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FACTORS AFFECTING SEED GERMINATION AND SEEDLING GROWTH OF TERRESTRIAL ORCHIDS CULTURED IN VITRO

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The effect of two sterilization substances (calcium hypochlorite and sodium hypochlorite) on the germination rate, and the effect of nitrogen and growth regulators on seedling growth and development, was studied in three critically endangered species of terrestrial orchids (*Dactylorhiza incarnata* subsp. *serotina*, *Dactylorhiza maculata* subsp. *maculata*, *Liparis loeselii*). Surface sterilization of mature seeds using 7.2% calcium hypochlorite (until decolorization from brown to ivory) stimulated the germination rate. Addition of peptone at 1 g l^{-1} concentration or auxin 3-indoleacetic acid (IAA; 1.43 μM) with cytokinin zeatin (0.72 μM) and 1-naphthylacetic acid (NAA; 1.34 μM) to the culture medium significantly increased the growth parameters of seedlings after 12 months.

Key words: Terrestrial orchids, in vitro generative propagation, seed sterilization, nitrogen form, growth regulators.

INTRODUCTION

Terrestrial orchids (Orchidaceae) have become objects of concern to conservationists due to the plants' high sensitivity to changes in its environment (Rasmussen, 1995). Surface sterilization of mature seeds before germination eliminates barriers to germination; hypochlorites are most often used for this (Lindén, 1992). The barriers include an almost waterproof testa, and embryo integuments containing suberin or abscisic acid-type (ABA) endogenous inhibitors. Embryo suberinization is species-specific and depends on the degree of seed maturity (van Waes and Debergh, 1986a).

Nitrogen is supplied to medium in inorganic form as the anion NO_3^- or the cation NH_4^+, and in organic form as polypeptides or free amino acids. Some species cannot use inorganic nitrogen, which can be explained by mycorrhizal symbiosis, when the requirements for amino compounds are most probably satisfied by transport of these substances with a symbiotic fungus (Dijk and Eck, 1995). Malmgren (1996) found that the ammoniated form of nitrogen is more appropriate than the nitrate form, and reported the fastest growth of *Dactylorhiza* seedlings at 50–100 mg l^{-1} NH_4NO_3. Mitra (1989) verified that most amino acids act as inhibitors or do not increase the growth of seedlings. Only arginine and aspartic acid support growth like NH_4NO_3.

As for growth regulators, auxins and cytokinins are most frequently used for nutrient media, to increase the percentage of germination or to stimulate protocorm and seedling development. According to De Pauw et al. (1995), their importance in germination is limited but increases during the development of protocorms. As reported by Mitra (1989), cytokinins as such or in combination with auxin indoleacetic acid (IAA) stimulate germination. On the other hand, Hadley (1970) found that 1–10 ppm kinetin with or without auxin IAA can inhibit seed germination in *Dactylorhiza purpurella*. Gibberellins are used as media additives only marginally. According to Hadley (1970), gibberellic acid induced abnormal elongation of protocorms and etiolation of leaves initiated after formation of shoots in *Dactylorhiza purpurella*. In *Dactylorhiza maculata* and *Listera ovata*, the use of gibberellic acid caused a decrease in the germination percentage (van Waes and Debergh, 1986b).

This work examined factors that influence in vitro germination and seedling growth of terrestrial orchids. Mature seeds of the critically endangered species *Dactylorhiza incarnata* subsp. *serotina* (Hausskn.) Soó et D.M. Moore (tax.), *Dactylorhiza*

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maculata (L.) Soó subsp. *maculata* and *Liparis loeselii* (L.) L. C. Richard (IUCN, 1994) were used to study the effect of the sterilization substances calcium hypochlorite and sodium hypochlorite on the germination rate, and the effect of nitrogen and growth regulators on growth parameters of seedlings.

MATERIALS AND METHODS

PROTOCORM CULTURE AND LONG-TERM STORAGE OF SEEDS

After collection at localities in northern Bohemia (Czech Republic), treated seeds were incubated in a growth chamber in the dark at 22±1°C, and protocorms with green leaf primordia were cultured in a room with a 22/18°C thermoperiod, light intensity 55 μmol m^{-2} s^{-1} and 16 h photoperiod. The remaining seeds were dried in Petri dishes with anhydrous $CaCl_2$ for 2 weeks at 5°C. Dried seeds were put into Eppendorf microtubes and stored in hermetically closed containers with silica gel at –20°C.

