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Effects of fungicides and bactericides on orchid seed germination and shoot tip cultures in vitro

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Abstract. Amphotericin B, benomyl, gentamycin, nystatin, quintozene penicillin G, sodium omadine, and vancomycin singly and in several combinations have no deleterious effects on the germination of orchid seeds, but inhibit the growth in vitro of shoot tip explants.

Introduction

Contamination by fungi and bacteria from the air, water, tools, glassware, and other sources may occur in orchid seedling and tissue cultures. Surface sterilization of the seeds or tissues can prevent initial contamination, but microorganisms often find their way into cultures at a later date. Incorporation of amphotericin B, benomyl (as the pure compound or the commercial preparation Benlate), gentamycin, nystatin (Mycostatin), quintozene (formerly known as PCNB), penicillin G, sodium omadine, and vancomycin in several combinations can prevent or inhibit contamination of *Cattleya aurantiaca* and *Stanhopea occulata* seedlings in vitro and *Phalaenopsis* (all three are orchids) flower stalk node cultures without adversely affecting the plants [20, 23, 24]. However, the effects of these compounds on the germination of orchid seeds, growth of excised shoot tips, and plantlet development of a larger number of species is not known. The work described here was undertaken to obtain information on the effects of bactericides and fungicides on seed germination and the subsequent growth of seedlings as well as shoot tip development in vitro. As in our previous work [20, 23, 24], the expectations are that combinations of these compounds can prevent or inhibit introduced contamination.

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Table 1. Bactericides and fungicides screened for their effects on orchid seed germination and excised shoot tips in culture

Compound[a]	Property	Concentrations screened (ppm)				Stock solution in 70% ethanol[a]	Volume of stock solution per liter of medium (ml)	Source
		Seeds		Shoot tips				
		Single compounds	In mixtures[b]	Single compounds	In mixtures[c]			
Amphotericin B (92.7% purity)	Fungicide	1, 5, 10	3	10	10	107.9 mg/10 ml	0.1, 0.3, 0.5, 1	SJ Luciana, Squibb Institute for Medical Research, PO Box 400, Princeton, NJ
Benomyl (Benlate,[d] 50% purity)	Fungicide	5, 25, 50[b]	15	50	50	500 mg/100 ml	1, 3, 5, 10	HJ Thome, Agrichemicals, Dupont Co, Wilmington DE 48640
Dowicide (97% purity)	Fungicide and bactericide	1, 5		5		51. 5 mg/20 ml	0.2, 1	Dow Chemical Co, PO Box 1847 2045 Dow Center, Midland, MI 48640
Ethirimol (100% purity)	Fungicide	25, 50, 100				1 g/10 ml	0.25, 0.5, 1	R Fox, Imperial Chemical Industries Ltd, Jealott's Hill Reserach Station, Bracknell, Berkshire RG12 GEY, UK
Gentamicin (purchased in sterile solution in ampule)	Bactericide	5, 25, 50	15	50	50		0.1, 0.3, 0.5, 1	Walter Prozman and TS Schaffer, Schering Corp, 60 Orange Street, Bloomfield, NJ 07003[a]

Nystatin (4020 units/mg)	Fungicide	5, 25	5	25	25	250/ mg/10 ml	0.2, 1	See amphotericin B[a]
Quintozene (PCNB, 99% purity)	Fungicide	25, 50, 100	25	100	100	101 mg/10 ml	2.5, 5, 10	LR Faulkner, Olin Corp, PO Box 991, Little Rock, AR 72203
Penicillin G (1595 units/mg)	Bactericide	25, 50, 100	25	100	100	1000 mg/ml	0.25, 0.5, 1	See amphotericin B[a]
Sodium omadine (90% purity)	Fungicide and bactericide	1, 5	5	5	5	55.5 mg/10 ml	0.2, 1	See quintozene
Vancomycin (97.8% purity)	Bactericide	5, 25, 50	5	50	50	511 mg/10 ml	0.1, 0.5, 1	RJ Hosley, Lilly Research Laboratories, Indianapolis, IN 46206[a]

[a]Also purchased from Sigma Chemical Corporation, PO Box 14508, St Louis, MO 63178
[b]See Tables 4 and 5
[c]See Table 6
[d]Available from retail nurseries as systemic fungicides under a variety of trade names. These fungicides contain 50% active ingredient; therefore, the amounts used should be 10, 50, 100 mg/liter

