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Successful disinfection protocol for orchid seeds and influence of gelling agent on germination and growth

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ABSTRACT

Artificial propagation of endangered orchid species is one of the most important actions of conservationists often jeopardized by low numbers of acquired seed, its contamination and viability. Disinfection and chemical composition of media are two of the most important factors contributing to better germination in temperate orchid species. The article deals with three world genera (*Epidendrum nocturnum, Prosthechea garciana, Maxillaria rufescens)* and one commercial hybrid (*Zygopetalum*) and describes an effective method of orchid seed disinfection carried out in a centrifuge. Germination percentages of all three genera and one hybrid were between 60 and 90 % from which we concluded that the risk of physical damage to the seeds by centrifugation is not significant. The time needed for disinfected seeds (*E. nocturnum, P. garciana, M. rufescens)* to swell-form protocorms was 10 days shorter compared to undisinfected seeds (*Zygopetalum* hybrid - green capsule method) and some other studies. Adequate wetting and stratification of the seed is very important for successful germination, which resembles processes in natural environment. Additionally, this method solves the problems of collecting and transferring the seeds after disinfection. It is also important that the time needed for disinfection is shorter, which is desirable for some sensitive species. Our study also focuses on importance of gelling agent, namely Gellan gum and agar, since we noticed an obvious superiority of the former in all phases of *in vitro* development.

Key words: orchids, seed, disinfection, wetting, gelling agent, agar, Gellan gum, germination morphological stage

IZVLEČEK

USPEŠNA METODA RAZKUŽEVANJA SEMEN ORHIDEJ IN VPLIV STRJEVALCA NA KALITEV IN RAST

Razmnoževanje ogroženih vrst orhidej je ena od najpomembnejših dejavnosti konservatorjev teh rastlin, ki jih pogosto ogrožajo majhne količine dostopnega semena, okuženost s patogeni in viabilnost. Razkuževanje in kemična sestava gojišča sta najpomembnejša dejavnika, ki vplivata na boljšo kalivost semena orhidej iz območij z zmernim podnebjem. V raziskavo so bili vključeni trije rodovi orhidej iz Srednje in Južne Amerike (*Epidendrum nocturnum, Prosthechea garciana, Maxillaria rufescens)* ter komercialni križanec (*Zygopetalum*). V delu je prikazana uspešna metoda razkuževanja semena orhidej z uporabo centrifuge. Kalivost semen vseh štirih rodovov orhidej je bila med 60 in 90 %, kar potrjuje, da so posledice poškodb zaradi vrtilnega momenta pri centrifugiranju zanemarljive. Čas, ki so ga razkužena semena (*E. nocturnum, P. garciana, M. rufescens*) potrebovala za razvoj protokormov, je bil za 10 dni krajši v primerjavi z nerazkuženim semenom (*Zygopetalum* - zelena semenska glavica) in s primerljivimi študijami. Za uspešno kalitev je pomembna zadostna omočitev semenske ovojnice in stratifikacija semena, s čimer se približamo procesom v naravnem okolju. Olajšano je tudi rokovanje s semenom, ki se zaradi centrifugalne sile sesede na dno mikrocentrifugirke. Zaradi učinkovitosti metode je seme manj časa izpostavljeno razkuževalnemu sredstvu, na katerega so semena nekaterih orhidej občutljiva. Proučevali smo tudi vpliv dveh strjevalcev gojišč in ugotovili, da je v vseh fazah *in vitro* kalitve in razvoja rastlin Gellan gum v primerjavi z agarjem učinkovitejši.

Ključne besede: orhideje, semena, razkuževanje, omočitev, strjevalci gojišč, agar, Gellan gum, kalivost, rast

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1 INTRODUCTION

The gateway to massive hybridization and conservation goals was opened when Knudson (1922) described and demonstrated his breakthrough method of non-symbiotic germination of orchid seed. Many technical and technological problems have been solved since, mainly understanding and satisfying the needs of individual genera and species of orchids. The existence of increasingly high number of orchid species around the globe is threatened (Koopowitz, 2001; Johnson et al., 2007). Artificial propagation of endangered species is therefore one of the most important actions of conservationists often jeopardized by low numbers of acquired seeds, their contamination and viability.