SURFACE STERILIZATION OF SEEDS

The effect on seed germination of two sterilization substances, 7.2% $Ca(OCl)_2$ and 0.5% NaOCl, alone or in combination with 70% ethanol treatment, was determined. The combination treatment included pretreatment with 70% ethanol in the presence of the given sterilization substance for 3 min. In all treatments the time of exposure to calcium hypochlorite filtrate or sodium hypochlorite solution was 40–50 min, until decolorization of seeds from brown to ivory. Each variant was performed in 44 replicates. One replicate consisted of one test tube containing 100–150 seeds. After 15 weeks, the number of protocorms was counted under a binocular microscope, and their percentages calculated.

NITROGEN CONTENT IN CULTURE MEDIUM

Fifteen weeks after germination, the protocorms were transferred to culture media containing ammonium ions as the source of nitrogen (control), and comparative treatments with the addition of organic peptone or amino acids. The results were evaluated after 12 months of culture. We determined the effect of the inorganic salt $(NH_4)_2$ SO_4 at 0.132 g l^{-1} concentration, and the effect of addition of organic peptone nitrogen (1 g l^{-1}) or amino acids (RPMI 1640, Sigma) on the growth parameters of seedlings. Composition of amino acid mixture (g l^{-1}): arginine, 10; asparagine, 2.5; aspartic acid, 1.0; cystine, 3.26; glutamic acid., 1.0; glycine, 0.5; histidine, 0.75; pro-

Fig. 1. Protocorm formation in *Dactylorhiza maculata* subsp. *maculata*. ×3.

Fig. 2. *Dactylorhiza incarnata* subsp. *serotina* seedlings. ×3.

Fig. 3. *Liparis loeselii* seedlings. ×3.

TABLE 1. Composition of germination and culture nutrient media according to Vejsadová et al. (2002)

	Germination medium (g l^{-1})	Culture medium (g l^{-1})
KH_2PO_4	0.216	0.216
$MgSO_4 . 7 H_2O$	0.246	0.246
KCl	0.150	0.150
$CaCl_2 . 2 H_2O$	0.029	0.029
$(NH_4)_2SO_4$	0.132	0.132
Citric acid	0.192	0.192
Biotin	0.001	0.001
Pyridoxine	0.001	0.001
Inositol	0.001	0.001
Folic acid	0.001	0.001
Niacinamide	0.001	0.001
Ca pantothenate	0.001	0.001
$Na_2EDTA . 2 H_20$	–	0.019
$Fe SO_4 . 7 H_20$	–	0.014
Charcoal	1	1
Agar ROTH	12	–
Agar Sigma	–	7.5
Casein hydrolysate	0.5	0.5
Yeast extract	0.5	0.5
Sucrose	20.0	25.0
Growth regulators	–	+
pH	5.3	

line, 1.0; leucine, 2.5; lysine, 2.0; methionine, 0.75; phenylalanine, 0.75; serine, 1.5; threonine, 1.0; tryptophane, 0.25; tyrosine, 1.4; valine, 1.0. In all the media, yeast extract and casein hydrolysate were standard components (Tab. 1).

GROWTH REGULATORS IN CULTURE MEDIUM

The protocorms were transferred to culture media containing 0.132 g l^{-1} $(NH_4)_2SO_4$ and growth regulators: 1.34 μM NAA (1-naphthylacetic acid), 1.43 μM IAA (1-indoleacetic acid), 1.11 μM BA (benzyladenine) and 0.72 μM zeatin (hydroxymetyltransbutenylaminopurine). Seedling subculture on identical medium was performed every 3 months.

DATA ANALYSIS

Two-way ANOVA was used to determine the individual and interactive effects between factors and orchid species. The comparative Duncan's multiple range test ($p = 0.05$) was used to determine differences between treatments.