Materials and Methods

Seeds

Mature seeds of *Cattleya elongata, Laelia tenebrosa,* and *Vanda tricolor* were used for the screening of single compounds (Table 1). *Brassavola nodosa, Cattleya aurantiaca, Cymbidium madidum, Dedrobium speciosum* var. *hillii, Epidendrum nocturnum, Grammatophyllum elegans, Laelia lobata, Phajus tankervilliae* x *P. maculatus, Odontoglossum citrosmum,* and *Zygopetalum mackay* were employed in germination experiments with mixtures of fungicides and bactericides (Table 1). The seeds were produced in the University of California, Irvine Orchid Collection (UCIOC), and stored over dessicant ($CaCl_2$) at 4°C. Availability of seeds determined their use in the screening of single compounds or mixtures.

The seeds were surface sterilized with saturated calcium hypochlorite (7 g/100 ml water) for 15 min in glass tubes and washed with sterile distilled water before placing them in culture [11].

Knudson C solution containing 0.2% graphite [20, 23, 24] was used as the basal culture medium. It was sterilized by autoclaving and the fungicides and bactericides were added in stock solutions (Table 1) to the medium before it solidified and were mixed by swirling. Prescription bottles, 100-ml capacity, containing 20 ml medium, were used as culture vessels.

Germination was evaluated five months after placing the seeds in culture on media containing single compounds and 10–12 weeks for mixtures by the growth index method [12, 21]. According to this method, seedlings are classified into six developmental stages (which are numbered one through six as in Fig. 1). Random samples of 100 seedlings are observed under a dissecting microscope and classified into the six stages (for example, 10% stage 1; 20% stage 2; 20% stage 3; 10% stage 4; 20% stage 5; and 20% stage 6). The percentage of seedlings in each stage is then multiplied by its number and the totals are added to produce the growth index $[(10 \times 1) + (20 \times 2) + (20 \times 3) + (10 \times 4) + (20 \times 5) + (20 \times 6) = 370]$. This growth index measures normal development rather than mere increases in size (i.e., appearance of meristems, leaves, and roots and not only increases in diameter, length, or weight of protocorms, for example), and can be subjected to statistical analysis [12, 21].

Shoot tips

New growths were taken from *Cymbidium* hybrids in the UCIOC, rinsed with tap water, and freed of dead, damaged, or excessively hard external tissues and parts. The shoots were then immersed in a mixture of household bleach–distilled water (1:1; vol/vol). Excision of explants was carried out under a dissecting microscope on an open laboratory bench washed with 95% ethanol. External leaves and leaf primordia were removed to expose

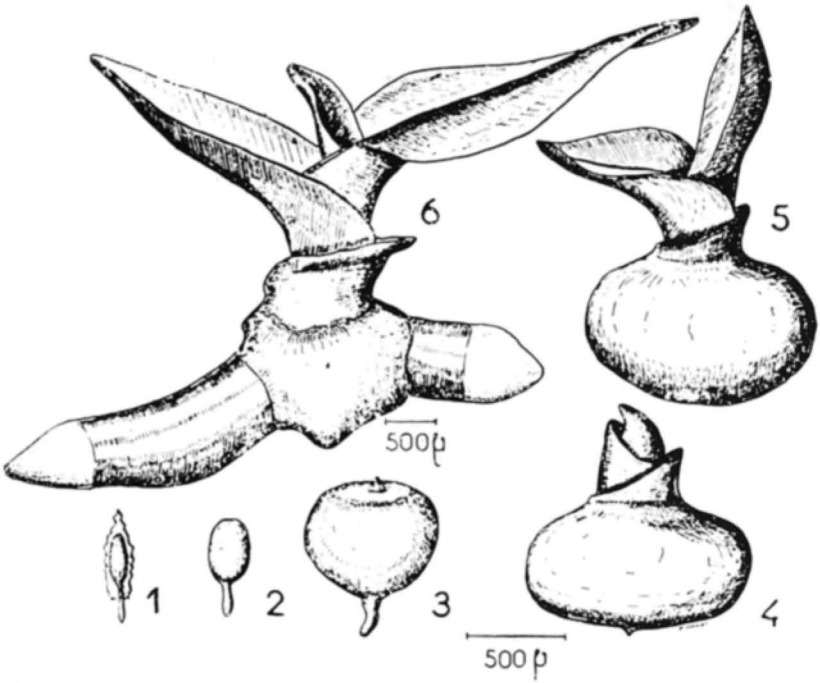


Figure 1. Stages in the germination of a *Cattleya* orchid [2].

