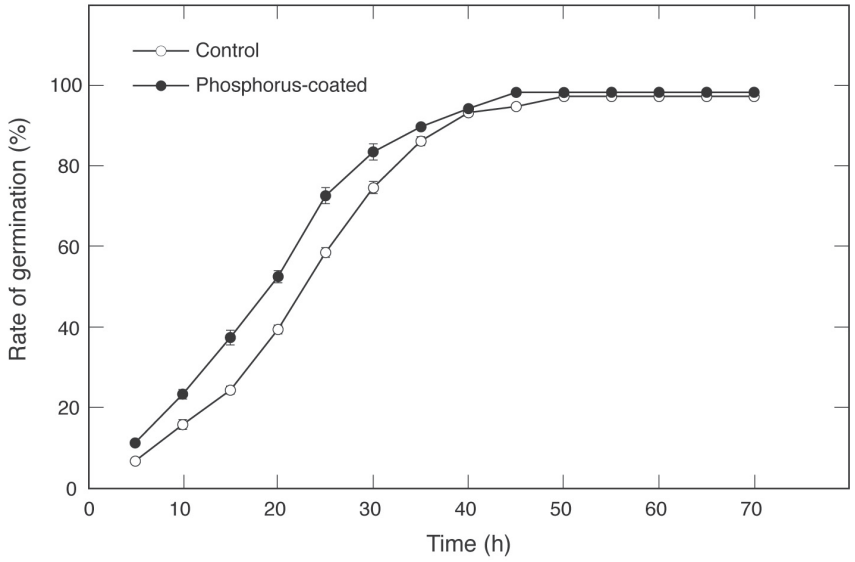

Fig. 2. After-effect of phosphorus coating on germination rate of barley (*Hordeum vulgare* L.) seeds of the second generation.

Results

Effect of phosphorus coating on germination characteristics and pigment content

The germination of non-treated barley seeds started within 22 h after the start of the experiment. The first signs of germination of phosphorus-treated seeds were visible only after 44 h. However, at the end of the experiment, the germination percentage of

phosphorus treated barley seeds reached that of control seeds (Fig. 1).

Germination of the second generation obtained from barley seeds treated with phosphorus was considerably more intense than the control (Fig. 2). In 2003 the second generation of seeds both for the phosphorus variant and control began intensive germination (Fig. 2), in contrast with the germination in 2002 (Fig. 1). This may be due to the different origin of seeds: in 2002 seeds were from Finnish company "Kemira Grow How", but in 2003 from Scientific Agricultural Centre of Latgale.

The germination energy of control barley seeds was 2.3 times higher than the germination energy of phosphorus-treated seeds. However, the germination energy of the second generation obtained from phosphorus treated seeds was 3.3 times higher than germination energy of control seeds (Fig. 3). Significant differences were not observed for indicators of germination power (data not shown).

In order to determine how phosphorus treatment of seeds affected the content of photosynthetic pigments in newly established barley seedlings, concentrations of chlorophyll *a*, chlorophyll *b* and carotenoids were determined in 7-day-old shoots. Both the chlorophyll *a* and chlorophyll *b* levels were significantly higher in the shoots of barley grown from phosphorus-treated seeds (Fig. 4). The concentrations of chlorophyll *a* (32 %) and chlorophyll *b* (41%) were higher in the leaves of shoots from phosphorus-treated seeds. Significant differences in carotenoid content were not observed (Fig. 4).

The concentrations of chlorophyll *a* (46 %), chlorophyll *b* (41 %) and carotenoids (43 %) were higher in the shoots of the second generation arising from seeds coated with phosphorus than in the control shoots (Fig. 4).

Effect of phosphorus coating on barley yield

The field tests demonstrated that when the barley 'Anabell' was fertilised with different nitrogen, phosphorus and potassium fertilisers, the plants germinated and grew better if

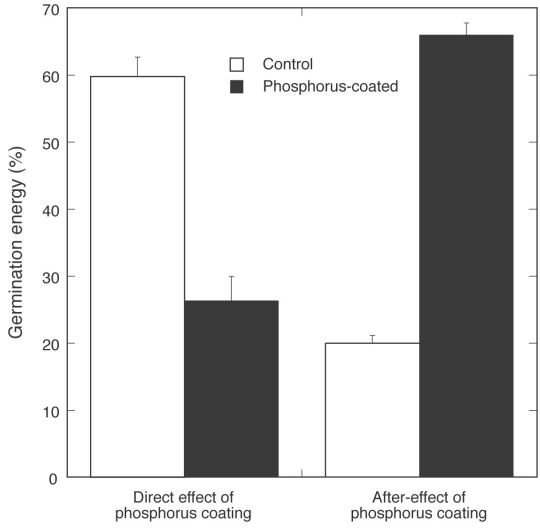

Fig. 3. Effect and after-effect of phosphorus coating on germination energy of barley (*Hordeum vulgare* L.) seeds.

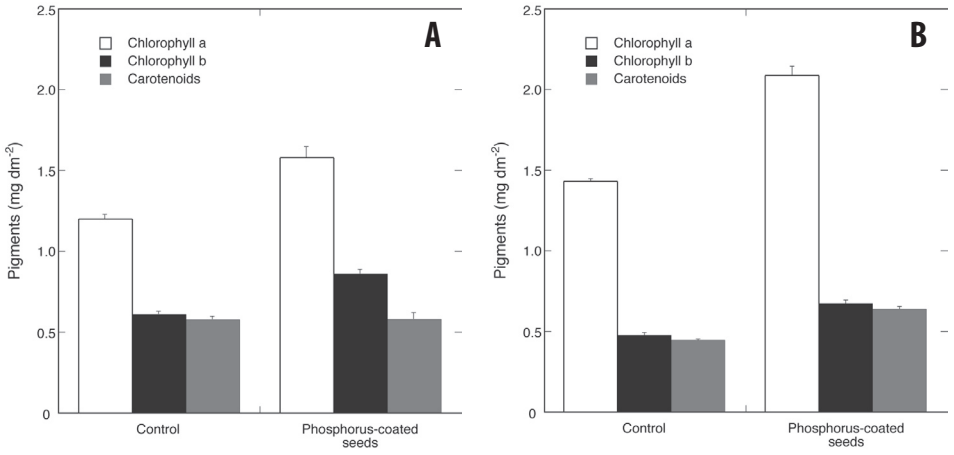

Fig. 4. Effect (A) and after-effect (B) of phosphorus coating on the total amount of pigments of green plastids of barley (*Hordeum vulgare* L.) seeds.

the seeds were coated with phosphorus. The barley yield increased by 3 to 91 %, when the seeds were treated with phosphorus coupled with nitrogen, phosphorus and potassium fertiliser application, compared to the control test (Table 1). The highest yield of the barley seeds 'Anabell' (7.21 t ha^{-1}) was obtained from plants grown from the phosphorus-treated seeds and using mineral nutrition with the general amount of nitrogen 120 kg ha^{-1} consisting from the basic fertiliser 90 kg ha^{-1} and supplement fertiliser 30 kg ha^{-1}: N_{120} (N_{90} 18:9:9 – 500 kg ha^{-1} + N_{30} – 220 kg ha^{-1} ammonium nitrate).

The yield of barley for control variants was greater in 2002 in comparison to 2003 which can be explained by meteorological factors. Nitrogen, phosphorus and potassium fertilisation had a much more greater effect on grain yield in 2003 in comparison to 2002, when there was insufficient amount of rain. Estimations of the standard error of difference showed that the increase in barley grain yield by phosphorus coating was significant, except in the case of phosphorus-treated seeds without fertiliser in 2002.

Discussion

Barley germination of control seeds was 1 to 22 % more intensive than for seeds coated with phosphorus. It is possible that the phosphorus concentration used in the present experiments for the barley seed coating was too high for optimal germination. A species-specific inhibitory effect of phosphorus seed coating has been shown earlier (Taylor 1997). Increased concentrations of phosphorus were shown to delay seedling emergence of lucerne (Scott, Blair 1989). Bieleski (1973) and Ragothama (1999) estimated that the concentration gradient from the soil solution to the plant root cell exceeds 2000-fold for phosphorus. In the soil solution the free phosphorus concentration is on average 1 μM. This concentration is well below the K_M for plant uptake. The possible cause of the poorer germination of phosphorus-treated seeds may be explained by the increased inhibiting impact of osmotic potential. An increased non-organic phosphorus (P_n) concentration in the direct vicinity of seeds may delay water uptake by change in osmotic potential, which affects germination process.

Table 1. Barley (*Hordeum vulgare* L.) cv. 'Anabell' grain yield as influenced by seed phosphorus coating and different levels of mineral nutrition in 2002 and 2003

Variants of fertilizer	Grain yield			
	2002		2003	
	t ha^{-1}	%	t ha^{-1}	%
Control	4.61	100.0	3.78	100.0
Control + P seeds (iSeeds™)	4.75	103.0	4.33	114.6
N_{120} (N_{90} 18:9:9 – 500 kg ha^{-1} + N_{30} – 88 kg ha^{-1} ammonium nitrate)	5.20	112.8	6.80	179.9
N_{120} (N_{90} 18:9:9 – 500 kg ha^{-1} + N_{30} – 88 kg ha^{-1} ammonium nitrate) + P seeds (iSeeds™)	5.48	118.9	7.21	190.7
N_{120} (N_{45} 18:9:9 – 250 kg ha^{-1} + N_{75} – 220 kg ha^{-1} ammonium nitrate)	5.75	124.7	5.42	143.4
N_{120} (N_{45} 18:9:9 – 250 kg ha^{-1} + N_{75} – 220 kg ha^{-1} ammonium nitrate) + P seeds (iSeeds™)	5.95	129.3	5.66	149.7
	$\gamma_{0.05}$ = 0.18		$\gamma_{0.05}$ = 0.20	

The sufficient store of phosphorus in seeds is vital for seed germination and successful seedling growth (Lott et al. 2000). It is possible that seed coating with phosphorus promotes the synthesis of storage compounds in seeds during the process of the formation of the yield.

The early vigour of barley seedlings was ensured by promotion of the formation of photosynthetic pigments and accordingly, higher rates of photosynthesis. This apparently supported for future development, as shown by an increase of grain yield (Table 1). Other studies have described an increase in dry matter and the amount of tillers which may be cosequence of increased photosynthesis and photosynthate production (Scott et al. 1985).

To summarize, the coating of seeds with well available phosphorus influenced germination, the content of the chlorophyll in shoots, and the yield of seeds of spring barley 'Anabell'. The yield of barley seeds increased by 3 to 91 % when the seeds were treated with phosphorus and different nitrogen, phosphorus and potassium fertilisers were used. Treatment of seeds with phosphorus effected positively the physiological activity of the next generation seeds: increased seed germination and germination power, anf higher concentrations of chlorophyll and carotenoids in the shoots.

In conclusion, the usage of phosphorus-treated barley seeds is effective: the physiological activity of the seeds increases as well as the yield of the seeds. Also, phosphorus usage is improved, which contributed to better environmental protection.

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Miežu sēklu fosfora apstrādes ietekme un pēcietekme uz dīgšanu, fotosintēzes pigmentiem un sēklu ražu

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Kopsavilkums

Fosfors ir nozīmīgs augu makroelements. Augsnē fosfors lielākoties ir augiem grūti pieejamā formā. Tāpēc kultūraugu sējumos lieto īpašas ar fosforu apstrādātas sēklas. Darba mērķis bija noskaidrot, kā miežu sēklu apstrāde ar fosforu ietekmē to dīgšanu, fotosintēzes pigmentu saturu lapās un graudu ražu; kā arī to, kāda ir sēklu apstrādes pēcietekme uz nākamās paaudzes sēklu dīgšanu. Nosakot sēklu dīgšanu procentos un pigmentu saturu dīgstos spektrofotometriski, konstatēja, ka sēklu apstrāde ar fosforu kavēja sēklu dīgšanu miežu augšanas sākumā, bet pigmentu daudzums dīgstos fosfora ietekmē palielinājās. Par 3 līdz 91% pieauga miežu graudu raža. Sēklu apstrāde ar fosforu pozitīvi ietekmēja nākamās paaudzes sēklu fizioloģisko darbību. Var secināt, ka ar fosforu apstrādāto miežu sēklu izmantošana ir efektīva: palielinās sēklu fizioloģiskā aktivitāte un sēklu raža, tiek uzlabota fosfora izmantošana un notiek vides saudzēšana.