RESULTS AND DISCUSSION

To enable successful generative propagation of terrestrial orchids in vitro, inhibiting substances from the mature seeds must be eliminated. This can be done by appropriate surface sterilization, inducing embryo germination and protocorm formation. Based on previous results (Vejsadová et al., 2002), two sterilization substances, 7.2% $Ca(OCl)_2$, and 0.5% NaOCl, were applied alone or in combination with 70% ethanol. The formation of protocorms (Fig. 1), expressed as percentages, was taken as the rate of seed germination. The results showed that calcium hypochlorite significantly stimulated the formation of protocorms in all species. Sodium hypochlorite significantly inhibited the germination rate: embryos were damaged and germination was significantly lower, as much as 50% lower in *D. incarnata* subsp. *serotina*. Pretreatment with 70% ethanol in the presence of $Ca(OCl)_2$ promoted the formation of protocorms; this effect reached statistical significance in *Liparis loeselii* (Tab. 2). The generally stimulating effect of surface sterilization with hypochlorite solutions can be explained in several ways. There may be a physiological effect of washing away the endogenous inhibitor abscisic acid (ABA) from the seeds, interrupting their dormant status and thus initiating germination. Another effect is dissolution of suberin substances from the embryo surface, which can then take in water more easily (Harvais and Hadley, 1967) The latter authors demonstrated that only seeds bleached white can swell and germinate, while brownish or brown seeds remained without any symptoms of swelling in culture. In contrast, however, the seeds did not germinate in our experiments if they had been sterilized to the point of whitening; this was due either to the higher concentration of the sterilization substance or to longer exposure time. The present results do not support the efficacy of long-term treatment of seeds using calcium hypochlorite (van Waes and Debergh, 1986b), but are in line with Rasmussen's (1995) finding that orchid seed germination frequencies are usually higher after sterilization with $Ca(OCl)_2$ than after NaOCl. The reason for this difference is not known. Miyoshi and Mii (1998) obtained the maximum germination rate irrespective of hypochlorite type or differences in available chlorine concentrations and hypochlorite exposure.

We also examined how the nitrogen form, growth regulators and orchid species influenced seedling growth. In 12 month-old seedlings, shoot growth expressed as average length was significantly increased (by up to 43%) by the addition of 1 g l^{-1} peptone in *D. maculata* subsp. *maculata*; no such significant effect was found in *D. incarnata* subsp. *serotina* (Tab. 3, Fig. 2). In *Liparis loeselii* (Fig. 3),

TABLE 2. Effect of sterilization substances on germination after 15 weeks

Species	Sterilization substance	Germination rate (%)
Dactylorhiza incarnata subsp. *serotina*	$Ca(OCl)_2$	43.18b
	NaOCl	21.33a
	70% ethanol + $Ca(OCl)_2$	46.25b
	70% ethanol + NaOCl	20.57a
Dactylorhiza maculata subsp. *maculata*	$Ca(OCl)_2$	71.28d
	NaOCl	42.51b
	70% ethanol + $Ca(OCl)_2$	78.89d
	70% ethanol + NaOCl	53.21bc
Liparis loeselii	$Ca(OCl)_2$	57.72c
	NaOCl	46.13b
	70% ethanol + $Ca(OCl)_2$	69.44d
	70% ethanol + NaOCl	45.10b

Mean germination rate values (of 44 repetitions) marked with the same letter do not significantly differ at p = 0.05 (Duncan's test).

TABLE 3. Effect of nitrogen form on seedling growth after 12 months

Species	Nitrogen form	Shoot length (mm) ± SD	Root length (mm) ± SD	Leaf number
Dactylorhiza incarnata subsp. *serotina*	$(NH_4)_2 SO_4$	75.50 ± 0.78c	33.40 ± 0.52b	4.00b
	$(NH_4)_2 SO_4$ + peptone	79.50 ± 0.85cd	34.20 ± 0.56ab	3.87b
	$(NH_4)_2 SO_4$ + RPMI 1640	71.00 ± 0.91c	33.14 ± 0.60b	3.80b
Dactylorhiza maculata subsp. *maculata*	$(NH_4)_2 SO_4$	82.20 ± 0.85d	73.80 ± 0.54c	4.20b
	$(NH_4)_2 SO_4$ + peptone	117.50 ± 0.71e	78.50 ± 0.61c	6.00c
	$(NH_4)_2 SO_4$ + RPMI 1640	79.40 ± 0.78cd	70.20 ± 0.55c	3.80b
Liparis loeselii	$(NH_4)_2 SO_4$	29.60 ± 0.46a	14.50 ± 0.50a	2.00a
	$(NH_4)_2 SO_4$ + peptone	41.80 ± 0.38b	19.10 ± 0.53a	2.66a
	$(NH_4)_2 SO_4$ + RPMI 1640	39.10 ± 0.52b	17.30 ± 0.58a	2.50a

Mean germination rate values (of 44 repetitions) marked with the same letter do not significantly differ at p = 0.05 (Duncan's test).

addition of amino acids significantly stimulated shoot growth. The nitrogen form in the studied concentrations had no effect on the growth of roots, while leaf formation significantly increased in *D. maculata* and *D. incarnata*. We did not find the inhibition effect reported by Mitra (1989), who found that most of 14 applied amino acids acted as inhibitors or had no growth effects. According to van Waes and Debergh (1986b), addition of serine, glutamic acid, peptone, casein hydrolysate and yeast extract only stimulates seed germination.