the shoot tip which was excised by making four incisions [2]. Explants were cultured on modified Tsuchiya [25, 27; also listed in 2] medium (Table 2). The fungicides and bactericides used were the same as those employed with seeds (Table 1). Erlenmeyer flasks, 25- or 50-ml capacity containing 5 or 12.5 ml medium, respectively, were used as culture vessels for shoot tips. Flasks containing explants in liquid media were placed on a reciprocating shaker (60 oscillations/min). Shoot tip cultures were evaluated visually and are described in subjective terms.

All cultures were maintained under 22° ± 2°C, 0.8 mW/cm^2 and 18-h photoperiods provided by a mixture of Wide Spectrum Gro Lux tubes and incandescent lamps.

Results

In experiments with single compounds, there were no major differences between the growth indices of controls and of seeds germinated on fungicide and bactericide containing media (Table 3). Only the highest concentrations of benomyl, nystatin, quintozene, and penicillin G prevented germination of *Cattleya elongata*. All concentrations of amphotericin B and the highest level of gentamycin reduced germination of *Vanda tricolor* (Table 3). The solvent (70% ethanol) had no deleterious effects on germination (Table 3).

Table 2. Tsuchiya medium as used for the culture of *Cymbidium* shoot tips [2, 25, 27]

Component	Amount per liter
Macroelements	
Potassium nitrate, KNO_3	525 mg
Dicalcium phosphate, $CaHPO_4$	200 mg
Potassium phosphate, KH_2PO_4	250 mg
Ammonium sulfate, $(NH_4)_2SO_4$	500 mg
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	250 mg
Ferric tartrate, $Fe_2(C_4H_4O_6)_3$	30 mg
Tryptone	2 g
Sucrose	20 g
Water, distilled	to 1000 ml

Combinations of compounds had no negative effects on the germination of *Brassavola nodosa, Cattleya autrantiaca, Laelia lobata, Phajus tankervilliae* x *P. maculatus,* and *Zygopetalum mackay* (Table 4). In these experiments, too, 2 ml 70% ethanol per liter of medium had no inhibitory effects except on the germination of *Odontoglossum citrosmum* (Table 4). Appearance of roots in seedlings of *Brassavola nodosa* and *Cattleya aurantiaca* was enhanced by all combinations. Most of these combinations also enhanced the percentage of seedlings of these species and *Laelia lobata* which developed leaves (Table 5). These three species belong to the subtribe Laeliinae. Only combination 5 increased the growth indices of *Phajus* (subfamily Epidendroideae) and *Zygopetalum* (subfamily Vandoideae). All other combinations were without effect on the growth indices of these orchids (Table 4). There were no seedlings of *Odontoglossum citrosmum* past stage 1 (Fig. 1-1) on any of the combinations of bactericides and fungicides. On the basal medium, seedlings of *O. citrosmum* did not develop past stage 2 (Fig. 1-2). There were no seedlings of *Zygopetalum mackayi* past stage 3 (Fig. 1-3) on combinations 1, 4, and 5, and the two controls. On media 2 and 3, the seedlings did not develop past stage 2 (Fig. 1-2).

All combinations accelerated the appearance of roots and leaves in *Brassavola nodosa.* Only combinations 3–5 had a similar effect on *Cattleya aurantiaca.* In *Laelia lobata,* the appearance, but not the expansion, of leaves was accelerated by combinations 1–3 and 5 (Table 5).

None of the combinations screened could prevent contamination of the cultures of *Dendrobium specisum* var. *hillii.* Only one flask (of three) on combination 5 (Table 4) remained uncontaminated and its growth index was 336. Of the seedlings, 1% had roots (average length 0.9 mm) and 18% had leaves (average length of the first leaf 2.21 ± 0.68 mm; of the second 1.34 ± 0.6 mm).

When used singly, the compounds we screened either delayed or entirely prevented the development of excised *Cymbidium* shoot tips (Table 6). All combinations had deleterious effects on these explants.