Sterilization and chemical composition of media are two of the most important factors contributing to better germination in temperate orchid species (Rasmussen, 1995), with the embryo itself being afforded little protection therefore vulnerable to physical damage (Hicks, 2000). Common technique for disinfection of orchid seeds that has been used by breeding companies, research institutions and gene banks is agitation of disinfection solution containing seeds. Additionally, some authors (Arditti, 1982; Snow, 1985; Hicks, 2000) describe a method called presoak, before adding a disinfectant, in a sugar or honey solution at various time intervals, which improves disinfection efficiency of heavily contaminated seeds and also enhances germination. Sugar or honey solution causes bacteria and fungal spores that are hidden in porous seed surface to germinate, consequently making them vulnerable to disinfection solution. It is important to understand other mechanisms behind this treatment and its influence in relation to the structure of the seed and the process of germination that takes place in natural environment. Orchid seeds are difficult to wet because the outer walls of their testa cells (outer integument) are hard, lignified and covered with a lipid cuticle. As the seeds ripen, testa cells lose moisture and their walls curve inwards. Both the cells of testa and as interior of seeds are filled with air. When seeds that fall into water or drop onto a moist substrate are chilled by cool water or reduced atmospheric temperatures the air inside testa contracts. This creates suction that draws water into seed through micropylar opening (Arditti and Ghani, 2000). Up to this point, presence of water is a crucial factor but after initial swelling of protocorms the fungus (symbiotic method) or constituents of media (asymbiotic method) play the most important role.

This paper describes a reliable protocol for sterilization of orchid seeds regardless of the level of contamination by various pathogens and signifies the importance of adequate wetting of the seed, which might often be the reason for low germination rates. Our studies also focus on importance of gelling agent, Gellan gum and agar, since we noticed an obvious superiority of Gellan gum in all phases of *in vitro* development in three genera and one commercial hybrid.

2 MATERIALS AND METHODS

The seeds of botanical genera *Epidendrum nocturnum* Jacq., *Prosthechea garciana* Garay & Dunst. (syn. *Encyclia garcianum* Carnevali & I. Ramírez) and *Maxillaria rufescens* Lindl. were kindly provided by Dr. Michel Vacherot, France in a form of dry seeds extracted from capsules. Additionally, a capsule of a commercial hybrid of *Zygopetalum* was used as control.

2.1 Disinfection and inoculation of seeds on the medium

Disinfection of seeds was carried out in 1.5 ml microcentrifuge tubes. Approximately 20 mg of isolated seeds were disinfected in 16.6 g/l Na_2 salt of dichloroisocyanuric acid (Sigma) dissolved in sterile distilled water with a drop of wetting agent Tween 20 (Sigma).

Seeds were soaked in disinfection agent for 8 min at room temperature, and then centrifuged for 2 min at 4000 rpm (1900x g) in a Beckman J2-HS centrifuge at 4 °C. A supernatant was removed and washed three times in sterile water using the following procedure: supernatant was removed and seeds were mixed in sterile water for few seconds and sedimented by centrifugation at the same conditions as described. Finally 0.8 ml of sterile water was added to each microcentrifuge tube and after resuspending seeds were inoculated using 1000 µl pipete in Petri dishes (90 x 15 mm) on media. Glass spreader was used to spread the seeds on the medium surface. Seed capsule from *Zygopetalum* was picked few days before dehiscence of the capsule. The whole capsule was disinfected for 15 min. in 16.6 g/l Na_2 salt of dichloroisocyanuric acid including Tween 20 and washed for three times. Seeds were extracted and immediately inoculated.

2.2 Subcultivation and culture conditions

Further subcultivations were carried out in bigger petri dishes (90 x 20 mm) and baby jars (55 x 72 mm). All vessels were sealed with Parafilm and cultured in a growth chamber under 16/8 photoperiod at 25^{+}/- 1 °C and illumination of 40 µmol m^{-2} s^{-1}.

