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Seed Germination and Further Development of Plantlets of *Paphiopedilum ciliolare* Pfitz. In Vitro

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ABSTRACT

Pierik, R.L.M., Sprenkels, P.A., Harst, van der B. and Meys, van der Q.G., 1988. Seed germination and further development of plantlets of *Paphiopedilum ciliolare* Pfitz. in vitro. *Scientia Hortic.*, 34: 139–153.

Seeds of the difficult-to-germinate *Paphiopedilum ciliolare* Pfitz. were sown asymbiotically on a modified Thomale medium. Germination was evaluated by determining the germination percentage and the germination index, which were found to have a strong correlation. Germination of seeds was studied on different culture media and under various environmental conditions. Germination was strongly promoted by 12 weeks of darkness in comparison to light, and by lowering the macro- and micro-salt concentration. Optimal germination occurred at a pH of 6.0 and a low sugar concentration. Germination was not affected by adding the vitamins nicotinic acid, biotin or pyridoxin. Auxin had a slight promotive effect, but neither cytokinin nor gibberellin affected germination. The presence of tryptone appeared to be essential, whereas banana homogenate was slightly inhibitory.

Optimal conditions for seed germination and seedling growth were different. Although the effect of active charcoal and banana homogenate were inhibiting during germination, they were promoting during further development of the seedlings.

Keywords: development; germination; growth; in vitro; *Paphiopedilum ciliolare*.

Abbreviations: BA, 6-benzylaminopurine; GA_3, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog (1962) medium; NAA, 1-naphthaleneacetic acid.

INTRODUCTION

Many orchid species have been successfully raised in vitro from seeds since Knudson's (1922) discovery that orchid seeds (actually pro-embryo-like structures) can germinate asymbiotically in vitro on artificial media. However,

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a few genera (e.g. *Paphiopedilum* and *Cypripedium*) are often considered difficult to germinate (Stimart and Ascher, 1981). Since there were no basic studies on the germination of *Paphiopedilum*, it was decided to study in detail the germination behaviour of one wild species of this genus, *Paphiopedilum ciliolare*. This species was chosen as the experimental plant because it appeared to be a particularly "hard-to-germinate" *Paphiopedilum*, and because sufficient seed capsules of this species became available.

During 1981–1982 numerous preliminary experiments were carried out (Pierik et al., 1982) to determine the germination requirements in preparation for our final experiments in 1983–1985. Based on the results of the preliminary experiments, the medium and other germination conditions were chosen as described in the next section.

The present paper summarizes the effects on germination of the following factors: time of sowing after opening the seed capsule; irradiance; nutritional factors (macro-salts, micro-salts, NaFeEDTA and sugars); pH; vitamins; regulators; and some miscellaneous compounds often added to nutrient media (tryptone, banana homogenate, and active charcoal).

MATERIALS AND METHODS

Capsules of *Paphiopedilum ciliolare* Pfitz. were obtained from a commercial grower (Sharon Orchids, Sprang Capelle) in The Netherlands. To obtain sufficient seed-set, artificial cross-pollination was carried out within a large population of plants grown in a greenhouse. Capsules were harvested as soon as they turned from green to brown (about 8 months after pollination). The percentages of possibly viable seeds (with visible embryos) in the capsules varied greatly for unknown reasons; both variation between plants and between the different years of harvest occurred. Seeds were sown 1 day after harvesting the closed capsules. Before opening the capsules with a sterile scalpel, they were surface-sterilized by dipping in ethyl alcohol (96%) and flaming twice. Seeds were sown on agar slopes to facilitate inoculation and spread over the surface of the culture medium with the aid of a moistened (in sterile agar) inoculation needle.