Table 3. Effects of separate bactericides and fungicides on the germination of orchid seeds

Compounds	Concentration (ppm)	Growth index[a]		
		Cattelya elongata[b]	*Laelia tenebrosa*[b]	*Vanda tricolor*[c]
Amphotericin B	1		344/11	330
	5		382/51	278
	10		321/51	324/55
Benomyl	5	370/75	410/26	480
	25	346/23	412/22	443/94
	50	NG[d]	NG[d]	NG[d]
Dowicide	1		381/42	375/66
	5	408/101	405/40	532/25
Ethirimol	25	450	403/45	430/158
	50		346/30	
	100	300/87	384/41	398/140
Gentamicin	5	421/18	350/21	326/44
	25	317/29	336/17	381/139
	50	313	346/76	302/2
Nystatin	5	298/259	412/9	402/16
	25	NG[d]	343/22	357/80
Quintozene	25	367/29	399/35	465/148
	50	361/75	364/57	428/111
	100	NG[d]	362/20	600
Penicillin G	25	377/41	382/9	456/63
	50	335/14	319/9	326/29
	100	NG[d]	366/38	355
Sodium omadine	1	350/50	339/15	447/150
	5	372/43	435/33	562/23
Vancomycin	5	392/12	378/24	484/30
	25	382/31	330/32	537/37
	50	341/21	373/24	494/57
Basal control		397/5	365/9	442/56
Solvent control		338/33	393/22	467/67

[a] The values given are grow index/standard deviation
[b] Subfamily Epidendroideae, tribe Epidendreae, substribe Laeliinae [7]
[c] Subfamily Vandoideae, Tribe Vandeae, Substribe Sarcanthinae [7]
[d] NG, no germination

Discussion

Orchid embryos lack cotyledons, leaf initials, and rudimentary roots. As a result, orchid seeds do not germinate like those of other plants. The dry seed swells (Fig. 1-1–2), forming a spherical body called a protocorm (Fig. 1-3), a term first used in 1890 by Melchior Treub to describe bodies formed when lycopods germinate [3]. Apical meristems on the tops of protocorms form leaves (Fig. 1-4) which elongate (Fig. 1-5) generally prior to, but at

Table 4. Effects of combination of fungicides and bactericides on the germination of orchid seeds

No.	Combinations[b]	Growth index[a] *Brassavola nodosa*[c]	*Cattleya aurantiaca*[c]	*Laelia lobata*[c]	*Phajus tankervilliae*[d] × *P. maculatus*[d]	*Odontoglossum citrosmum*[e]	*Zygopetalum mackayi*[f]
1	Benomyl, nystatin penicillin G, gentamycin	415/30	274/43	195/69	114/5	100/0	200/70
2	Benomyl, nystatin, penicillin G, gentamycin, sodium omadine	390/0	269/20	225/23	111/14	100/0	137/4
3	Benomyl, nystatin, penicillin G, gentamycin, amphotericin B, vancomycin	436/27	211/69	137/35	126/10	100/0	151/2
4	Benomyl, quintozene penicillin G, amphotericin B, sodium omadine	436/39	271/64	125/19	114/4	100/0	213/13
5	Benomyl, nystatin, amphotericin B, penicillin G, sodium omadine	408/40	246/55	199/125	148/23	100/0	259/16
Controls							
Basal medium only			259/9	191/37	118/15	125	169/39
Basal medium plus 2.0 ml 70% ethanol/ liter		230	173/25	169/45	153/23	100/0	193/34

[a]The values given are growth index/standard deviation
[b]See Table 1 for concentrations
[c]Subfamily Epidendroideae, tribe Epidendroideae, subtribe Laeliinae [7]
[d]Subfamily Epidendroideae, tribe Epidendrodeae, subtribe Blentiinae [7]
[e]Subfamily Vandoideae, tribe Cymbidieae, subtribe Oncidiinae [7]
[f]Subfamily Vandoideae, tribe Maxillarieae, subtribe Zygopetalinae [7]

times together with, the appearance of roots (Fig. 1-6). Consequently, substances which affect seedlings may do so at any one of several stages, including swelling of the seeds, formation of an apical meristem, as well as initiation and elongation of leaves and roots. Because of this, germinating orchid seeds are very suitable for studying the effects of fungicides and bactericides in culture media.