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Assessment of the condition of freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus 1758) populations in Latvia

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Abstract

The freshwater pearl mussel *Margaritifera margaritifera* is a species threatened throughout the world. Pear mussel populations in Latvia were assessed using criteria developed in Sweden. The population size, distribution and density of the four largest *Margaritifera margaritifera* populations in four rivers were determined. For the estimation of pearl mussel population age structure, classes of mussel shell length were used. The most important threat was from dams and other beaver activities, which decreased the viability of the pearl mussel population. Two of the pearl mussel populations of Latvia, according to the Swedish scoring system, correspond to class II with a high nature conservation value, five populations to class I with a lower nature conservation value, and none to the class III, a very high nature conservation value.

Key words: conservation value, *Margaritifera margaritifera*, Latvia, population age structure.

Introduction

Freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus 1758) is a species threatened of becoming extinct throughout the world. Populations with normal chance of reproduction can be found only in a few locations (Araujo, Ramos 2000; Bauer 1989). It is generally accepted that the condition of the European pearl mussel populations are declining due to the isolation of separate local populations (Geist, Kuehn 2005).

The freshwater pearl mussel populations of Latvia are also completely isolated between each other. The pearl mussel populations of Latvia were surveyed in 1999 and 2000. The populations are in the ageing phase (Rudzite 2001; Rudzite 2004). Possible reasons of the population decline are: pearl fishing in the 17th and 18th centuries; eutrophication and siltation of the rivers caused by intensive agriculture during the 1950s and 1960s, and drainage management. In all of the known pearl mussel populations in Latvia, the water quality is too low for the survival of juvenile pearl mussels (Rudzīte 2004). An additional threat to the mussel populations of Latvia is the activities of European beaver *Castor fiber* L. – a reintroduced species in Latvia. Beavers were reintroduced since 1927, and during the Soviet period their dispersal was specially planned and stimulated (Balodis 1990). During recent years, beavers have spread to all of the pearl mussel rivers.

Six criteria are used to assess the long-term viability of the *M. margaritifera* population used in Sweden (Erikson et al. 1998): (i) population size; (ii) population density; (iii)

distribution – length of stream or river inhabited by a coherent population; (iv) smallest size of found mussel; (v) the proportion of mussels shorter than 2 cm; (vi) the proportion of mussels shorten than 5 cm. Each criterion is scored 0 to 6 points. It is desirable to develop the model and test it on other mussel populations, in Sweden as well as in other countries (Erikson et al. 1998).

In Latvia it is necessary to determine the density of the population and population age structure for evalution of the conditions of the pearl mussel populations of Latvia. For this purpose, the criteria worked out in Sweden (Erikson et al. 1998) was used, which was previously applied only in Sweden. The aim of the present paper was to evaluate the conditions of seven pearl mussel populations in Latvia and to estimate their probability of survival.

Materials and methods

From 1999 to 2004 pearl mussels in seven rivers were studied using the method of total counting of mussels, where all the mussels were counted in a part of the particular river (Rudzīte 2001; Rudzīte 2004). This method is also used in Sweden (Erikson et al. 1998). In four of the largest populations, in Pērļupe, Ludze, Tumšupe, Rauza, the census was done in a five-meter-long river stretches selected in the middle part of the population area . In every stretch, the average river width was measured and all the mussels where counted. The population density (mussels per m^2) for every single river part was calculated. For the whole river, the average population density, standard deviation and standard error were calculated. In three smaller populations, found in the rivers Dadžupe, Dzirnupe, Mergupe, the density of populations was calculated using the total number of mussels and the average river width. The length of the river was estimated from topographical maps 1:10 000.

For the estimation of pearl mussel population age structure, classes of mussel shell length were used, according to the method of Erikson et al. (1998). Sliding calliper and ruler were used for measuring. In total, 2731 mussels were measured in the Rauza river basin.

There are six criteria of importance regarding sustainability of long-term viability of the pearl mussel population in Sweden. Each criterion is scored 0 to 6 points (Erikson et al. 1998; Table 1).

Table 1. Criteria and scores for the assessment of nature conservation value for *Margaritifera margaritifera* populations (Erikson et al. 1998)

No	Criterion	Score (points)					
		1	**2**	**3**	**4**	**5**	**6**
1	Population size (× 1000)	<5	5 - 10	11 - 50	51 - 100	101 - 200	>200
2	Mean density (mussels m^{-2})	<2	2 - 4	4.1 - 6	6.1 - 8	8.1 - 10	>10
3	Distribution (km)	<2	2 - 4	4.1 - 6	6.1 - 8	8.1 - 10	>10
4	Smallest mussel found (cm)	>50	41 - 50	31 - 40	21 - 30	11 - 20	≤10
5	Proportion of mussels <2 cm (%)	1 - 2	3 - 4	5 - 6	7 - 8	9 - 10	>10
6	Proportion of mussels <5 cm (%)	1 - 5	5 - 10	11 - 15	16 - 20	21 - 25	>25

Table 2. The density of *Margaritifera margaritifera* populations in four rivers of Latvia

River	Mean width of the surveyed part (m)	Surveyed area (m^2)	Individuals found (number)	Population density (mussels m^{-2})		
				Mean	Minimum in 5-m strech	Maximum in 5-m strech
Pērļupe	3.6	545.1	112	0.21	0	1.51
Ludze	7.9	1190.0	2584	2.17	0.32	6.26
Tumšupe	7.0	1047.5	318	0.30	0	1.96
Rauza	8.8	1313.0	1486	1.13	0.06	2.99

With reference to the total number of points, the populations are classified, to evaluate their conservation value. The total number of points is used for classifying the investigated mussel populations into three classes. A score at 1 to 7 points indicates class I "Site of nature conservation value", 8 to 17 points – class II "High nature conservation value", and 18 to 36 points – class III "Very high nature conservation value" (Erikson et al. 1998). The values of long-term viability criteria were calculated and the overall population condition was assessed for seven pearl mussel populations in Latvia.

The following maps were used: Latvia Republic Satellite map of scale 1 : 50 000, the Soviet Union army topographical maps of scale 1 : 50 000 and 1 : 10 000.

Results

The density of *Margaritifera margaritifera* was determined for the four largest populations, in four rivers. The recording was conducted in 5-meter-long stretches. The mean width and surveyed area in the four investigated rivers varied from 3.6 m and 545.1 m^2 to 8.8 m and 1313.0 m^2, respectively (Table 2). The lowest number (112) of mussel individuals was found in Pērļupe and the highest (2584) was found in Ludze, which also had the highest population density (0.32 to 6.26 mussels m^{-2}, mean 2.17 mussels m^{-2}, in 5-m stretches). In Pērļupe the mean density was 0.21 mussels m^{-2}, in Tumšupe 0.30 mussels m^{-2}, and in Rauza 1.13 mussels m^{-2} (Table 2). Additional study was carried out on the population age structure in the middle part of Rauza population (Rauza A, Fig. 1), in the lower reaches of the river (Rauza B, Fig. 1), as well as at the middle of the tributary (Ludze A, Fig. 1) and at the upper reaches inhabited by beavers (Ludze B, Fig. 1).

The upper part of Ludze river is strongly affected by beavers. The beaver dams cause silting, warm water, increased eutrofication and shading. There is a lower influence of beaver in the other three parts.

Seven pearl mussel populations of Latvia here evaluated using the population criteria developed in Sweden (Erikson et al. 1998; Table 1). The average *M. margaritifera* population density in Latvia was 0.58 mussels m^{-2} (Table 3). The maximum observed density was 2.27 mussels m^{-2}, and the lowest – less than 0.00001 mussels m^{-2}. None of the pearl mussel populations corresponded to the third class, two corresponded to the second class, with a good viability level and probability of survival (Table 4). The others corresponded to the first class and their existence is endangered.

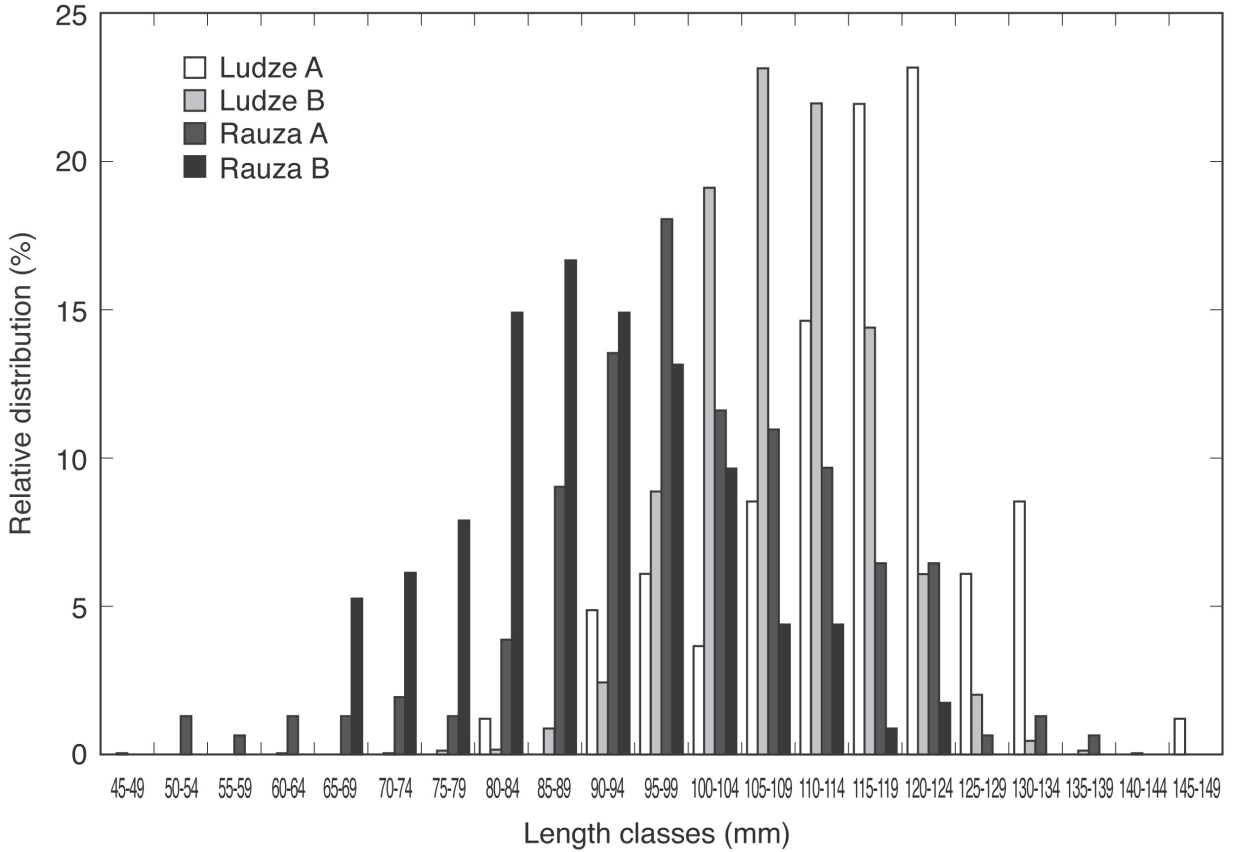

Fig. 1. The population age structure of *Margaritifera margaritifera* in Rauza river basin (in length classes). Ludze A - upper part of Ludze river; Ludze B - lower part of Ludze river; Rauza A - upper part of Rauza river; Rauza B - lower part of Rauza river. Classes of length were used, because there is a linear connection between the age of individuals and their length (Eriksson, et al. 1998).