The basic culture medium, serving as a control (indicated in the tables with an asterisk), consisted of: $(NH_4)_2SO_4$, 60; KNO_3, 400; KH_2PO_4, 300; $Mg(NO_3)_2 \cdot 6\ H_2O$, 110; NH_4NO_3, 250 (all in mg l^{-1}; modified macro-salts according to Thomale GD1, 1957); micro-salts according to Murashige and Skoog (1962) at half strength except iron; NaFeEDTA, 25 mg l^{-1}; fructose, 7.5 g l^{-1}; glucose, 7.5 g l^{-1}; nicotinic acid, 0.5 mg l^{-1}; pyridoxin, 0.5 mg l^{-1}; biotin, 1.0 mg l^{-1}; Difco tryptone, 2.0 g l^{-1}; Difco Bacto-agar, 8.0 g l^{-1}. Since tryptone (a non-synthetic mixture of substances, mainly amino acids and vitamins) plays such a dominant role in the germination (Thomale, 1957) and development of *Paphiopedilum*, this complex was added despite the disadvan-

tages (unknown composition, not synthetic). The pH was adjusted to 6.0 with 1.0 M KOH before autoclaving. Pyrex test-tubes were used, each containing 22 ml of medium. All media, including added regulators, vitamins and complex compounds (banana homogenate, tryptone, etc.), were autoclaved at 120°C for 20 min; slants were made during cooling. To avoid dehydration during the long culture period and to reduce infections, the test-tubes were "closed" with cotton plugs, aluminium foil and Vitafilm (Goodyear); no aeration problems occurred due to the closures.

Seed cultures were incubated in a growth chamber in continuous darkness at 25°C. In one experiment seeds were germinated partially in light at 25°C, under a 16-h photoperiod, provided by fluorescent light (Philips TL 57/38 W), irradiance 3 W m^{-2}. After 12 weeks all cultures were transferred to a growth chamber at 25°C, under the same light conditions as described above. After germination in the dark, it was necessary to stimulate plant development in the light.

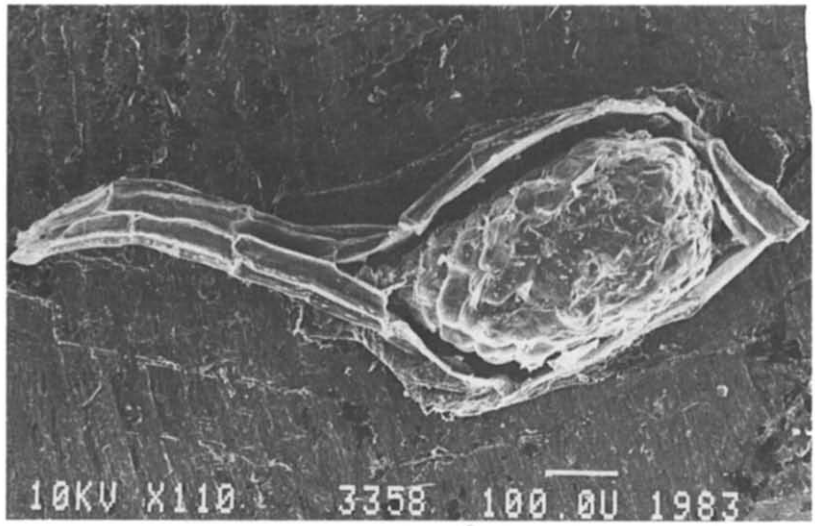
Germination was determined after 12 weeks incubation in darkness, since it took at least 4–6 weeks before germination started and not all the seeds germinated simultaneously. The parameters considered for germination were germination percentage and germination index. To evaluate the germination results more accurately, the index given below (modified after Arditti, 1967) was used to describe germination qualitatively after 12 weeks.

The following class values were given to the seeds: seeds containing an embryo but not germinating (swollen), 0 (a); swollen seeds, germinating, but not yet rupturing the seed coat, 1 (b); seeds with embryos just rupturing the seed coat, 2 (c); seeds with embryos completely out of the seed coat, 3 (d). The letters a, b, c and d indicate the frequency of each class of seeds. The germination percentage is calculated by dividing $100(b+c+d)$ by $a+b+c+d$. The germination index is calculated by dividing $(1b+2c+3d)10$ by $a+b+c+d$. When no germination occurs at all, the germination index is 0; when all seeds germinate and reach the class value of 3, the germination percentage is 100% and the germination index is 30.