The orchid species used in these experiments were affected differently by the compounds screened. These differences did not always correlate with the systematic affinities [7] of the orchids (Tables 3–5). Growth of *Cattleya aurantiaca* and *Stanhopea occulata* seedlings was not affected deleteriously by 70% ethanol [23, 24], but germination of *Odontoglossum citrosmum* was inhibited. The very low growth index of the latter on basal medium, however, suggested that the seeds may have been of low viability and therefore very susceptible to inhibition (the amount of work and time factors associated with germinating orchid seeds are such that viability tests are not practical). Previous reports regarding the use of bactericides and fungicides on seed germination indicated that both the effects of the compounds and the tolerance of different plant species may vary. For example, benomyl was less toxic to cottonseeds than 5-ethoxy-3-(trichloromethyl)-1, 2, 4-thiadiazole and carboxin [6]. On the other hand, 'benomyl gave a slightly stunting action to cotton seedlings at 160 ppm' [1] and inhibited germination, length of radicles, and allantoinase activity in seeds of *Arachis hypogaea, Glycine max., Phaseolus aureus,* and *P. mungo* [16]. Benomyl [methyl-1(butylcarbamoyl)-2-benzimidazole carbamate] and BAS 3460F (methyl-2-benzimidazole carbamate], but not thiabendazole, can break the heat-induced seed dormancy in celery seeds [22].

Temik had a deleterious effect on the germination of Egyptian cotton, but vitavax/captan and disyston or a combination of both frequently enhanced it [10]. Benomyl, thiram, and captan either enhanced or had no deleterious effects on the germination of wheat [8] and peas [13]. Combinations of penicillin G, nystatin (Mycostatin, Squibb), and amphotericin B (Fungizone, Squibb) prevented in vitro contamination of seedlings of *Hibiscus esculentus, Vigna sinensis,* and *Dolichos lablab* [9]. Our findings with *Cattleya aurantiaca* and *Stanhopea occulata* seedlings and cultures of *Phalaenopsis* flower stalk nodes also demonstrated that some bactericides and fungicides may enhance growth somewhat or at least have no deleterious effects, whereas others may be inhibitory [20, 23, 24]. Thus our findings are similar in principle to previous reports.

Benomyl is a systemic fungicide which is taken up and translocated by plant cells and organs [19]. This means that in the presence of benomyl not only the culture medium but also the plant material will be protected from fungal contamination. In addition, benomyl has been reported to have a cytokinin-like effect in wheat leaf [26], and soybean callus and radish cotyledon [18] bioassays, as well as a growth-enhancing effect on shoot and

Table 5. The effects of combinations of fungicides and bactericides on root and leaf formation and growth[a]