Table 3. The characteristics of *Margaritifera margaritifera* populations in seven localities of Latvia

No	Criterion	Pērļupe	Ludze	Tumšupe	Rauza	Dadžupe	Dzirnupe	Mergupe
1	Population size	570	20000	1200	3000	200	20	7
2	Mean density	0.21	2.17	0.30	1.13	0.01	0	0.00001
3	Distribution (km)	2	7	4	24	2.5	1.2	1
4	Smallest mussel found (cm)	8.4	4.8	6.3	5.3	8.6	7.6	7.0
5	Proportion of mussels < 2 cm (%)	0	0	0	0	0	0	0
6	Proportion of mussels < 5 cm (%)	0	0	0	0	0	0	0

Table 4. The assessment of seven pear mussel *Margaritifera margaritifera* populations of Latvia after the population evaluation system used in Sweden (Erikson et al. 1998)

No	Criterion	Pērļupe	Ludze	Tumšupe	Rauza	Dadžupe	Dzirnupe	Mergupe
1	Population size (× 1000)	1	3	1	1	1	1	1
2	Mean density (mussels m^{-2})	1	2	1	1	1	1	1
3	Distribution (km)	1	4	2	6	2	1	1
4	Smallest mussel found (cm)	1	2	1	1	1	1	1
5	Proportion of mussels < 2 cm (%)	0	0	0	0	0	0	0
6	Proportion of mussels < 5 cm (%)	0	0	0	0	0	0	0
	Total points	4	11	5	9	5	4	4
	Class	I	II	I	II	I	I	I

Discussion

From 1999 to 2004 about two-thirds of the former pearl production area in Latvia (Rudzīte 2004) was surveyed systematically. None of the pearl mussel populations demonstrated good probability of survival, as they did not correspond to the third class in the population evaluation system. Additionally, all populations were in the aging phase (Rudzīte 2001).

The most aged populations were found in the localities with a beaver population (Ludze A, Fig. 1). The poor conditions likely existed already 60 to 70 years ago as this river is one of the first beaver reintroduction locations (Balodis 1990).

The population density of pearl mussels in Latvia is very low compared with the typical population density of 1000 to 2000 mussels m^{-2} (Baumgärtner, Heitz 1995). In Sweden, for

example, the maximum density is 33.7 mussels m^{-2}, and minimum 0.2 mussels m^{-2}, with a mean value 5.2 mussels m^{-2} (Erikson et al. 1998). However, other studies in Sweden mention population densities as low as 0.032 and 0.045 mussels m^{-2}, compared to a mean 0.52 mussels m^{-2} in Central Europe (Bauer 1988). In Finland, 100 mussels m^{-2} is a high density (Valovirta 1998). In Sweden, a high density of population is considered to be above 10 mussels m^{-2}, which corresponds to a value of 6 points (Erikson et al. 1998; Table 1). In Latvia there is only one population with a population density corresponding to two points, and the others – only one point (Table 3, Table 4). The condition of the two most highly valuable populations – in Rauza and its tributary Ludze – differ. The Rauza population is smaller but occupies a larger part of the river than the population in the tributary, which supports the smallest, youngest pearl mussels in Latvia (Table 4).

By 1999, beavers have dispersed to all freshwater pearl mussel rivers of Latvia (Rudzīte, unpublished data). Beavers destroy the habitat of pearl mussels and salmon fish by building dams. Behind the dam, a pond with still and warm water is formed, with raised nitrogen concentrations. Silting of substrates for mussel attachment is enhanced and the water quality does not correspond to suitable living conditions for the mussels (Rudzīte 2004).

The population age structure in the Rauza river basin (Fig. 1) is similar to that of other populations in Latvia (Rudzīte 2001), but is the largest in Latvia and includes also younger pearl mussels. This increases the value of this population greatly according to the evaluation criteria (Table 4).

The main reason for the bad condition of pearl mussel population is obviously the high level of dissolved inorganic nitrogen, which does not allow the survival of mussels in the first years (Lande, Lande 2000; Moorkens et al. 2000; Buddensiek 2001; Rudzīte 2004). Juvenile pearl mussels smaller than 2 and 5 cm are not found in Latvia (Table 2, Table 4). The age structure of Rauza population in 2004 is the same as in 1999 (Rudzīte 2001). It can be expected that, in the Rauza as well at its tributary, in ten years the pearl mussel population will still exist and some separate mussels may live till 80 or even 100 years. However, the population age structure indicates gradual extinction. Therefore actions must be taken to lover the nitrogen level to create favourable conditions for juvenile pearl mussels. The low density of juvenile pearl mussels (Table 3, Fig. 1) indicates that even when the probability of survival has worsened some individuals are still able to find favourable conditions for survival.

Conclusions

The population density of pearl mussels of Latvia is very low compared with other populations in Europe, with the maximum density of 2.27 mussels m^{-2} and minimum 0.00001 mussels m^{-2}, mean 0.58 mussels m^{-2}. Additional studies on the age structure of the population does not change the previous conclusion from a study in 1999 where it was found that the population is severely aging and is becoming extinct. Beaver is a threat for the pearl mussel population and therefore unacceptable in streams inhabited by *M. margaritifera*. The pearl mussel populations of Latvia, according to the Swedish evaluation system, corresponds to the classes I and II. The condition of two populations corresponding to class II may have high nature conservation value, and there is a high probability that it will survive.

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Ziemeļu upespērlenes *Margaritifera margaritifera* (Linnaeus 1758) stāvokļa novērtējums Latvijā

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Kopsavilkums

Ziemeļu upespērlenes *Margaritifera margaritifera* (Linnaeus 1758) Latvijas populāciju stāvokļa vērtēšanai izmantota Zviedrijā izstrādātā metode. Pētīts populācijas blīvums četrās lielākajās upespērleņu populācijās. Analizēta arī populāciju vecuma struktūra izmantojot garuma klases. Visās pērleņu upēs ir sastopami bebri, vērtēta to negatīvā ietekme uz pērleņu populācijas vecuma struktūru. Atbilstoši Zviedrijā izmantotajai populāciju vērtēšanas sistēmai neviena no Latvijas pērleņu populācijām neatbilst visaugstākajam vērtējumam – III klasei (populācija ar ļoti augstu saglabāšanās pakāpi). Divas atbilst II klasei (populācija ar augstu saglabāšanšs pakāpi), pārējās piecas atbilst I klasei (populācija ar zemāku saglabāšanās pakāpi), tātad, to izdzīvošanas iespējas ir apdraudētas.

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Organogenesis in callus cultures of *Linum usitatissimum* L.

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Abstract

Linseed flax cultivars 'Lirina', 'Barbara' and 'Szaphir' were tested for their regeneration capacity through adventitious shoot organogenesis from hypocotyl-derived and stem segment-derived callus. Shoots number per explant was strongly influenced by genotype, culture medium and application of growth regulators. The highest shoot number per explant was obtained from hypocotyl-derived callus of cultivar 'Szaphir' on combined MSB_5 media, supplemented by 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine. Hypocotyl-derived callus gave the better results than stem segment-derived callus. Four-week-old callus showed the maximum shoot regeneration frequency.

Key words: callus, genotype, growth regulators, linseed flax, shoot organogenesis.

Introduction

Flax belongs to the family *Linaceae*, with more than 200 species, and only one species – common flax (*Linum usitatissimum* L.) has a practical use. Common flax is a plant with a long history of cultivation and breeding in Lithuania (Bacelis 2001), although it is attracting new interest in Europe. The fibre flax cultivars are mostly grown in Lithuania, but recently the interest in oil cultivars is also increasing. The breeders of the Lithuanian University of Agriculture have started to work on breeding of linseed flax using tissue culture techniques.

The development of procedures for efficient regeneration of plants from cultured cells, tissues and organs are a prerequisite for application of *in vitro* culture techniques to plant gene manipulation for crop germplasm enhancement (Zhang et al. 2004). The capacity of cells to regenerate via different morphogenic programmes is a result of cell dedifferentiating to become competent to the stimulus. This is then followed by induction for the developmental programme and eventual development into the organ (Nhut et al. 2003). Regeneration methods frequently depend on the type of tissue used to initiate cultures, with the generation or acquisition of starting material potentially becoming a limiting factor (Koroch et al. 2003).

Flax improvement has not developed at the same rate as in other crops (mainly cereals) in recent years. Tissue culture can speed up the novel breeding which can lead to flax improvement and even to incorporation of valuable and desirable traits into flax cultivars

(resistance to fungal diseases, oil quality improvement and herbicide tolerance) through somatic hybridization and somaclonal variation (Basiran et al. 1987).

Although tissue culture of *Linum usitatissimum* L. has been carried out for more than 20 years information about what controls organogenesis and embryogenesis in this species and its cultivars is still scarce (Dedicova et al. 2000).

In the present paper we report the organogenetic response of linseed flax callus to different media, supplemented by growth regulators.

Materials and methods

The investigation was carried out with three linseed varieties: 'Lirina', 'Barbara', and 'Szaphir'. To study linseed morphogenesis, hypocotyls and stem segments were used as explants. Tissue culture protocols were the same as described previously (Blinstrubiene et al. 2004). Linseed tissue was plated on basal Murashige and Skoog (1962; MS) medium, B_5 medium (Gamborg 1975) and on combined medium MSB5 (MS macro salts and B_5 micro salts with vitamins), 0.75MSB_5 (75 % MS macro salts and B_5 micro salts with vitamins), 0.5MSB_5 (50 % MS macro salts and B_5 micro salts with vitamins) supplemented with sucrose 30 g l^{-1} and Difco-Bacto agar 6 g l^{-1}. The following growth regulator combinations were used: 1.0 mg l^{-1} kinetin + 0.1 mg l^{-1} indole-3-acetic acid (IAA), 1.0 mg l^{-1} 6-benzylaminopurine (BAP) + 0.05 mg l^{-1} α-naphtylacetic acid (NAA), 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine (2iP), 1.0 mg l^{-1} kinetin. The media pH was 5.7 ± 0.1, illumination – 5000 lx, photoperiod – 16 h, temperature 25 ± 2 °C. Each variant consisted of 50 explants and four replications were used. Induced callus were transferred to the same fresh medium every four weeks using 50 ml media into 200 ml glassware.

The morphogenic potential of tissues was evaluated by analyzing morphological parameters of the structures formed on the explants. The evaluation was based on the relative frequency of callus producing shoots (%) as well as the average number of shoots per explant.

Significant differences were evaluated using a computer program (Tarakanovas 1996) for ANOVA. The mean value and SE for each genotype were calculated.

Results

The shoots were produced spontaneously from the green soft callus of common flax with or without subculture onto fresh medium. Organogenesis of shoots from hypocotyl-derived callus that had been grown for four weeks on the culture media is shown in Table 1.

The number of shoots per explant was significantly affected by the culture media. The use of combined MSB_5 medium resulted in significantly more shoots per explant than any other medium and this was observed for all three cultivars. However, there were differences in shoot formation on MSB_5 medium supplemented with different growth regulators. Kinetin resulted in less shoot regeneration frequency in all cultivars, whereas 2iP had a stimulative effect on shoot formation in all cultivars tested. Cultivars differed significantly in the number of shoots per explant. Hypocotyl-derived callus from the cultivar 'Szaphir' gave the best results, while the cultivar 'Lirina' had the lowest organogenous response. MSB_5 medium supplemented with 2iP gave a regeneration rate of 4.67 shoots per explant for cultivar 'Szaphir'; 3.84 for 'Barbara' and 2.96 for 'Lirina'. This growth regulator appeared

to give the best regeneration frequency for all three cultivars tested.

The observed effects of medium composition on shoot organogenesis from stem segment-derived callus are summarized in Table 2.