2.3 Medium

The culture medium used for germination and subculturing was composed of Gamborg B5 macro-salts (Gamborg et al., 1976) and full strength MS micro-salts (Murashige and Skoog, 1962). Iron ions were added as 25 mg/l Na_2Fe-EDTA and 25 g/l sucrose were added as carbohydrate source. Media were solidified using either 8 g/l agar (Difco Bacto), mark A or 2.6 g/l Gellan gum (Sigma), mark G. The pH was adjusted to 5.4 prior to autoclaving.

3 RESULTS

3.1 Disinfection

The method used in our study, including preliminary tests, has proven to be 100 % efficient in disinfecting seeds of four epiphytic orchids (Table 1). Washing of seeds in the centrifuge was also efficient, since we observed no abnormalities caused by possible remnants of dichloroisocyanuric acid at germination. Centrifugation forced the seeds to gather at the bottom of microcentrifuge tubes, which enabled transfer to Petri dishes without difficulties, using a pipette.

Table 1: Efficiency of disinfection of three orchid genera and one hybrid

Genotype	Material for disinfection	Disinfection efficiency (%)	Germination (%)
Epidendrum nocturnum	isolated seeds	100	70
Prosthechea garciana	isolated seeds	100	70
Maxillaria rufescens	isolated seeds	100	60
Zygopetalum hybrid	intact capsule	100	90

3.2 Germination

We estimated the germination percentage visually with the use of stereomicroscope. Presented percentages should be considered approximate and are as follows: 70 % for *E. nocturnum* and *P. garciana*, 60 % for *M. rufescens* and above 90 % for *Zygopetalum* hybrid (Figs. 1, 2 and 3). Individual genus needed the same time for germination on medium A as well as on medium G. After 7 days we observed first swollen embryos - protocorms in *E. nocturnum* and *P. garciana*. The actual day of germination is considered to be after 9 days since by that day more than half of the embryos formed protocorms (Fig. 1). *M. rufescens* formed protocorms after 11 days *in vitro*. The longest period needed for germination (21 days)

was observed in *Zygopetalum* hybrid, which was the only seed material that was not disinfected in the centrifuge (Table 2).

Protocorms of *E. nocturnum* and *P. garciana* left the testa after 21 days and covered themselves with rhizoids. *Zygopetalum* protocorms reached this phase after 30 days.

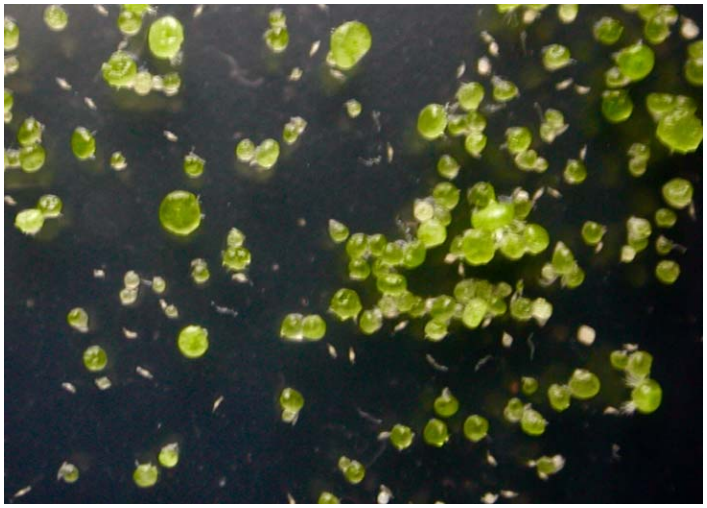

Figure 1: Developing protocorms of *Epidendrum nocturnum* 11 days after inoculation on medium G