All experiments had one variable factor (nutritional, hormonal or physical). Each treatment consisted of 12 test-tubes each containing at least 60 seeds. All experiments reported here were carried out in duplicate spread over different years (1983–1984–1985). Data presented in the tables are the mean of at least 2 experiments. The germination indices (in principle the most important parameter) were statistically analyzed (at $P<0.10$) using Tukey's H.S.D. test. Since a very strong correlation existed (cf. results, Fig. 6) between germination percentage and germination index, germination percentages were not analyzed statistically.

RESULTS

General. — Dry seeds of *Paphiopedilum ciliolare* are extremely small, and the embryos consist only of a clump of undifferentiated cells surrounded by a seed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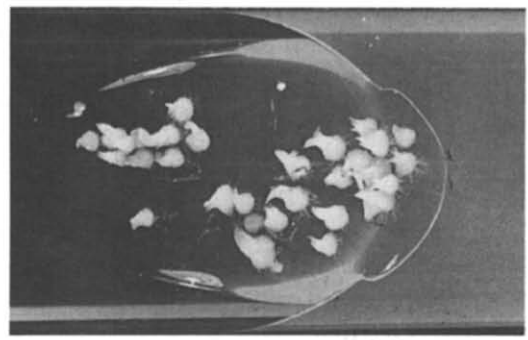

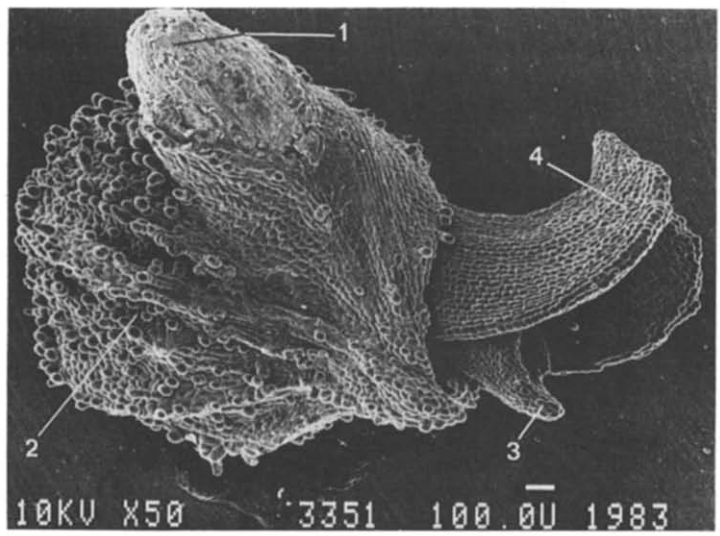
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coat. The process of germination starts approximately 4 weeks after isolation with a swelling of the embryos (Fig. 1). Six weeks after isolation the embryos become so swollen that the seed coat begins to rupture. A shoot tip then becomes visible at one side of the white protocorm (no chlorophyll formation in darkness), and rhizoids at the other (Fig. 2). When the protocorm reaches a size of approximately 2 mm, the shoot tip forms the first leaf and straight away the first root can be distinguished at the base of the first leaf (Fig. 3).

Generally the first roots become visible when the cultures are transferred from darkness to light, where the protocorms rapidly turn green. As the root starts to develop, the protocorm is gradually consumed and leaf development strongly enhanced. However, plant growth and development are rather slow, since 1.5 years after sowing the seeds the leaf size does not exceed 3 cm.

After 1.0–1.5 years the seedlings were transferred to our standard sowing medium devoid of tryptone, but enriched with 50 g l^{-1} banana homogenate and 2.0 g l^{-1} active charcoal; the latter two compounds strongly enhanced the growth and development of *P. ciliolare* plantlets. If maintained on the original medium for more than 1.5 years, many plants died.

Time of sowing. — To eliminate any difference in germination behavior due to the time of sowing, the following experiment was carried out. The capsules were opened after sterilization and seeds taken randomly were sown either immediately or after 1 h, after 2 h, etc. Table I shows that the difference in the time of sowing had no significant effect on germination.