Generally, stem segment-derived callus response to medium composition was similar

Table 1. Effect of medium composition and growth regulators on number of shoots per hypocotyl-derived callus of three linseed flax (*Linum usitatissimum* L.) cultivars. Data are means ± SE within four weeks of culture. MS, Murashige and Skoog medium; B_5, Gamborg medium; IAA, indole-3-acetic acid; BAP, 6-benzylaminopurine; NAA, naphthylacetic acid; 2iP, N^6-Δ^2-isopentenyl adenine

Geno-type	Growth regulators (mg l^{-1})	Number of shoots per explant				
		MS	B_5	MSB_5	$0.75MSB_5$	$0.5MSB_5$
'Lirina'	kinetin 1.0 + IAA 0.1	0.68 ± 0.08	1.30 ± 0.11	1.59 ± 0.14	0.34 ± 0.02	0.15 ± 0.01
'Lirina'	BAP 1.0 + NAA 0.05	1.84 ± 0.10	1.98 ± 0.12	2.52 ± 0.15	0.50 ± 0.03	0.23 ± 0.02
'Lirina'	2iP 2.0	2.39 ± 0.10	2.49 ± 0.10	2.96 ± 0.13	0.59 ± 0.03	0.29 ± 0.02
'Lirina'	kinetin 1.0	0.49 ± 0.12	1.17 ± 0.10	1.20 ± 0.11	0.26 ± 0.02	0.10 ± 0.01
'Barbara'	kinetin 1.0 + IAA 0.1	2.25 ± 0.13	2.66 ± 0.15	3.10 ± 0.18	0.75 ± 0.02	0.30 ± 0.01
'Barbara'	BAP 1.0 + NAA 0.05	2.28 ± 0.15	2.96 ± 0.17	3.24 ± 0.20	0.86 ± 0.02	0.42 ± 0.02
'Barbara'	2iP 2.0	2.60 ± 0.17	3.15 ± 0.20	3.84 ± 0.22	0.90 ± 0.03	0.49 ± 0.01
'Barbara'	kinetin 1.0	1.85 ± 0.14	2.34 ± 0.11	2.45 ± 0.10	0.64 ± 0.02	0.33 ± 0.01
'Szaphir'	kinetin 1.0 + IAA 0.1	2.39 ± 0.20	2.79 ± 0.23	3.38 ± 0.21	1.15 ± 0.10	0.60 ± 0.01
'Szaphir'	BAP 1.0 + NAA 0.05	2.67 ± 0.24	3.18 ± 0.20	3.90 ± 0.27	1.27 ± 0.11	0.68 ± 0.03
'Szaphir'	2iP 2.0	3.26 ± 0.26	3.62 ± 0.24	4.67 ± 0.30	1.53 ± 0.10	0.75 ± 0.05
'Szaphir'	kinetin 1.0	2.09 ± 0.15	2.47 ± 0.18	3.15 ± 0.20	1.09 ± 0.07	0.46 ± 0.02

Table 2. Effect of medium composition and growth regulators on number of shoots per stem segment-derived callus of three linseed flax (*Linum usitatissimum* L.) cultivars. Data are means ± SE within four weeks of culture. MS, Murashige and Skoog medium; B_5, Gamborg medium; IAA, indole-3-acetic acid; BAP, 6-benzylaminopurine; NAA, naphthylacetic acid; 2iP, N^6-Δ^2-isopentenyl adenine

Geno-type	Growth regulators (mg l^{-1})	Number of shoots per explant				
		MS	B_5	MSB_5	$0.75MSB_5$	$0.5MSB_5$
'Lirina'	kinetin 1.0 + IAA 0.1	0	1.20 ± 0.10	1.47 ± 0.14	0.26 ± 0.02	0.10 ± 0.01
'Lirina'	BAP 1.0 + NAA 0.05	1.73 ± 0.10	1.81 ± 0.16	2.00 ± 0.20	0.40 ± 0.09	0.15 ± 0.01
'Lirina'	2iP 2.0	2.24 ± 0.11	2.30 ± 0.10	2.37 ± 0.12	0.44 ± 0.08	0.20 ± 0.02
'Lirina'	kinetin 1.0	0	1.10 ± 0.10	1.15 ± 0.12	0.20 ± 0.02	0.07 ± 0.01
'Barbara'	kinetin 1.0 + IAA 0.1	1.92 ± 0.16	2.34 ± 0.20	2.79 ± 0.22	0.63 ± 0.07	0.22 ± 0.01
'Barbara'	BAP 1.0 + NAA 0.05	2.15 ± 0.18	2.51 ± 0.21	2.86 ± 0.22	0.68 ± 0.08	0.30 ± 0.04
'Barbara'	2iP 2.0	2.46 ± 0.21	2.89 ± 0.23	3.00 ± 0.21	0.75 ± 0.06	0.32 ± 0.03
'Barbara'	kinetin 1.0	1.40 ± 0.13	2.00 ± 0.16	2.19 ± 0.15	0.54 ± 0.05	0.16 ± 0.01
'Szaphir'	kinetin 1.0 + IAA 0.1	2.11 ± 0.15	2.48 ± 0.10	2.94 ± 0.16	0.85 ± 0.06	0.38 ± 0.02
'Szaphir'	BAP 1.0 + NAA 0.05	2.49 ± 0.17	2.89 ± 0.20	3.21 ± 0.23	0.92 ± 0.05	0.46 ± 0.03
'Szaphir'	2iP 2.0	2.97 ± 0.20	3.46 ± 0.24	3.76 ± 0.26	1.10 ± 0.07	0.53 ± 0.04
'Szaphir'	kinetin 1.0	1.86 ± 0.16	2.21 ± 0.18	2.65 ± 0.21	0.77 ± 0.05	0.28 ± 0.02

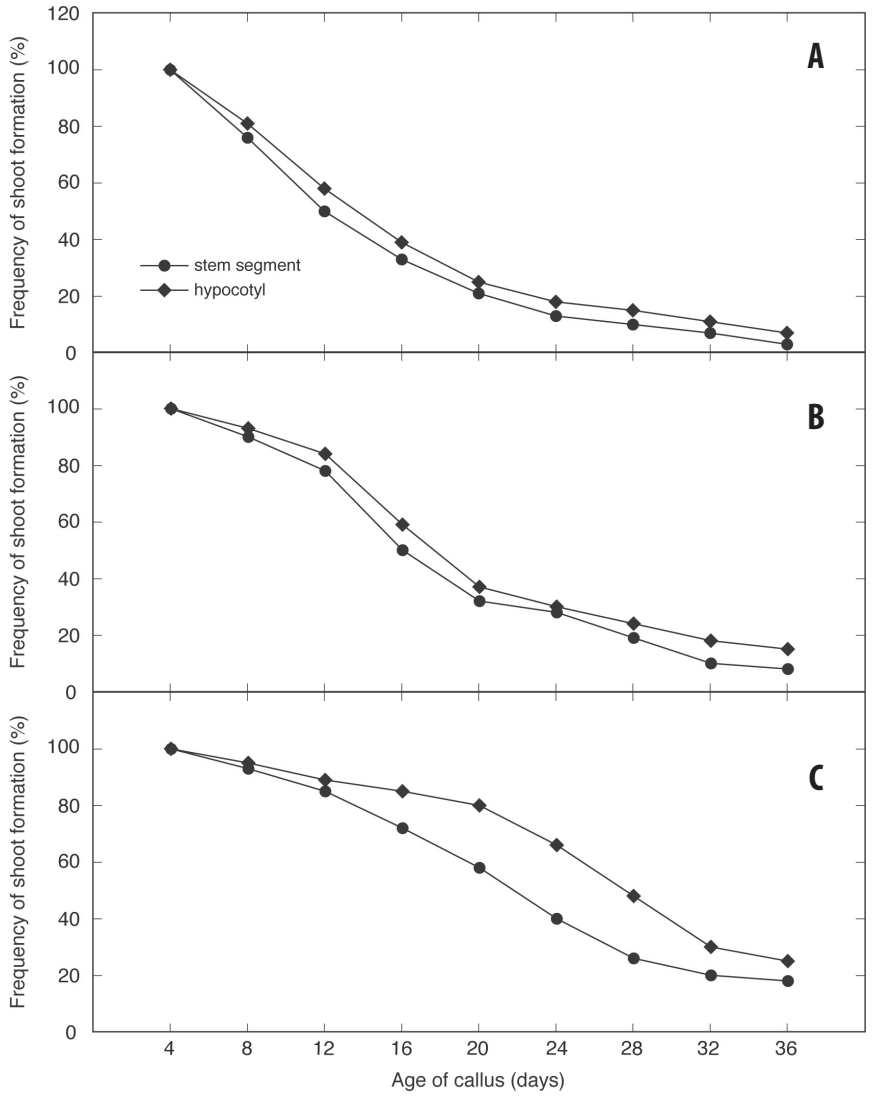

Fig. 1. Effect of callus age on frequency of shoot formation of linseed flax cultivars 'Lirina' (A), 'Barbara' (B) and 'Szaphir' (C).

to the hypocotyl-derived callus. The highest shoot number per explant was obtained on combined MSB_5 medium supplemented with 2iP.

There were significant differences in response to shoot regeneration among the tested cultivars. The number of shoots produced per stem segment-derived callus ranged from 3.76 for cultivar 'Szaphir' to 2.37 for cultivar 'Lirina' (Table 2). The results indicate that shoot regeneration ability is strongly influenced by the genotype used.

The age of callus also affected shoot regeneration in oilseed flax. Four-week-old callus showed the maximum shoot regeneration frequency (Fig. 1).

The shoot regeneration frequency decreased with an increase in callus age from 4 to 36 weeks, similarly for stem segment-derived and hypocotyls-derived callus, excepting cultivar 'Szaphir'. The organogenesis potential of hypocotyl-derived callus of 'Szaphir' declined less with age: the shoot formation frequency from twenty-weeks-old callus was only 20 % less than that from four-week-old callus. Among the tested cultivars, the morphogenic potential was most rapidly lost with age for callus of cultivar 'Lirina'.

Discussion

The process of organogenesis appears to be complex, involving multiple internal and external factors. The reinitiation of cell division, considered to be one of the key factors during regeneration, appears to be controlled differently depending on the model system. The type of first division under inductive conditions can be different (Blervacq et al. 1995), often depending on growth regulators in the culture medium and the type of the primary explant used (Dedicova et al. 2000).

Studies spanning the past 50 years have shown the effects of plant hormones on cell proliferation. However, because most hormones also provoke morphogenic effects, the cell-cycle consequences may be direct or part of the morphogenetic response. Cytokinins and auxins are indispensable for maintaining undifferentiated cells in proliferation during in vitro culture and are directly linked to cell proliferation (Dewitte, Murray 2003). Many plant species require both exogenous auxin and cytokinin in a suitable proportion in order to induce shoot formation. Chen et al. (1998) observed that a medium containing the combination of 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 1 mg l^{-1} BAP significantly improved shoot regeneration in an anther culture of flax. In another study, the maximum shoot regeneration frequency in *Brassica* species was obtained in medium supplemented with 3.0 mg l^{-1} BAP and 0.15 mg l^{-1} NAA (Tang et al. 2003). Hypocotyls of *Beta vulgaris* showed the best response of adventitious shoot regeneration in medium supplemented by BAP and NAA (Zhang et al. 2004). However, other studies have shown that shoot formation on hypocotyls of *Linum* seedlings was marginally promoted by a cytokinin (BAP) or thidiazuron (Mundhara, Rashid 2002).

Although the use of low auxin/cytokinin ratios is common for *in vitro* shoot induction of flax (Marchall, Courduries 1992; Cunha, Ferreira 1996), the development of adventitious shoots of the tested linseed cultivars seems to be determined by a low 2iP concentration. The present results suggest that kinetin, at least at concentration above 1.0 mg l^{-1}, inhibits shoots regeneration from oilseed flax callus. In fact, the kinetin in combination with 0.1 mg l^{-1} IAA had lowest inhibitory effect on the development of shoots.

A single media was employed by Blinstrubiene et al. (2004) to induce callus and regeneration from stem segments and hypocotyls. Generally there was no difficulty in the induction of callus, with the stem segments producing more callus than the hypocotyls. However, the present results show that hypocotyl-derived callus had better morphogenic ability in comparison with stem segment-derived callus. Successful shoot regeneration was found to depend on genotype and culture media. This is in agreement with the results of other published work with flax cultivars (Chen et al. 1998; Nichterlein et al. 1991).

In conclusion, this study demonstrates that hypocotyls and stem segments of linseed flax can be used for adventitious shoot organogenesis and provide a regeneration method using a convenient and abundant tissue source that will facilitate the application of

biotechnology for the continued study and improvement of this plant species.

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Organoģenēze *Linum usitatissimum* kallusa kultūrā

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Kopsavilkums

Pārbaudīja sēklu linu šķirņu 'Lirina', 'Barbara' un 'Szaphir' reģenerācijas spēju adventīvo dzinumu veidošanā no kallusiem, kas iegūti no hipokotila un stumbra segmentiem. Uz vienu eksplantu iegūto dzinumu daudzumu ievērojami ietkmēja genotips, kultivēšanas barotne un izmantotie augšanas regulatori. Vislielāko skaitu dzinumu uz eksplantu ieguva uz hipokotila kallusa šķirnei 'Szaphir', kultivējot uz kombinētās barotnes MSB_5 2.0 mg l^{-1} N^6-Δ^2-isopentenil adenīna klātbūtnē. Hipokotila kallusu izmantošana ļāva iegūt labākus rezultātus, nekā stumbra segmentu kallusu izmantošana. Maksimālā dzinumu reģenerācijas frekvence bija četras nedēļas veciem kallusiem.