After 12 months of auxin exposure, shoot growth was significantly higher (by 12–14%) in the presence of IAA (1.43 μM) in seedlings of *D. maculata* subsp. *maculata*. In *D. maculata* and *D. incarnata*, root growth was significantly stimulated by 1.34 μM NAA. The cytokinin zeatin (0.72 μM) stimulated shoot growth by up to 33%, but not root growth or the number of leaves. Shoot growth was highest in the presence of auxin IAA and cytokinin zeatin in all species studied (Figs. 4–6). Roots were stimulated at concentrations of 1.34 μM NAA and 1.11 μM cytokinin BA, with their growth increased by ~30%. *D. maculata* seedlings had the highest shoot and root growth rates.

Generally, auxins stimulate root formation and cytokinins enhance shoot development and cell division. Rasmussen (1995) reported a positive effect of adding cytokinins (e.g., benzyladenine or kinetin) on protocorm development; Hadley (1970) observed a negative effect of kinetin. In our experiments, orchid seedlings had significantly higher growth in the presence of auxin and cytokinin combinations. The growth differences in response to auxins and

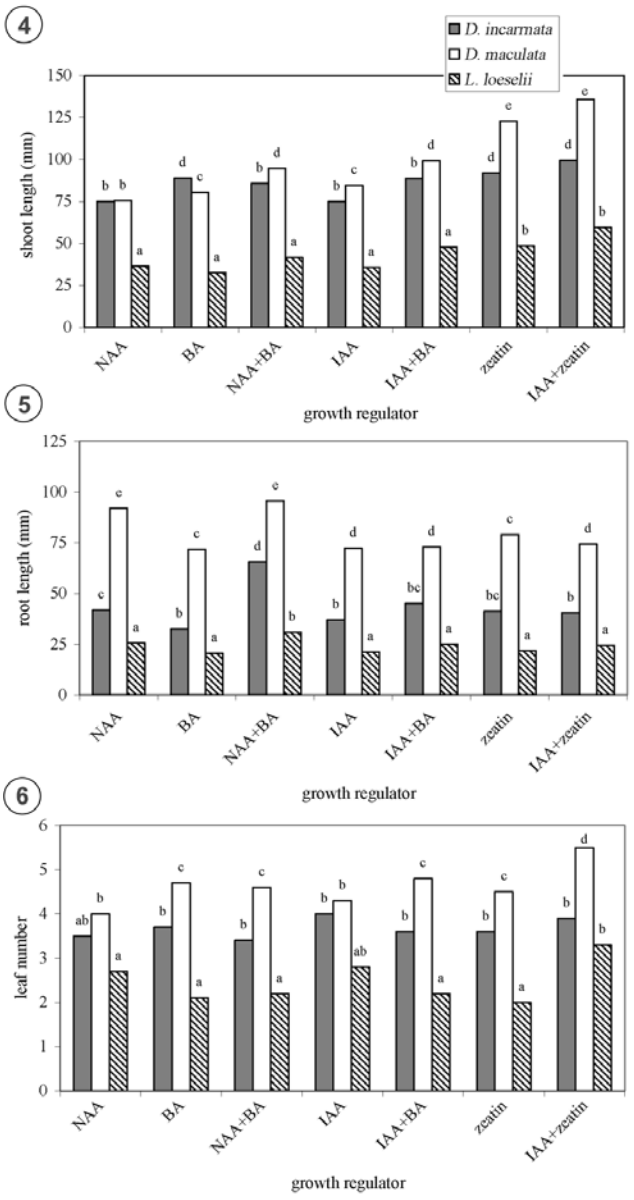

Fig. 4. Effect of auxins and cytokinins on shoot growth in 12-month-old seedlings. **Fig. 5**. Effect of auxins and cytokinins on root growth in 12-month-old seedlings. **Fig. 6**. Effect of auxins and cytokinins on shoot multiplication in 12-month-old seedlings.

cytokinins do not suggest strong species-specificity in the species studied.

Our study showed that treatment of mature seeds with 7.2% calcium hypochlorite, peptone or auxin (IAA, NAA), and the addition of cytokinin (zeatin, BA) to the culture medium substantially improved germination and seedling growth in *Dactylorhiza incarnata* subsp. *serotina*, *Dactylorhiza maculata* subsp. *maculata* and *Liparis loeselii*.

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