Influence of light, darkness and temperature. — Since preliminary experiments (Pierik et al., 1982) showed that germination of *P. ciliolare* was favoured by darkness and partially inhibited by light, one group of seeds was incubated in darkness and another in continuous light of different intensities. Table I shows that the maximum germination occurred in darkness, whereas it was inhibited by light. The inhibition increased by increasing the irradiance from 0 to 3.0 W m^{-2} during the first 12 weeks, although only significant inhibition occurred at an irradiance of 3.0 W m^{-2}. The inhibiting effect of light was much more pronounced when all cultures were transferred to light (3.0 W m^{-2}) after 12 weeks; those which germinated in the dark developed quite well, whereas the proto-

Fig. 1. Germination starts approximately 4 weeks after isolation with a swelling of the embryos. Photograph taken with a scanning electron microscope. Bar represents 0.1 mm.

Fig. 2. Ten weeks after sowing, the protocorms reach a size of approximately 2 mm. A shoot tip with 2 leaf primordia is visible at the top of the protocorms, and rhizoids are present at the base.

Fig. 3. Protocorm (2) with 2 leaf primordia (3,4) and the development of the first root (1). Photograph taken with a scanning electron microscope. Bar represents 0.1 mm.

TABLE I

The influence of time of sowing (after opening the seed capsule) and irradiance on germination

Factor examined	Treatment	Germination (%)	Germination index
Time of sowing	Direct	84	14.4 a
	After 1 h*	83	14.4 a
	After 2 h*	82	14.2 a
	After 4 h*	85	14.8 a
	After 6 h	85	15.3 a
	After 8 h	86	14.8 a
Irradiance ($W\ m^{-2}$)	0 (darkness)*	61	9.9 a
	0.75	58	7.6 ab
	1.50	53	7.3 ab
	3.00	44	5.2 b

*Basic culture medium (see text).

corms formed in the light developed very poorly, and many died prematurely. Mortality was increased by increasing the irradiance during germination. The effect of temperature (21, 25 and 29°C) was only examined in preliminary experiments (not shown) which showed that highest germination occurred at 25°C.

Macro- and micro-salts. — The interpretation of experiments is often complicated by the use of very different macro-salt media. Therefore an experiment was designed to compare three very divergent media: (1) the classical Thomale GD1 (1957) medium for *Paphiopedilum*; (2) Knudson C (1922) medium which has been the basis for most of the solutions used to germinate orchid seeds; (3) the modern medium of Murashige and Skoog, MS (1962). Since the latter has an extremely high salt content, this medium was tested at 4 strengths (0.25, 0.50, 0.75 and 1.00). Results of this experiment are shown in Table II. It is quite clear that germination is not strongly affected by the macro-salts chosen; best results were obtained on Knudson C and MS 0.25, whereas germination on MS 1.00 was much lower. The most pronounced result showed in the MS-series, indicating that the best germination was reached at the lowest salt concentration. At a later stage of the development of the protocorms and plantlets, very pronounced effects of the macro-salts became apparent. Thomale's medium was the best, whereas Knudson C ultimately resulted in necrosis and death of the plants. On the MS-medium growth and further development decreased with increasing salt concentrations. Thomale's medium proved to be superior to MS 0.25.

The effect of the micro-salt concentration (Table II) was small, although the best results were obtained on a medium without, or with a low concentra-

TABLE II

The influence of minerals, pH and sugar concentration on germination

Factor examined	Treatment	Germination (%)	Germination index
Macro-salts	Thomale*	75	14.8 ab
	Knudson C	78	16.7 b
	MS 1/4	78	16.9 b
	MS 1/2	68	14.8 ab
	MS 3/4	58	11.4 ab
	MS 1/1	50	9.2 a
Micro-salts (except iron)	Without	85	25.1 a
	MS 1/4	81	23.0 ab
	MS 1/2*	77	22.2 ab
	MS 3/4	74	20.7 b
	MS 1/1	71	19.6 b
NaFeEDTA (mg l^{-1})	0	70	8.7 a
	6.25	65	9.4 a
	12.5	65	10.2 a
	18.75	59	9.8 a
	25.0*	63	10.1 a
pH	5.5	48	7.0 ab
	6.0*	54	7.8 b
	6.5	47	6.3 ab
	7.0	39	5.1 a
Glucose + fructose (%)	0.50 + 0.50	48	7.2 a
	0.75 + 0.75*	41	6.5 a
	1.00 + 1.00	37	5.5 b
	1.25 + 1.25	29	4.1 c

*Basic culture medium (see text).

tion of, micro-salts. Further growth and development of the seedlings (not shown in the tables) was not strongly affected by the addition of micro-salts. The best growth of seedlings occurred on a micro-salt concentration of 0.25 or 0.50.