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Induction of somatic embryos on *in vitro* cultured zygotic embryos of spring *Brassica napus*

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Abstract

Mature and immature zygotic embryos of three *Brassica napus* doubled haploid lines were examined for their ability to produce somatic embryos. Seedlings from mature seeds did not produce somatic embryos in all of the tested media. Induction of somatic embryogenesis directly from immature seeds of doubled haploid lines was obtained on plant growth regulator-free medium. There was no callus phase, thus, the time of induction was very short. Age of zygotic embryos was the most significant factor influencing somatic embryos induction and development into plantlets. Immature zygotic embryos at the age of 20 to 21 days after pollination were most suitable for direct somatic embryogenesis in the tested rapeseed doubled haploid lines.

Key words: *Brassica napus,* zygotic embryos, somatic embryogenesis.

Introduction

The oilseed *Brassica* species (*B. napus*, *B. rapa* and *B. juncea*) are among the most important vegetable oil and protein-rich meal crops producers in the world. Cultivation of oilseed *Brassica* has increased tremendously during the last decade and, by now, it is the second largest contributor to the world supply of vegetable oil. This success is largely attributed to continuous and intensive breeding efforts (Zhou 2001). Oilseed *Brassica napus* L. was the first crop species in which breeding was achieved by both traditional as well as modern methods.

Various plant tissues have been used for regeneration of *B. napus* shoots, including hypocotyls (Khehra et al. 1992), cotyledons (Narasimhulu et al. 1988), root segments (Sharma et al. 1989), stem and leaf segments of one-month old seedlings (Ovesna et al. 1993). The creation of novel germplasm through techniques of tissue culture and gene transfer holds great potential for improving the quality and agronomic characters of rapeseed. For this, *in vitro* regeneration of plants via organogenesis or embryogenesis is a prerequisite. Regeneration of somatic embryos from plant tissue has been reported for many plant species (Bajaj 1995). Mature and immature zygotic embryos have been used to initiate a regenerable culture in many plants, including *Arabidopsis thaliana* (Luo, Koop 1997), *Pisum sativum* (Tetu et al. 1990), and *Dalbergia latifolia* (Rao, Sita 1996), however there have been only a few reports on the induction of direct somatic embryogenesis in *B. napus* with seeds as the starting material (Graves et al. 1991; Koh, Loh 2000).

The aim of the present paper was to induce direct somatic embryogenesis from zygotic

embryos of spring rapeseed of different doubled haploid lines.

Materials and methods

The investigation was carried out with doubled haploids (DH) 07-133, 07-152, 07-196 of spring rape (*Brassica napus* L.). Donor plants were grown in the growth room with a light intensity of 5000 lx and a 16-h photoperiod. the age of zygotic embryos was recorded as the number of days after pollination (DAP). Both mature and immature seeds (age 14 to 29 DAP) were used as explants for producing somatic embryos.

Seed pods of various age were surface-sterilised in 70 % ethanol for 2 min and rinsed three times with autoclaved water. Seeds (10 per dish) were aseptically germinated in 90 mm Petri dishes containing 25 ml of Murashige and Skoog (1962; MS) basal medium supplemented with 2 % sucrose and solidified with 0.8 % Difco-Bacto agar. The MS medium was adjusted to pH 3.5, 4.0 or 5.0 before the addition of agar and autoclaving at 115 °C for 30 min.

The Petri dishes were sealed with Parafilm and incubated at 25 ± 2 °C temperature, a 16-h photoperiod, and 5000 lx light intensity. After three days the seedlings were transferred to fresh MS medium of the same pH and cultivated 28 days under similar lighting and temperature conditions.

For each treatment, 30 seeds were cultured and the experiment was repeated three times. The percentage of seeds forming somatic embryos and the mean number of somatic embryos per embryogenic seedling was recorded. Mean values and SE for each genotype were calculated.

Results

Many of the seedlings developed swollen hypocotyls within two weeks after germination. Seedlings from matured seeds did not produce somatic embryos in all of the tested media (data not shown).

Immature zygotic embryos cultured on media with various pH formed white nodular structures without an intervening callus phase. The white nodular structures developed into globular somatic embryos after four weeks of culture, indicating that the nodular structures were somatic embryos at an early developmental stage. The frequency of somatic embryo formation was significantly affected by the genotype, zygotic embryo age and the pH of the medium.

Seedlings that developed from zygotic embryos at the age of 14 to 15 DAP cultivated on pH 3.5 medium did not form any somatic embryos (Table 1). Higher medium pH (4.0) stimulated somatic embryogenesis of lines 07-196 and 07-133, however increasing the medium pH to 5.0 increased the embryogenic potential only of line 07-133. Seedlings developed from zygotic embryos of age 20 to 21 DAP had underwent somatic embryogenesis in all of the tested media. Explants of lines 07-196 and 07-133 exhibited the greatest frequency of somatic embryo formation at pH 5.0 medium, while a pH of 4.0 was more suitable for line 07-152. Zygotic embryos isolated 25 to 26 DAP positively responded on all tested media, except explants of line 07-152 cultivated at pH 3.5 medium. For lines 07-196 and 07-152 the highest somatic embryos induction rates were observed at pH 5.0 of medium, while a lower medium pH (4.0) raised somatic embryogenesis for line

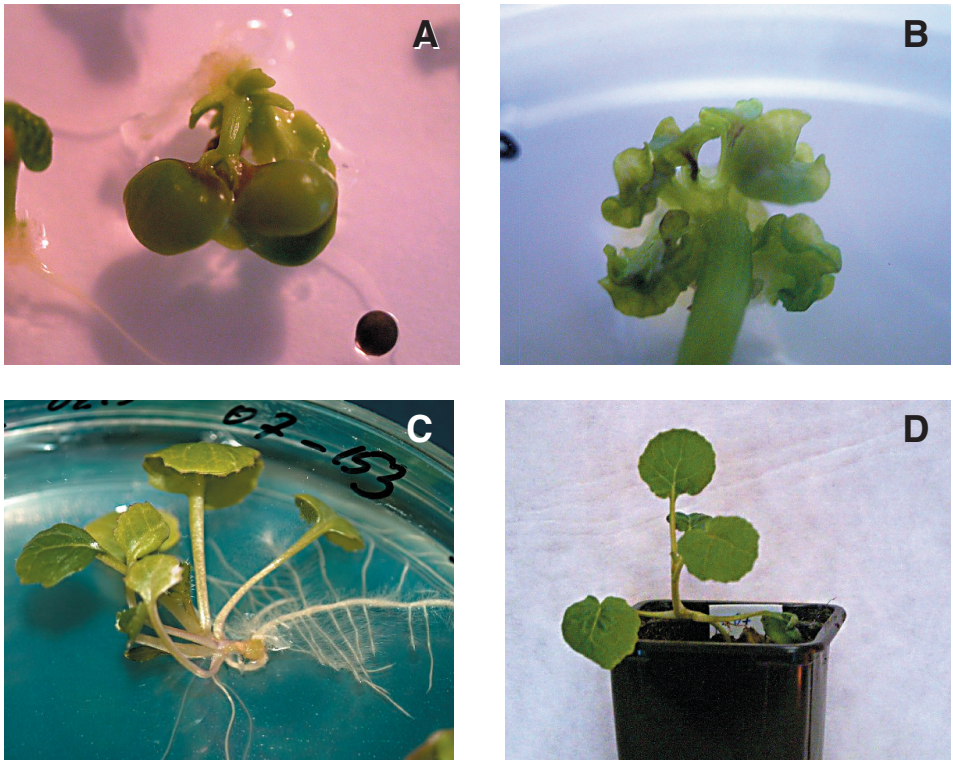

Fig. 1. Somatic embryogenesis of *Brassica napus*: A, somatic embryos on the hypocotyls; B, cotyledonary somatic embryos after four weeks of culture; C, plantlets in regeneration medium; D, rapeseed seedlings in greenhouse.

07-133 explants. Immature zygotic embryos at the age of 28 to 29 DAP had the greatest embryogenic potential at medium pH 5.0 (lines 07-196 and 07-152) and pH 4.0 (line 07-133).

Age of zygotic embryos was the most significant factor influencing somatic embryo development into plantlets. Zygotic embryos isolated 20 to 21 DAP showed the highest number of somatic embryos per explant (Table 2).

The frequency of somatic embryo formation decreased with an increase in zygotic embryo age. This suggests that zygotic embryos of 20 to 21 DAP are most suitable for direct somatic embryogenesis in the tested lines. The number of somatic embryos per embryogenic seedling also varied with the pH of medium. Culture medium of pH 4.0 appeared to be the best for somatic embryo formation, as it supported the highest number of somatic embryos per explant for DH 07-196 (18.2) and DH 07-152 (7.8), while DH 07-133 produced the highest number of SE per embryogenic seedling cultivated at pH 5.0 of medium.

Most somatic embryos were formed on the hypocotyls (Fig. 1A) of immature zygotic embryos and a few somatic embryos were formed on the cotyledon. No somatic embryos

Table 1. Effect of of zygotic embryo age and medium pH on percentage of somatic embryogenesis of *Brassica napus* doubled haploid DH lines

Genotype	Time after pollination (days)	Seedlings with somatic embryos (%)		
		pH 3.5	pH 4.0	pH 5.0
DH 07-196	14 - 15	0.0	4.7 ± 0.9	3.2 ± 0.4
DH 07-133	14 - 15	0.0	4.3 ± 1.1	12.3 ± 2.1
DH 07-152	14 - 15	0.0	0.0	0.0
DH 07-196	20 - 21	13.1 ± 2.3	4.6 ± 0.7	14.7 ± 2.3
DH 07-133	20 - 21	6.7 ± 1.1	9.1 ± 1.9	13.3 ± 1.9
DH 07-152	20 - 21	2.3 ± 0.4	14.8 ± 2.5	8.1 ± 1.6
DH 07-196	25 - 26	29.2 ± 3.6	25.2 ± 3.1	34.4 ± 4.2
DH 07-133	25 - 26	4.7 ± 0.9	31.3 ± 3.8	13.2 ± 2.3
DH 07-152	25 - 26	0.0	6.7 ± 1.5	30.7 ± 3.5
DH 07-196	28 - 29	47.3 ± 4.9	27.6 ± 3.2	89.8 ± 6.9
DH 07-133	28 - 29	4.7 ± 0.8	37.7 ± 4.1	11.1 ± 2.2
DH 07-152	28 - 29	0.0	0.0	14.3 ± 2.7

were formed on the radicle. Globular embryos derived from immature zygotic embryos developed into cotyledonary embryos after an additional four weeks of culture. Embryos and accessory cotyledons formed in a ring around the top of the hypocotyls, and on some embryos one to two accessory root poles were also initiated lower down near the original root pole. Most somatic embryos possessed two cotyledons, however, some had three or more cotyledons (Fig. 1B). Upon transfer to B_5 basal medium, cotyledonary embryos developed into plantlets (Fig. 1C) at a frequency of approximately 55 %. Plantlets were acclimatized to greenhouse conditions and resembled true rapeseed seedlings in growth habit (Fig. 1D).

Discussion

Direct somatic embryogenesis from hypocotyls of immature zygotic embryos has been reported for *Brassica campestris* (Maheswaran, Williams 1986), *Linum usitatissimum* (Pretova, Williams 1986), *Brassica napus*, *Brassica rapa* and *Brassica juncea* (Graves et al. 1991), and *Rosa hybrida* (Kim et al. 2003). Factors controlling the initiation process have been reviewed by Williams and Maheswaran (1986) and further discussed by Pretova et al. (2000). It has been suggested that initiation involves a weakening of the cell-cell interaction gradient, which coordinates normal bipolar development of the embryo. In the presence of a continuing stimulus for mitotic divisions, cells which are relatively undifferentiated and retain their internal pre-determination for embryo morphogenesis may escape from overall group control to re-initiate the embryogenic pathway independently as somatic embryoids. The external culture medium is believed to be permissive rather than determinative for somatic embryogenesis in this system.