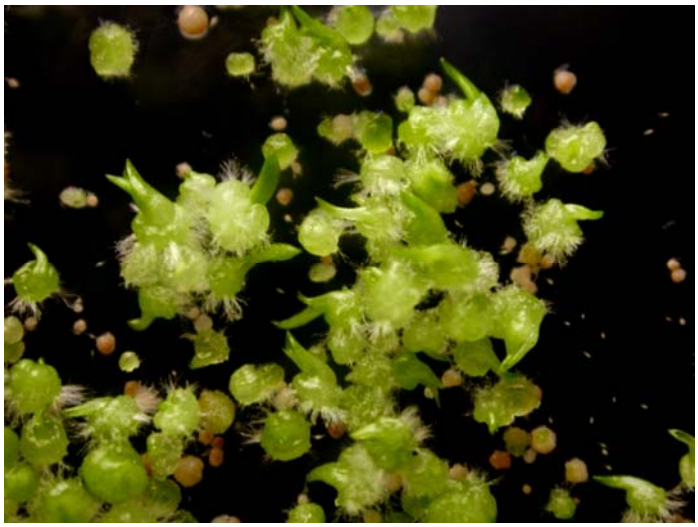

Figure 2: Uneven germination of *Zygopetalum* hybrid as a consequence of green capsule technique used

After initial development of protocorms *M. rufescens* entered stagnation and by the end of the experiment, after 223 days, only few formed structures as small as 1-2 mm that would eventually become first and second leaf. *E. nocturnum* reached the first leaf phase after 35 days, *Zygopetalum* and *P. garciana* reached it after 45 days (Fig. 2). After this period of time obvious differences between plants grown on medium A and G were observed (Table 2 and Figs. 3 and 4).

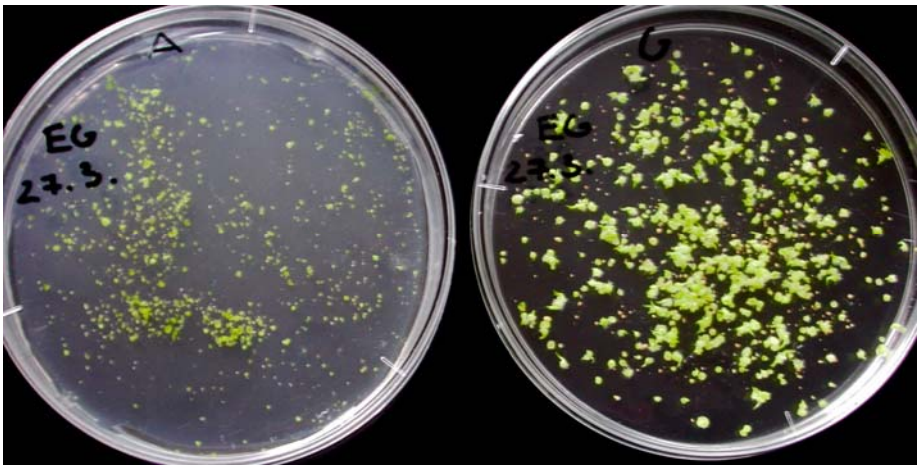

Figure 3: Differences in protocorm development between medium A (left) and medium G (right) 45 days after inoculation *Prosthechea garciana*