Table II demonstrates that germination and early development were not influenced by the NaFeEDTA concentration. However, during further development (not shown in the table) in the light, iron was of vital importance. Plant development was strongly promoted by iron, and all protocorms and plantlets died during the light treatment on a iron-free medium (Fig. 4); a NaFeEDTA concentration of 25 mg l^{-1} was optimal, a concentration 6.25 mg l^{-1} being less satisfactory.

pH. — The pH of the medium (Table II) had hardly any effect on germination,

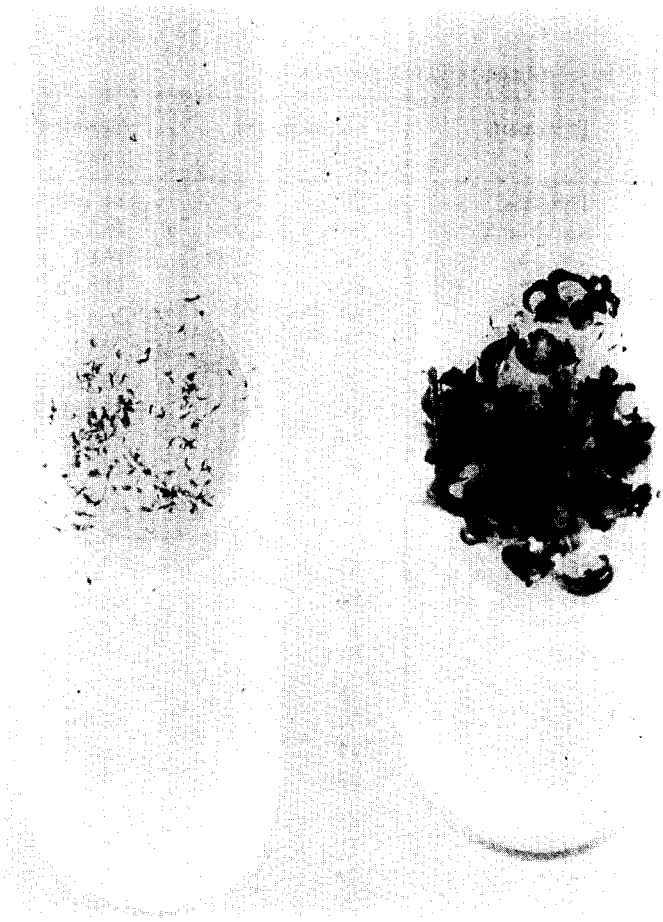

Fig. 4. Germination and development of seedlings on a medium (left) without NaFeEDTA and (right) with 25 mg l^{-1} NaFeEDTA. Although germination took place without iron, all protocorms died. Photograph taken 7 months after sowing.

the 2 lowest pH's reacting slightly better, although not significantly, than the 2 highest pH's. Further development in the light was clearly better at a low pH, and a pH of 7.0 strongly inhibited growth.

Sugar. — Preliminary experiments showed that germination did not occur without sugar in the medium. However, the highest germination occurred (Table II) on a medium with the lowest sugar concentration (0.50+0.50%; 0.75+0.75%) and decreased with further increases in the sugar concentration. In contrast to germination, the optimal sugar concentration for further development in the light appeared to be 0.75+0.75% or 1.0+1.0, whereas develop-

TABLE III

The influence of regulators on germination

Factor examined	Treatment	Germination (%)	Germination index
IAA ($mg\,l^{-1}$)	0*	53	6.5 a
	0.001	54	7.5 ab
	0.01	59	8.0 bc
	0.1	64	8.5 bc
	1.0	67	9.5 bc
BA ($mg\,l^{-1}$)	0*	97	17.6 a
	0.001	95	17.2 a
	0.01	95	17.7 a
	0.1	95	18.6 a
	0.5	95	18.9 a
	1.0	95	17.5 a
GA_{4+7} ($mg\,l^{-1}$)	0*	79	13.4 a
	0.001	80	12.1 a
	0.01	78	11.5 a
	0.1	75	11.2 a
	1.0	78	13.4 a

*Basic culture medium (see text).