It has been hypothesized that the addition of 6-benzylaminopurine in the process of

Table 2. Effect of zygotic embryo age and medium pH on intensity of somatic embryogenesis of *Brassica napus* doubled haploid (DH) lines

Genotype	Time after pollination (days)	Number of somatic embryos per embryogenic seedling		
		pH 3.5	pH 4.0	pH 5.0
DH 07-196	14 - 15	0.0	1.1 ± 0.1	8.4 ± 1.2
DH 07-133	14 - 15	0.0	3.2 ± 0.8	6.3 ± 1.3
DH 07-152	14 - 15	0.0	0.0	0.0
DH 07-196	20 - 21	1.1 ± 0.2	18.2 ± 2.7	10.3 ±2.4
DH 07-133	20 - 21	4.3 ± 0.9	6.1 ± 1.8	8.5 ± 2.1
DH 07-152	20 - 21	2.2 ± 0.7	7.8 ± 1.9	6.7 ± 1.1
DH 07-196	25 - 26	3.8 ± 1.1	4.4 ± 0.8	5.8 ± 0.9
DH 07-133	25 - 26	2.3 ± 0.8	3.2 ± 0.7	1.5 ± 0.1
DH 07-152	25 - 26	0.0	3.1 ± 0.5	3.3 ± 1.2
DH 07-196	28 - 29	0.2 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
DH 07-133	28 - 29	1.2 ± 0.3	0.3 ± 0.1	5.5 ± 0.8
DH 07-152	28 - 29	0.0	0.0	1.4 ± 0.2

direct somatic embryogenesis is responsible for preservation of the mitotic stimulus in the hypocotyl cells of the immature zygotic embryo (Pretova et al. 2000). The best embryogenic response in *Brassica napus* cultivar 'Regent' was obtained on a medium supplemented with 0.05 mg l^{-1} 6-benzylaminopurine, while somatic embryo production from 'Westar' zygotic embryos was improved two to three fold by decapitating the shoot apical meristem (Graves et al. 1991).

Auxin has been found to be essential for somatic embryo induction in many plant species (Merkle et al. 1995). However, immature zygotic embryos of some species require a relatively high concentration of cytokinin, which can reverse the arrest of pre-embryogenic cells, resulting in somatic embryogenesis (Norgaard, Krougstup 1991; Laurrain et al. 1996; Kim et al. 2003).

Unlike the results of many previous experiments, the induction and development of somatic embryos from immature zygotic embryos of *Brassica napus* in our study did not require an exogenous supply of plant growth regulators in the culture medium. Thus, we suggest that the immature zygotic embryos of the tested rapeseed lines are comprised of pre-embryogenic cells, which begin division in response to medium pH to differentiate into induced embryogenic determined cells. It has been hypothesized that low external pH may simulate auxin action during the induction of somatic embryos by lowering the cytosolic pH (Koh, Loh 2000). Another possible effect of low medium pH could be enhancement of the uptake of nutrients such as Fe compounds or NO_3^- (Polowick, Sawhney 1991), which could affect the subsequent development of the Brassica napus culture.

The procedure for somatic embryo induction from immature zygotic embryos described in this paper is efficient and can rapidly produce normal intact plants. This system may be of considerable potential for the study of plant embryogenesis, as it excludes the use of exogenous phytohormones and other complicated culture manipulations.

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Organoģenēze *Linum usitatissimum* kallusa kultūrā

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Kopsavilkums

Pārbaudīja nobriedušu un nenobriedušu *Brassica napus* trīs dažādu dubulto haploīdo līniju zigotisko embriju spēju veidot somatiskos embrijus. Dīgsti no nobriedušām sēklām neveidoja somatiskos mebrijus nevienā no pārbaudītajām barotnēm. Somatiskā embrioģenēze inducējās tieši no dubulto haploīdu sēklām barotnē bez augšanas regulatoriem. Tā kā attīstība notika bez kallusa fāzes, indukcijas laiks bija ļoti īss. Zigotisko embriju vecums bija būtiskākais faktors, kas ietekmēja somatisko embriju indukciju un to attīstību par mikroaugiem. Pārbaudītajām rapšu dubulto haploīdu līnijām vispiemērotākais nenobriedušo zigotisko embriju vecums tiešās somatiskās embrioģenēzes norisei bija 20 līdz 21 dienu pēc apputeksnēsanas.

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Mieturaļģu sugu nosaukumi un termini latviešu valodā

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Kopsavilkums

Darbā sniegti jaunizveidotie un Terminoloģijas apakškomisijas akceptētie mieturaļģu ģinšu un sugu latviskie nosaukumi un morfoloģiskie termini. Sniegts morfoloģisko terminu skaidrojums, ko papildina shematiski zīmējumi, kā arī vispārīgs mieturaļģu morfoloģijas raksturojums. Īpaša uzmanība pievērsta to morfoloģisko terminu skaidrojumam, kuri līdz šim latviešu literatūrā minēti maz vai nemaz, bet ir ļoti svarīgi sugu noteikšanā un aprakstu veidošanā. Vairākiem svarīgiem morfoloģiskajiem terminiem pirmo reizi dots skaidrojums latviešu valodā. Darbā apkopoti 25 sugu un četru mieturaļģu ģinšu latviskie nosaukumi, kā arī 27 morfoloģisko terminu skaidrojumi. Vārdnīcā apkopoti morfoloģiskie termini latviešu, angļu, vācu, latīņu un krievu valodā.

Atslēgas vārdi: *Chara*, mieturaļģes, morfoloģija, *Nitella*, *Nitellopsis*, termini, *Tolypella*.

Ievads

Mieturaļģes Latvijā ir bieži sastopamas, tās aug ezeros, dīķos, karjeros, upēs, jūrā un citur (Rudzroga 1995b). Neskatoties uz to lielo nozīmi dabā, mieturaļģes līdz šim Latvijā ir pētītas ļoti maz. Uzsākot pētījumus šajā jomā, konstatējām, ka vairumam mieturaļģu nav izveidoti sugu latviskie nosaukumi, kā arī trūkst daudzu mieturaļģu morfoloģiju raksturojošo terminu latviešu valodā.

Citu tautu valodās – angļu, vācu, zviedru, dāņu, somu, lietuviešu un igauņu valodās mieturaļģēm lieto nacionālos nosaukumus (Schubert, Blindow 2003), tādēļ tādi ir nepieciešami arī latviešu valodā.

Mieturaļģu uzbūve un morfoloģija ir savdabīga un būtiski atšķiras no citām aļģēm un vaskulārajiem augiem, tādēļ to morfoloģiskajiem aprakstiem nepieciešami vairāki termini, kādi netiek lietoti nedz citām aļģēm, nedz arī vaskulārajiem augiem. Iepazīstoties ar literatūru, konstatējām, to, ka trūkst vienprātības līdz šim lietotajos latviskajos mieturaļģu morfoloģiskajos terminos, kā arī to, ka vairāki termini, kuri apzīmē sugu noteikšanā svarīgas pazīmes, vispār nav ne reizi lietoti latviešu valodā publicētajā literatūrā. Tas ievērojami apgrūtina mieturaļģu sugu aprakstu un noteikšanas tabulu sastādīšanu un lietošanu.

Mūsu darba mērķis bija apzināt līdz šim lietotos sugu nosaukumus un morfoloģiskos terminus un visām Latvijā sastopamajām mieturaļģēm izveidot sugu un ģinšu latviskos nosaukumus, kā arī morfoloģiskos terminus.

Visiem morfoloģiskajiem terminiem dots skaidrojums, un tie apkopoti vārdnīcā angļu, vācu, krievu un latīņu valodā. Izmantojot apkopotos un jaunizveidotos morfoloģiskos terminus, izveidots mieturaļģu morfoloģiskais raksturojums.

Materiāls un metodes

Meklējot sugu un ģinšu latviskos nosaukumus, tika pārbaudīta zinātniskā un uzziņu literatūra.

Tika ierosināts trīs ģintīm izveidot jaunus ģinšu nosaukumu, kas būtu labskanīgi un latviskas cilmes. Sugu nosaukumus, kuri agrāk jau bija minēti literatūrā, tika saglabāti, bet pārējām sugām epiteti tika vai nu tieši tulkoti no latīņu valodas, vai arī tie tika izveidoti atbilstoši sugas raksturīgākajām morfoloģiskajām pazīmēm.

Izvērtējot līdz šim literatūrā minētos morfoloģiskos terminus, tika izvēlēti un saglabāti tie, kuri labāk atbilda aprakstāmajai pazīmei. Morfoloģiskajiem terminiem, kuriem līdz šim nebija minēti latviskie nosaukumi vai arī tie izrādījās nepiemēroti, tika izveidoti jauni, pamatojoties uz terminiem latīņu un citās valodās (Kirpichnikov, Zabinkova 1977; Vaczy 1980; Gollerbah, Krasavina 1986; Moore 1986). Veidojot jaunus terminus, pieturējāmies pie principa, lai mieturaļģēm specifiskie morfoloģiskie termini nebūtu identiski vaskulāro augu morfoloģiskajiem terminiem. Savukārt, termini, kas vienlīdz labi raksturo gan vaskulāros augus, gan mieturaļģes (piemēram, posms, mezgls, miza) netika mainīti.

Sugu, ģinšu nosaukumus un morfoloģiskos terminus apstiprināja Terminoloģijas apakškomisijas sēdē 2004. gada 11. martā.

Rezultāti un diskusija

Ģinšu un sugu latviskie nosaukumi

Ģints *Chara* pirmā ir minēta ar latviskotu nosaukumu "hāra" (chāra) (Galenieks 1929). Vēlākajā literatūrā ģints latviskais nosaukums lietots dažādi: gan "hāra" (Rudzroga 1984), gan "hara" (Hržanovskis, Ponomarenko 1986; Piterāns, Vimba 1987; Rudzroga 1995a; 1995b). Terminoloģijas apakškomisija ierosināja *Chara* ģinti turpmāk saukt par "mieturīti", jo tiešais zinātniskā nosaukuma latviskojums "hara" nav labskanīgs latviešu valodā.

Pirmais sugas epitets lietots mieturaļģei *Chara globularis* (sinonīms *C. fragilis*) – "trauslā hāra" (Rudzroga 1984). Vēl sugu latviskie nosaukumi literatūrā atrodami sugām *C. aspera* – "skarbā hāra", *C. vulgaris* – "parastā hāra", *C. canescens* – "iesirmā hāra" un *C. baltica* – "Baltijas hāra" (Rudzroga 1995a). Pārējām sugām latviskie nosaukumi līdz šim nav lietoti.

Ģintij *Nitella* ieteikts saglabāt līdz šim lietoto nosaukumu – "nitella" (Rudzroga 1996). Ģints *Nitella* sugu latviskie nosaukumi literatūrā nav minēti.

Ģintīm *Nitellopsis* un *Tolypella* un to sugām latviskie nosaukumi līdz šim nav lietoti. Ģints *Nitellopsis* ieteicamais nosaukums ir "nitellīte", bet *Tolypella* – "kamolīte".

Latvijā sastopamo mieturaļģu sugu latviskie nosaukumi sniegti 1. tabulā.

Mieturaļģu morfoloģiskie termini

Latvijā nav senas mieturaļģu pētīšanas vēstures, tādēļ visbiežāk mieturaļģes minētas dažādās botānikas mācību grāmatās (Galenieks 1929; Galenieks 1948; Piterāns u.c. 1975). Literatūrā latviešu valodā, aprakstot vienu aļģes lapoņa daļu, bieži vien izmantoti dažādi termini (2. tabula). Aplūkojot dažādos laikos publicēto literatūru, var secināt, ka tikai nedaudzi termini, tādi kā anterīdijs, oogonijs, oospora, mezgls, posms un rizoīds, kuri nav specifiski mieturaļģēm, lietoti vienādi. Pārējo morfoloģisko terminu lietojums dažādos darbos atšķiras.