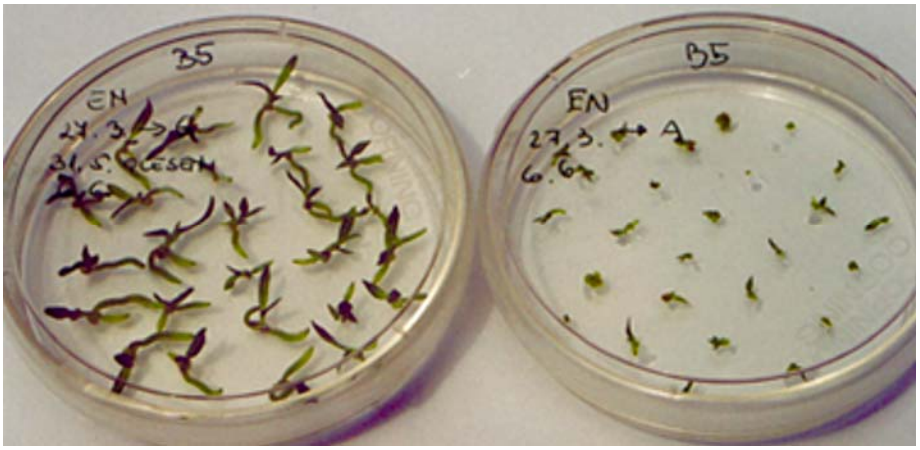

Figure 4: The difference in the growth and development of plants of *Epidendrum nocturnum*, which were at the stage of germination, cultivated on medium containing Gellan gum (left) and agar (right), after the first subcultivation

3.3 Subcultivation

By the time of first subcultivation for *E. nocturnum* and *P. garciana*, 65 days after inoculation, both genera that was cultured on medium G developed 3-4 mm big protocorms with first leaf that was 4 mm long. Protocorms on medium A were smaller (2-3 mm) and were covered with much less rhizoids than those cultured on medium G. Very few of them developed first leaf, which was only 1-2 mm long. We only subcultivated plants germinated on medium G (Table 2, Fig. 4).

Subcultivation for *Zygopetalum* was performed 80 days after inoculation, mainly because of uneven germination on both media (Fig. 2). Half of the seedlings (protocorms with one or more roots) were 4-8 mm big with two leaves 5-8 in length and a short root, the rest were smaller protocorms some still in early germination phases. Protocorms cultured on medium A were on average smaller, less developed and was consequently not subcultivated.

Protocorms of *E. nocturnum* and *P. garciana* became seedlings 11 days after first subcultivation. After second subcultivation in bigger Petri dishes (90 x 20 mm) on media were solidified with 2.6 g/l

Gellan gum, and 70 days *in vitro*, seedlings of *E. nocturnum* and *P. garciana* developed 1-2 leaves (0.5-1.5 cm in length) and 1-2 roots (1-2 cm in length) (Table 2 and Fig. 4). After another subcultivation in baby jars and 172 days *in vitro*, seedlings of *E. nocturnum* and *P. garciana* developed 4-6 leaves (2-4 cm in length) and 2-4 roots (3 cm in length) and were transferred to acclimatization (Table 2).

After another subcultivation in baby jars and 150 days *in vitro*, 50 % of *Zygopetalum* seedlings developed 3-4 leaves (4-5 cm in length), two roots (3 cm in length) and were transferred to acclimatization. Other half of the plants followed gradually, using approximately the same amount of time for growth, if we calculated the time from germination and not their overall *in vitro* presence. All results are presented in table 2.

Table 2: Number of days taken for *in vitro* germination and subsequent growth and development in three orchid genera and one hybrid

Genus/ morphological stages	Medium (number of days after inoculation)	
	A	B
Epidendrum nocturnum		
Germination	9	9
1 leaf initiation	45	35
2 leaf initiation	128	106
3 leaf initiation	/	135
4 leaf initiation	/	160
1 root initiation	106	76
2 root initiation	/	114
Prosthechea garciana		
Germination	9	9
1 leaf initiation	76	45
2 leaf initiation	128	92
3 leaf initiation	/	120
4 leaf initiation	/	158
1 root initiation	115	76
2 root initiation	/	158
***Zygopetalum* hybrid**		
Germination	21	21
1 leaf initiation	65	45
2 leaf initiation	102	80
3 leaf initiation	/	112
4 leaf initiation	/	150
1 root initiation	/	80
2 root initiation	/	112
Maxillaria rufescens		
Germination	11	11
1 leaf initiation	180	180
2 leaf initiation	/	223
3 leaf initiation	/	-
4 leaf initiation	/	-
1 root initiation	/	-
2 root initiation	/	-

Legend: / = Plants were not subcultured; - = Plants did not reach that phase

Table 3: Number and size of the leaves and roots of seedlings after subcultivation