TABLE IV

The influence of tryptone, banana homogenate and active charcoal on germination

Factor examined	Treatment	Germination (%)	Germination index
Tryptone (%)	0	40	5.9 a
	1.5	73	12.8 b
	2.0*	80	14.1 b
	2.5	79	13.3 b
Banana homogenate ($g\,l^{-1}$)	0*	48	8.9 a
	25	17	1.9 b
	50	11	1.3 b
Active charcoal (%)	0*	69	11.5 a
	0.1	73	9.9 a
	0.2	74	9.8 a

*Basic culture medium (see text).

ment was inhibited by increasing the sugar concentration to 1.25+1.25% or higher.

Vitamins. — Although promotive effects of the vitamins biotin, pyridoxin and

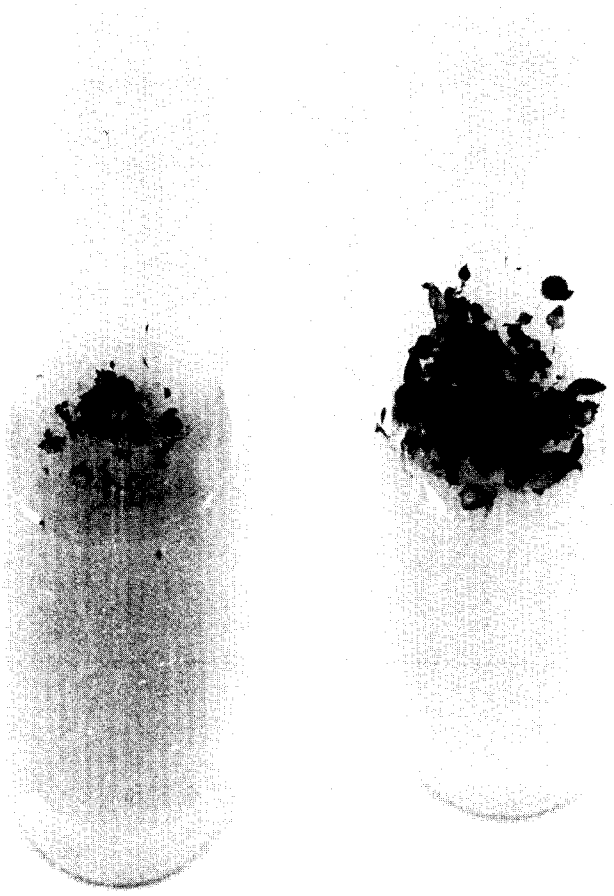

Fig. 5. The effect of tryptone on germination and further development. Left, without tryptone; right, with 2 g l^{-1} tryptone. Without tryptone, germination and development are strongly inhibited.

especially nicotinic acid had been observed (Withner, 1959; Arditti, 1967) on germination and further development of orchid seeds, these vitamins had no effect in our experiments (data not shown).

Regulators. — The influence of three types of regulators (auxins, cytokinins and gibberellins) is shown in Table III. IAA stimulated germination, and a similar result (experimental data not shown) was obtained with the auxin IBA. The cytokinins BA and kinetin (data not shown) and the gibberellins GA_{4+7} and GA_3 (data not shown) had no effect on germination. However, seedling growth and development in the light were increasingly inhibited and distorted by an increasing auxin concentration; IBA was more toxic than IAA. A similar effect on further seedling growth was induced by both cytokinins and

gibberellins; inhibition started at a concentration of 0.5 mg l^{-1} of cytokinin and at a concentration of 0.01 mg l^{-1} of gibberellin.

Complex mixtures. — Finally, the effects of tryptone, banana homogenate and active charcoal were analyzed. Table IV clearly shows that the addition of tryptone was of paramount importance for germination (Fig. 5). All the tested concentrations of this complex mixture of substances were equally effective. In light, growth and development of seedlings completely stopped on media without tryptone, and a concentration of 2.0 g l^{-1} was optimal; similar results were obtained with peptone. Addition of banana homogenate (Table IV) almost completely inhibited germination. Active charcoal (Table IV) hardly affected germination, but in light it inhibited the further development of the plants, which yellowed and subsequently died.