1. tabula. Mieturaļģu latviskie nosaukumi

Chara aspera	skarbā mieturīte
C. baltica	Baltijas mieturīte
C. canescens	iesirmā mieturīte
C. connivens	satuvinātā mieturīte
C. contraria	pelēkā mieturīte
C. filiformis	pavedienu mieturīte
C. globularis	trauslā mieturīte
C. hispida	dzeloņainā mieturīte
C. intermedia	vidējā mieturīte
C. rudis	raupjā mieturīte
C. strigosa	asā mieturīte
C. tomentosa	savītā mieturīte
C. virgata	slaidā mieturīte
C. vulgaris	parastā mieturīte
Nitella batrachosperma	kuplā nitella
N. flexilis	lokanā nitella
N. gracilis	slaidā nitella
N. mucronata	smailā nitella
N. opaca	blāvā nitella
N. syncarpa	gļotainā nitella
N. tenuissima	smalkā nitella
N. translucens	caurspīdīgā nitella
Nitellopsis obtusa	strupā nitellīte
Tolypella nidifica	ligzdainā kamolīte
T. prolifera	pušķu kamolīte

Atsevišķos agrākajos darbos (Galenieks 1948; Piterāns u.c. 1975) vairāki mieturaļģēm izmantotie termini, piemēram, vainags un bumbulis, ir identiski ar tiem, ko lieto vaskulārajiem augiem. Šiem orgāniem ir līdzīga funkcija, taču izcelsme un uzbūve ir dažāda, tāpēc attiecībā uz mieturaļģēm nebūtu pareizi lietot šādus terminus.

Salīdzinot mieturaļģu morfoloģiskos terminus dažādās valodās (3. tabula), redzams, ka angļu un vācu valodās vairums terminu ir pārņemti no latīņu valodas. Pretēji tam, krievu valodā gandrīz visi termini ir krieviskoti, neizmantojot latīņu valodas terminus, daļa pat izvērsta paskaidrojumā, lai lietotājs labāk saprastu vienu vai otru morfoloģisko pazīmi.

Mieturaļģu morfoloģiskais raksturojums

Mieturaļģes ir makroskopiskas daudzšūnu aļģes ar sarežģītu uzbūvi. To uzbūve un morfoloģija ir savdabīga un būtiski atšķiras no citām aļģēm un vaskulārajiem augiem, tādēļ to morfoloģiskajiem aprakstiem nepieciešami vairāki termini, kas netiek lietoti aprakstot citas aļģes un augus. Visu morfoloģisko terminu skaidrojums, kas nepieciešams mieturaļģu morfoloģijas raksturojuma veidošanai, sniegts 4. tabulā.

Visām mieturaļģu ģintīm laponis sastāv no galvenās ass, no kuras atiet sānzari un īszari

2. tabula. Morfoloģisko terminu salīdzinājums literatūrā un ieteicamais termins

Augu sistemātika (Galenieks 1948)	Zemāko augu sistemātika (Piterāns u.c. 1975)	Zemāko augu sistemātikas praktikums (Piterāns, Vimba 1987)	Botānika (Hržanovskis, Ponomarenko 1986)	Izplatītāko Latvijas aļģu noteicējs (Rudzroga 1995)	Ieteicamais termins
anteridijs	anterīdijs	anterīdijs	anterīdijs	anterīdijs	anterīdijs
posma šūna	posma šūna	posma šūna	centrālā šūna	gara, cilindriska šūna	centrālā šūna
				divrindu miza	divkārša miza
				dzelonītis	dzelonītis
	galvenā ass, gardzinums	galvenā ass	lapoņa ass	galvenā ass, stublis	galvenā ass
	dzinumu bumbulis	bumbuļveida izaugums, bumbulis	bumbulis	gumiņš	gumiņš
atzarojums	īsdzinums	sānzars ar ierobežotu augšanu, īsais sānzars	atzarojums	sānu dzinums, „lapas"	īszars
mezgls	mezgls	mezgls	mezgls	mezgls	mezgls
	miza	miza	ārējās šūnas	miza	miza
oogonijs	oogonijs	oogonijs	oogonijs	oogonijs	oogonijs
mizas šūna	mizas šūna	mizas šūna		mizas šūna	oogonija segšūna
	oospora	oospora	oospora	oospora	oospora
				pielapa	pielapa
	pielapām līdzīgs vainags				pielapu gredzens
posms	posms	posms	posms	posms	posms
	rizoīds	rizoīds	rizoīds	rizoīds	rizoīds
lapai līdzīgais zars			vienšūnas zarojums		sānšūna
	otrās pakāpes gardzinums, sānass	sānzari ar neierobežotu augšanu	sānu zarojums		sānzars
				trīsrindu miza	trīskārša miza
vainags	vainags	vainadziņš	vainadziņš	vainadziņš	vainadziņš
				vienrindas miza	vienkārša miza

3. tabula. Latviešu – angļu – vācu – latīņu – krievu morfoloģisko terminu vārdnīca

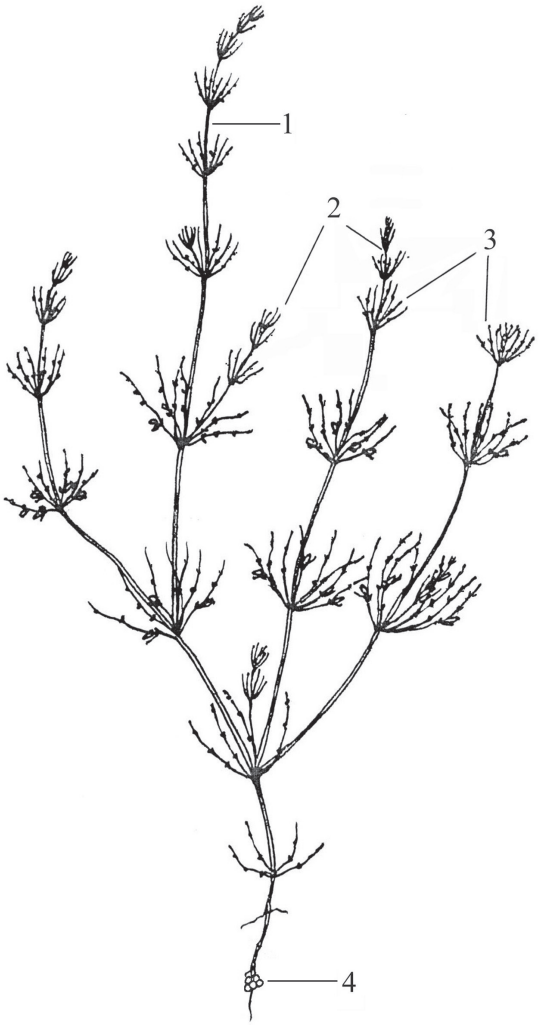
Latviešu	Angļu (Moore 1986)	Vācu (Krause 1997)	Latīņu (Kirpichnikov, Zabinkova 1977; Vaczy 1980)	Krievu (Gollerbah, Krasavina 1986)
anterīdijs	antheridium	Antheridium	*antheridium*	антеридий
aulakanta miza	aulacanthous cortex	aulacanthe Rinde	*cortex aulacanthus*	первичная коровая полоса меньше вторичной
centrālā šūna	internode cell	Internodialzelle	*internode cell*	вытянутая клетка
dažādjoslu miza	heterostichous cortex	heterostiche Rinde	*cortex heterostichus*	кора с неравновеликими коровыми трубками
divkārša miza	diplostichous cortex	diplostiche Rinde	*cortex diplostichus*	двухполосная кора
dzelonītis	spine cell	Stachel	*aculeus*	шип
galvenā ass	main axis	Achse	*caule*	стебель
gumiņš	bulbil	Bulbille	*bulbillus*	клубенёк
īszars	branchlet	Ast	*folia verticillorum*	лист
mezgls	node	Knoten, Nodium	*nodus*	узел
miza	cortex	Rinde	*cortex*	кора
oogonijs	oogonia	Oogonium	*oogonium*	оогоний
oogonija segšūna	peripheral cell	Hüllzelle	–	обволакивающая клетка
oospora	oospore	Oospore	*oospora*	ооспора
pielapa	stipulode	Stipulare	*stipula*	прилистик
pielapu gredzens	ring of stipulodes	Stipularkranz	–	венчик прилистков
posms	internode	Internodium	*internodium*	междоузлие
primārā mizas josla	primary row	primäre Reihe	–	первичная коровая полоса
rizoīds	rhizoid	Rhizoide	*rhizoides*	ризоид
sānšūna	bract-cell, bracteole, bractlet	Brakteolen Blättchen	*bractea, bracteola*	лисовой членик
sānzars	branche	Ast	*foliola*	боковой побег
sekundārā mizas josla	secondary row	sekundäre Reihe	–	вторичная коровая полоса
tilakanta miza	tylacanthous cortex	tylacanthe Rinde	*cortex tylacanthus*	первичная коровая полоса крупнее вторичной
trīskārša miza	triplostichous cortex	triplostiche Rinde	*cortex triplostichus*	трехполосная кора
vainadziņš	coronula	Krönchen	*coronula*	коронка
vienādjoslu miza	isostichous cortex	isostiche Rinde	*cortex isostichus*	кора с равновеликими коровыми трубками
vienkārša miza	haplostichous cortex	haplostiche Rinde	*cortex haplostichus*	однополосная кора

4. tabula. Mieturaļģu morfoloģisko terminu skaidrojums

Termins	Termina skaidrojums
anterīdijs	vīrišķais gametangijs, kurā attīstās vīrišķās dzimumšūnas
aulakanta miza	miza, kurā primārās mizas joslas ir mazākas nekā sekundārās mizas joslas; dzelonīši atrodas uz iegrimušajām mizas šūnu joslām
centrālā šūna	gara cilindriska šūna, kura veido posmu un ģintī *Chara* ir klāta ar mizas šūnām
dažādjoslu miza	miza, kurā primārās un sekundārās mizas joslas ir dažāda lieluma
divkāršā miza	miza ar divkāršu mizas joslu skaitu attiecībā pret īszaru skaitu
dzelonītis	vienšūnas veidojums uz primārās mizas; dzelonīši var būt novietoti pa vienam vai pušķos pa 2 vai 3
galvenā ass	centrālā lapoņa ass, ko veido mezgli un posmi; ārēji līdzīga kosas stumbram
gumiņš	balts vai bezkrāsains, vienšūnas vai daudzšūnu, cieti saturošs veidojums uz rizoīdiem un sānzaru lejasdaļas; gumiņš ir veģetatīvās vairošanās un pārziemošanas orgāns
īszars	lapoņa sānass ar ierobežotu augšanu, kas novietoti mieturos galvenās ass un sānzaru mezglos
mezgls	īszara piestiprināšanās vieta mieturī
miza	gareniski izstieptas šūnu joslas ap centrālo šūnu, piešķirot asīm svītrainu un rievainu izskatu; joslas var būt primāras vai sekundāras; raksturīga *Chara* ģints mieturītēm
oogonijs	sievišķais gametangijs, kurā attīstās olšūna
oogonija segšūna	šūna, kas spirāliski aptver oogoniju
oospora	apaugļota olšūna (zigota)
pielapa	vienšūnas veidojums, kas veido pielapu gredzenu
pielapu gredzens	pielapu rinda zem īszaru mietura
posms	ass daļa starp diviem mezgliem
primārā mizas josla	mizas šūnu josla ar dzelonīšiem
rizoīds	bezkrāsains, pavedienveida izaugums; ar rizoīdiem mieturaļģes nostiprinās substrātā un uzņem barības vielas
sānšūna	vienšūnas veidojums uz īszariem; sānšūnas var atrasties starp gametangijiem, uz sterilajiem īszariem un divmāju aļģēm uz oogonijiem anterīdiju vietā; raksturīga *Chara* un *Nitellopsis* ģintīm
sānzars	lapoņa sānass ar neierobežotu augšanu, ko veido mezgli un posmi; ārēji atgādina augu zarus
sekundārā mizas josla	mizas šūnu josla bez dzelonīšiem
tilakanta miza	miza, kurā primārās mizas joslas ir lielākas nekā sekundārās mizas joslas; dzelonīši atrodas uz izvirzītajām mizas šūnu joslām
trīskāršā miza	miza ar trīskāršu mizas joslu skaitu attiecībā pret īszaru skaitu
vainadziņš	5 - 10 šūnas oogonija augšpusē
vienādjoslu miza	miza, kurā primārās un sekundārās mizas joslas ir vienāda lieluma
vienkāršā miza	miza ar vienādu mizas joslu skaitu attiecībā pret īszaru skaitu

(1. attēls). Īszari uz galvenās ass piestiprināti mieturos mezglu vietās. Īszari ģintīm *Chara* un *Nitellopsis* ir nezaroti, bet ģintīm *Tolypella* un *Nitella* - zaroti. Īszaru zarojuma veids ir svarīga pazīme *Nitella* ģints mieturaļģēm. Zarojums ir regulārs *Nitella* ģints mieturaļģēm, savukārt *Tolypella* sterilie īszari ir nezaroti, bet fertilie īszari var zaroties līdz pat četrām reizēm. Starp mezgliem atrodas posmi, posmus veido viena gara centrālā šūna. Visām Latvijā sastopamajām ģints *Chara* sugām centrālo šūnu apņem miza, ko veido vairākas šūnu joslas. *Tolypella*, *Nitella* un *Nitellopsis* aļģēm nav mizas.