Genotype/ days after inoculation	No. of leaves		Length of leaves		No. of roots		Length of roots	
	A	G	A	G	A	G	A	G
Epidendrum nocturnum								
65	1	1	1-2 mm	4 mm				
172		4-6		2-4 cm		2-4		3 cm
Prosthechea garciana								
65	1	1		4 mm				
172		4-6		2-4 cm		2-4		3 cm
***Zygopetalum* hybrid**								
80	1	2	2-3 mm	5-8 mm		1 or more		3 mm
150		3-4		4-5 cm		2		3 cm

Legend: A – medium with agar; G – medium with Gellan gum

4 DISCUSSION

Various approaches have been reported for orchid seed disinfection. Among them are agitation of disinfection solution with seeds, filtration (vacuum induced passing of the solution through the seeds), gas disinfection and others. They employ different chlorine based gases and solutions (various hypochlorites, Na_2 salt of dichloroisocyanuric acid), Virkon S, hydrogen peroxide, etc. (Hicks, 2000). In addition to conventional disinfection, Hicks (2000) describes a method called presoak in water or any sugar-based solution. This method is effective in invoking fungus vulnerability but it also wets the seed, which is an important aspect of germination process (De Pauw and Remphrey, 1993; Rasmussen, 1995; Arditti, 2000). The latter author describes five important factors enhancing germination of *Cypripedium* sp., one of many hard-to-germinate terrestrial orchids. These are disinfection, cold stratification, soaking, appropriate light regime and chemical constitution of the medium. Method described in this paper successfully satisfies first three factors with the use of centrifuge. We emphasize the stratification at 4 °C, causing micropylar suction of water, which occurs in natural circumstances. Additionally, this method solves the problems of collecting and transferring the seeds after disinfection. It is also important that the time needed for disinfection is shorter, which is desirable for some sensitive species.

For the limited amount of species studied, the storage characteristics of orchid seeds are classified as 'orthodox' in the sense that seed longevity is enhanced by reducing moisture contents (from around 20 %, wet basis to 5 %) and decreasing storage temperatures (from 62 to 0 °C) (Pritchard and Seaton, 1993). When stored dry at 5-8 °C, the time taken for viability to fall to 50 % can be 8-14 years, assuming high initial seed quality (Koopowitz, 2001). At cryogenic temperatures (-196 °C), all metabolic processes and physicochemical changes are arrested which provides a possibility for storing these materials alive in a state of anabiosis for decades (Nikishina, 2001). Thorough wetting and stratification offered by this method might raise the percentage of viable seeds after desiccation and prolonged period of time in seed storage banks. More comprehensive study on the subject is needed to confirm this hypothesis.

Germination percentages of all four genotypes were between 60 and 90 % from which we

concluded that the risk of physical damage to the seeds by rotation moment is not significant. Elevated rotation speeds would probably reduce seed viability, although this was not established in our research.

The time needed for embryos to swell-form protocorms was much lower compared to some studies reporting 20 and up to 160 days needed to reach this phase (Arditti, 1992; Bhattacharjee et al., 1999a; Bhattacharjee et al., 1999b). *Zygopetalum* seeds were not subjected to sterilization procedure, which explains longer time needed for germination compared to *E. nocturnum*, *P. garciana* and *M. rufescens*. This confirms our hypothesis about importance of adequate wetting and stratification of the seeds prior to inoculation on the medium.

After germination phase, constitution of the medium plays the most important role, which can be compared to natural conditions where appropriate fungus invades germinating seeds and their rhizoids. Our experiment showed retarded growth in medium solidified with agar, whereas the same medium with Gellan gum enabled very good development in *E. nocturnum*, *P. garciana* and *Zygopetalum* hybrid. None of the used media, regardless of the gelling agent suited *M. rufescens*. Post experiments showed normal growth of that genus, using commercially available medium (Sigma P-1056) solidified with Gellan gum. Uneven germination of *Zygopetalum* was probably due to green capsule technique using immature seed capsule.

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