DISCUSSION

Little work has been done on *Paphiopedilum* seed germination. The reasons for this are (1) the period from pollination to seed ripening is extremely long (9–12 months), (2) the regular supply of seeds is difficult, and (3) germination is often affected by many unknown factors. This study shows (compare the control treatments, indicated with an asterisk in the tables) that the germination varied strongly from one seed capsule to another. Part of the variation can be explained by differences in the period between pollination and harvesting of the seed capsules, since unripe seeds of *Paphiopedilum* generally germinate better than ripe ones (Fast, 1971). It remains quite difficult to predict the germination behaviour, as was also found by Flamée (1978). By determining the correlation between the germination percentage and the germination index (Fig. 6), it became evident that both need not be determined since the correlation coefficient between them is very high ($R=0.95$).

Results presented in Table I clearly show that the best germination occurred in darkness. Under a photoperiod of 16 h an increase in irradiance resulted in a decrease in germination, and later caused high mortality and growth inhibition. Our observations on the inhibitory effect of light during the first 12 weeks is in agreement with earlier publications on *Paphiopedilum* and *Cypripedium* seed germination (Harvais, 1973; Reyburn, 1978; Stimart and Ascher, 1981). Some authors (Thomale, 1957; Lucke, 1971; Flamée, 1978; Ernst, 1980) studied germination only in light, and did not make a comparison between light versus dark germination. Since in several experiments with other species and hybrids of *Paphiopedilum* (R.L.M. Pierik et al., unpublished results) an inhibitory effect of light on germination was observed, we completely agree with Stimart and Ascher's (1981) conclusion that germination in *Paphiopedilum* is optimal in complete darkness.

Although it must be presumed that present-day macro-salts of orchid media

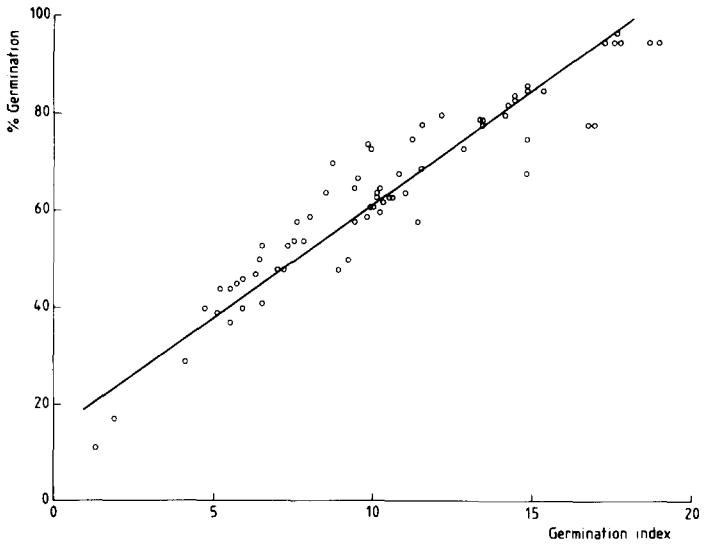

Fig. 6. Correlation between the germination percentage and the germination index. $R = 0.95$.

have been chosen in response to experimental data, the complete lack of these data in the literature means that the criteria taken into account remain unknown (Withner, 1959). The Knudson C medium (1922) has been the basis for most of the macro-salt solutions used to germinate orchid seeds, although Thomale's (1957) and Murashige and Skoog's (1962) media are completely different from Knudson C. Our conclusion that Thomale's macro-salt medium has to be preferred over the other tested media is supported by the work of Flamée (1978), Stimart and Ascher (1981) and Fast (1971). This conclusion is based not only on a relatively high germination rate, but also on the good subsequent growth of young seedlings in light and during sub-culturing. There is no doubt that germination is progressively inhibited with increasing concentrations of macro- and micro-salts (Table II). Although in our experiments (there is no literature known) germination was not significantly affected by the NaFeEDTA concentration, protocorm development and further growth of the seedlings was completely inhibited on media without iron or with a low iron concentration This remarkable effect of iron can be partially explained by the fact that *P. ciliolare* grows naturally on soils in the north of the Phillipines which have an extremely high iron content (B. Singer, personal communication, 1983).