Ģints *Chara* un *Nitellopsis* mieturaļģēm uz īszariem atrodas sānšūnas. Latviešu valodā iesakām lietot vienu terminu "sānšūna", kas raksturo veidojumus uz īszariem, un kuriem



1. attēls. *Chara aspera* kopskats. 1 – galvenā ass, 2 – sānzari, 3 – īszari, 4 – rizoīds ar gumiņiem.
Fig. 1. General morphology of *Chara aspera*. 1 – main axis, 2 – branches, 3 – branchlets, 4 – rhizoid with bulbils.

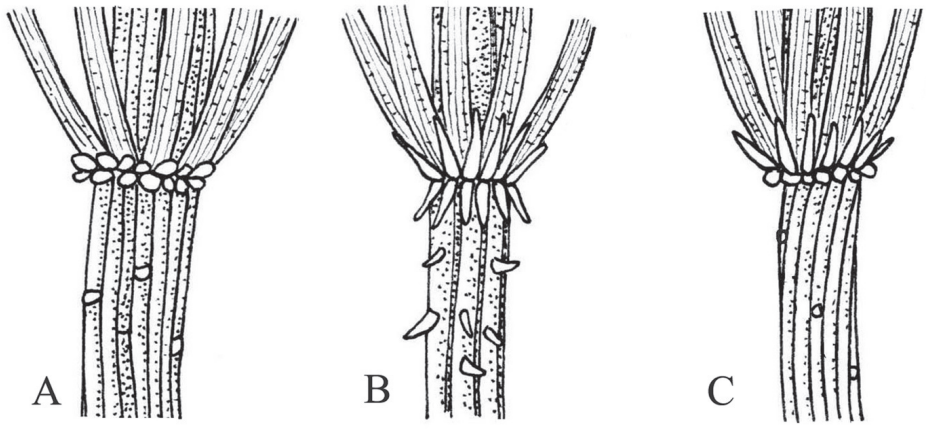
angļu valodā lieto vairākus apzīmējumus – "bract-cell", "bracteole" un "bractlet"; atkarībā no to novietojuma un funkcijām. Sānšūnas var atrasties ap gametangijiem, uz sterilajiem īszariem un anterīdiju vietā zem oogonijiem.

Chara ģintī zem mezgliem atrodas pielapu gredzens, kas sastāv no divām rindām. Pielapu forma ir svarīga sugas pazīme. Pielapu gredzena abas rindas var būt vai nu labi attīstītas, vai arī reducētas, kā arī viena no tām var būt reducēta, bet otra attīstīta (2. attēls).

Chara mizas šūnu joslu skaits attiecībā pret īszaru skaitu ir svarīga pazīme sugu noteikšanā. Miza var būt vienkārša, divkārša vai trīskārša (3. attēls). Vienkāršai mizai mizas joslu skaits ir vienāds ar īszaru skaitu, tāda miza raksturīga tikai vienai Latvijā sastopamai sugai – iesirmajai mieturītei (*C. canescens*). Savukārt divkāršai un trīskāršai mizai mizas joslu skaits ir attiecīgi divas vai trīs reizes lielāks nekā īszaru skaits. Šādas mizas ir raksturīgas visām pārējām sugām.

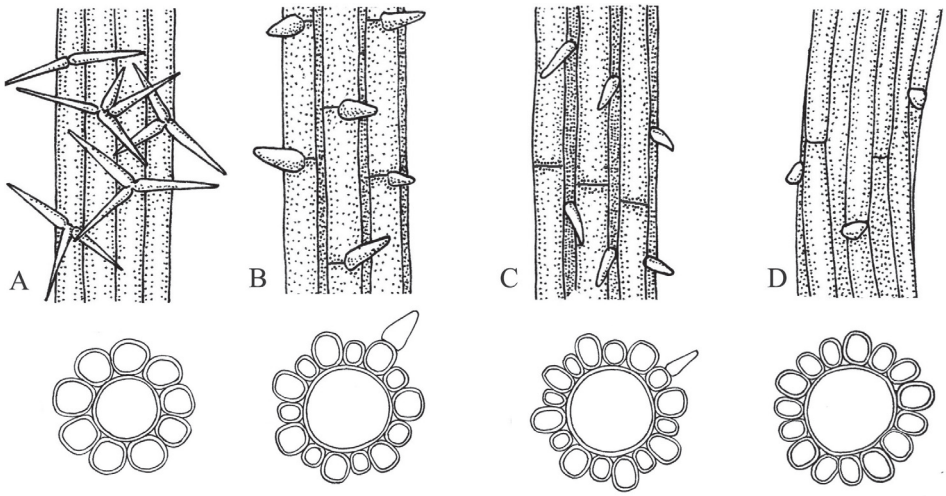
Uz primārajām mizas šūnām atrodas dzelonīši, kuru forma, izmērs un novietojums ir svarīga sugas pazīme. Vienkārša miza sastāv tikai no primārām mizas joslām, un dzelonīši atrodas uz visām joslām. Divkāršai mizai dzelonīši atrodas uz katras otrās, bet trīskāršai mizai – uz katras trešās joslas. Mizas joslas, uz kurām neatrodas dzelonīši, sauc par sekundārajām mizas joslām. Divkāršai mizai starp primārajām joslām atrodas viena sekundārā mizas josla, bet trīskāršai – divas sekundārās mizas joslas.

Pēc primāro un sekundāro mizas joslu novietojuma izšķir divu veidu mizas. Ja primārās mizas šūnu joslas ar dzelonīšiem ir izvirzītas (lielākas par sekundārajām mizas joslām bez dzelonīšiem), šādu mizu sauc par tilakantu. Tilakanta miza ir Latvijā bieži satopamajai savītajai mieturītei (*C. tomentosa*), kā arī jūrā sastopamajai Baltijas mieturītei (*C. baltica*). Savukārt, ja primārās mizas joslas ar dzelonīšiem ir iegrimušas (mazākas par sekundārajām mizas joslām), tādu mizu sauc par aulakantu mizu. Aulakanta miza ir, piemēram, parastajai mieturītei (*C. vulgaris*).

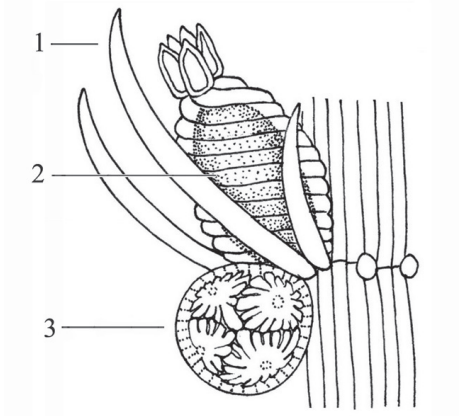


2. attēls. *Chara* ģints galvenā ass ar pielapu gredzeniem. A – reducētas pielapas, *C. globularis*; B – attīstīti abi pielapu gredzeni, *C. baltica*; C – augšējais pielapu gredzens attīstīts, apakšējais reducēts, *C. virgata*.

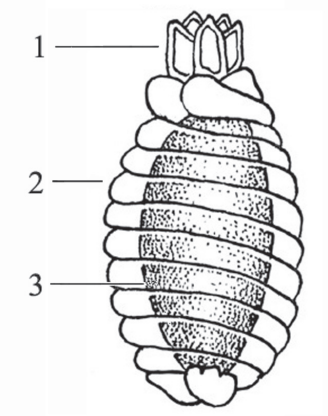
Fig. 2. Main axis and ring of stipulodes of genus *Chara*. A – reduced stipulodes, *C. globularis*; B – both rings of stipulodes developed, *C. baltica*; C – upper ring of stipulodes developed, lower – reduced, *C. virgata*.



3. attēls. *Chara* ģints mizas veidi. Augšējā rindā – galvenās ass sānskats, apakšējā – šķērsgriezums. A – vienkārša miza, *C. canescens*; B – divkārša tilakanta miza, *C. baltica*; C – divkārša aulakanta miza, *C. vulgaris*; D – trīskārša vienādjoslu miza, *C. globularis*.
Fig. 3. Types of cortification in genus *Chara*. Upper row – main axis sideview, lower row – cross-section of the main axis. A – haplostichous cortex, *C. canescens*; B – diplostichous tylacanthous cortex, *C. baltica*; C – diplostichous aulacanthous cortex, *C. vulgaris*; D – triplostichous isostichous cortex, *C. globularis*.



4. attēls. *Chara* ģints gametangiji un sānšūnas ap gametangijiem. 1 – sānšūna, 2 – oogonijs, 3 – anterīdijs.
Fig. 4. Gametangia and bractlets in genus *Chara*. 1 – bractlet, 2 – oogonia, 3 – antheridium.



5. attēls. Oogonija uzbūve. 1 – vainadziņš, 2 – oogonija segšūnas, 3 –oospora.
Fig. 5. Structure of oogonia. 1 – coronula, 2 – peripheral cell, 3 – oospore.

Vīrišķie gametangiji – anterīdiji un sievišķie gametangiji – oogoniji ir novietoti uz īszariem (4. attēls). Vairums mieturaļģu ir vienmājas, bet dažas – divmāju augi. Vienmājas *Chara* ģints aļģēm anterīdiji atrodas zem oogonija, bet ģintī *Nitella* un *Tolypella* anterīdiji novietoti virs oogonija. Nogatavojušies anterīdiji parasti ir koši oranžā vai sarkanā krāsā, bet oogoniji – melni vai tumši brūni. Oogonija iekšpusē atrodas oospora (5. attēls).

Oogonija augšpusē atrodas vainadziņš, ko veido piecas vai desmit nelielas šūnas. Oogoniju no ārpuses spirāliski apņem oogonija segšūnas.

Mieturļģes substrātā nostiprinās ar rizoīdiem. Dažām mieturaļģēm uz rizoīdiem, kā arī uz substrātā iegrimuāajiem sānzariem, atrodas balti vai bezkrāsaini, vienšūnas vai daudzšūnu gumiņi. Gumiņu esamība un forma ir sugas pazīme. Skarbajai mieturītei (*C. aspera*) raksturīgi nelieli, apaļi, balti gumiņi, bet strupajai nitellītei (*Nitellopsis obtusa*) balti, staraini, zvaigžņveida gumiņi, kādi nav nevienai citai Latvijā sastopamai sugai.

Daudzas mieturaļģu morfoloģiskās pazīmes ir ļoti svarīgas sugu noteikšanā. Tādēļ, lai nerastos pārpratumi un kļūdas, aprakstot mieturaļģes latviešu valodā, ieteicams lietot vienotu terminoloģiju.

Pateicības

Pētījums veiksts ar Eiropas Sociālā fonda atbalstu. Izsakām lielu pateicību zīmējumu autorei Rūtai Kazākai. Pateicamies kolēģiem, kuri palīdzēja mieturaļģu nosaukumu radīšanā un raksta tapšanā – Ģertrūdei Gavrilovai, Viesturam Šulcam, Pēterim Evartam-Bunderam un visiem Terminoloģijas apakškomisijas locekļiem.

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Latvian names and associated morphological terms of the *Charophytes*

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Abstract

This paper provides the Latvian names of the *Charophytes* and the associated morphological terms in Latvian language, newly made and now accepted by the Subcommission of the Terminology. Explanation of morphological terms and shematical drawings as well as general morphological descriptions of the *Charophytes* are given. Special attention is drawn to explain morphological terms which have been not or have been seldomly used in the literature in Latvian, but are very important for use in species determination and description. The interpretation of many neccesary morphological terms is given for the first time in Latvian. The paper summarizes the Latvian names of 25 species and four genera of *Charophytes* as well as explanation of 27 morphological terms. Translations of morphological terms are given in English, German, Latin and Russian languages.

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