There is little literature concerning the effects of the pH on orchid seed germination. Withner (1959) concluded that the pH does not appear to have a definite effect. However, Reyburn (1978) observed in *Cypripedium* (a closely related genus of *Paphiopedilum*) that dark germination was optimal at pH 5.5–6.0, whereas a pH of 7.0 was strongly inhibitory. This agrees with the con-

clusion from our study. In most studies on *Paphiopedilum*, germination was obtained at a pH of 5.0–5.6 (Flamée, 1978; Ernst, 1975, 1980; Fast, 1971; Thomale, 1957).

Since germination of *P. ciliolare* did not occur without sugar, it is certainly heterotrophic for sugar, as are other species or hybrids of *Paphiopedilum* (Fast, 1971; Lucke, 1971). Orchids must have an external supply of carbohydrates to continue their growth and differentiation. An extraordinary low sugar concentration was optimal for germination; higher concentrations being inhibitory. At 2.5% sugar, germination was dramatically inhibited.

The addition of the vitamins biotin, nicotinic acid and pyridoxine independantly brought about no increase in germination and further development even during the light phase, although Thomale (1957) and Lucke (1971) reported positive effects of these vitamins. However, since in our experiments tryptone, which contains these vitamins, was always present in the basic medium, a positive effect of additional vitamins could not be expected (Arditti, 1977).

Among the regulators tested (Table III), only the addition of auxins resulted in a slight stimulatory effect on germination. In the original Thomale GD1 medium (1957) a compound called "Wuchsstoff 66f" (growth substance 66f) was present. Although the identity of this substance could not be traced, we presume that it was an auxin, since cytokinins and gibberellins were not known in 1957. Literature on the effect of regulators on orchid seed germination is rather scarce. Withner (1959) reported that several orchids react positively to IAA, IBA or NAA, and Hegarty (1955) observed a stimulatory effect of IBA on germination of *Paphiopedilum* hybrids.

The most important compound in our medium is certainly tryptone (or peptone), which is composed (Arditti, 1977) of vitamins and amino acids as well as other compounds. Fast (1971), Flamée (1978) and Thomale (1957) came to the same conclusion. Tryptone has a very beneficial effect not only on germination in the dark, but also on further development of *Paphiopedilum*. Stimart and Ascher (1981) suggested the use of the Norstog (1973) embryo culture medium (which is completely defined) instead of Thomale GD1 (1957) with tryptone or peptone.

Addition of banana homogenate significantly reduced germination (Table IV), which is in agreement with earlier findings of Fast (1971). However, Ernst (1980) advised the addition of banana homogenate to the media for germination of *Paphiopedilum*, and suggested that the seeds were sown on a medium with both active charcoal and banana homogenate present. This contrasts completely with our results, where the addition of banana homogenate significantly reduced germination and that of active charcoal slightly reduced germination. However, the significant increase of shoot and root development in *P. ciliolare* when active charcoal ($2\ g\ l^{-1}$) and banana ($50\ g\ l^{-1}$) are added after protocorm formation agrees completely with earlier findings of Ernst (1974, 1975, 1980) and Fast (1971).

TABLE V

Factors which have a different influence on germination and plant development. The optimum conditions for the 2 processes are given

Factors	Germination	Plant development
Irradiance	Darkness	Weak light (3 W m^{-2})
Macro-salts	Knudson C	Thomale
NaFeEDTA	No effect	25 mg l^{-1}
Glucose + fructose	0.5 + 0.5%	1.0 + 1.0%
IAA	0.001-1.0 mg l^{-1}	Negative
Tryptone	1.5-2.0 g l^{-1}	Negative
Banana homogenate	Negative	50 g l^{-1}
Active charcoal	No effect	2 g l^{-1}

Finally, it should be concluded that certain conditions optimal for seed germination of *P. ciliolare* appeared to be dissimilar to those required for optimal further growth and development of plantlets (Table V summarizes our findings).

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