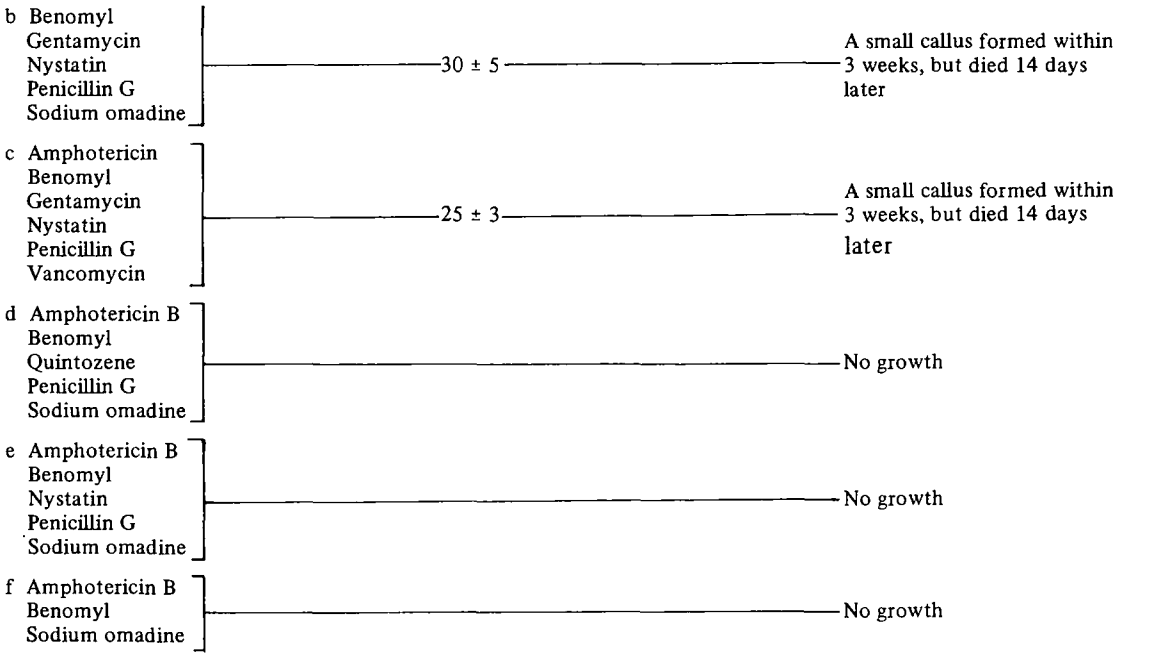
Mixtures		*Brassavola nodosa*				
		Roots[b]		Leaves[c]		
Combinations[b]	No.	Seedlings with roots (%)	Length (mm)	Seedlings with leaves (%)	Length (mm)[d] 1st leaf	2nd leaf
Benomyl, nystatin penicillin G, gentamycin	1	1.7/0.6	0.56/0.13	20.0/13.9	1.32/0.49	0.75/0.26
Benomyl, nystatin penicillin G, gentamycin, sodium omadine	2	1.0/1.7	0.62/0.33	31.3/11.2	1.91/11.2	0.95/0.46
Benomyl, nystatin, penicillin G, gentamycin, amphotericin B, vancomycin	3	2.3/2.5	0.39/0.15	47.0/21.5	1.88/0.63	0.93/0.44
Benomyl, quintozene penicillin G, amphotericin B, sodium omadine	4	1.0/1.0	0.58/0.14	51.3/18.6	1.93/0.90	0.87/0.49
Benomyl, nystatin, amphotericin B, penicillin G, sodium omadine	5	5.7/8.1	0.45/0.07	57.7/1.5	2.72/1.48	1.38/0.91
Controls						
Basal medium only		–	–	–	–	–
Basal medium plus 2.0 ml 70% ethanol/liter		0[e]	–	15	1.50/0.41	0.94/0.30

Cattleya aurantiaca[e]			*Laelia lobata*[e]			*Phagus tankervilliae* × *P. maculatus*
Leaves[c]			Leaves[c]			
Seedlings with leaves (%)	Length (mm) 1st leaf	2nd leaf	Seedlings with leaves[f] (%)	Length (mm)[d] 1st leaf	2nd leaf	Seedlings with leaves (%)
0[g]	–	–	11.30/16.3	–	–	There were no seedlings past stage 3
3.30/4.0	1.36/0.29	0.92/0.40	10.70/3.1	–	–	There were no seedlings past stage 3
14.00/11.5	2.34/1.22	1.12/0.39	9.00/6.1	–	–	1.0/1.0[f]
16.00/21.0	2.27/1.01	1.03/0.37	1.30/2.3	2.89/0.73	1.36/0.57	1.0/1.7[f]
22.00/13.0	2.85/1.56	1.33/0.86	13.0/18.4	–	–	There were no seedlings past stage 2
2.70/3.8	1.69/0.54	1.23/0.14	1.0/1.7	1.5/0.62	1.17/0.63	There were no seedlings past stage 2
0.23/0.40[g]	–	–	There were no seedlings past stage 3[h]			0.3/0.6[g]

[a]Average of all replicas/standard deviation; see Table 4 for systematic affilations of the orchids
[b]By definition, roots are preset only on stage 6 seedlings (Fig. 1)
[c]Stages 5 and/or 6 have leaves (Fig. 1)
[d]The 1st (lower) leaf is usually longer
[e]None of the seedlings formed roots
[f]There were no seedlings past stage 4 (Fig. 1), which means that none had expanded leaves

Table 6. Effects of bactericides and fungicides on the growth and development of excised *Cymbidium* (subfamily Vandoideae, tribe Cymbideae, subtribe Cyrtopodiinae [7]) shoot tips

No.	Compound	Time for explants to turn green (days)	Remarks
	Control	12 ± 2	Normal growth, proliferation
	Single compounds		
1	Amphotericin B	17 ± 2	Except for a delay, normal growth, proliferation, and plantlet formation
2	Benlate	42 ± 3	Weak and inhibited growth and development; no shoot formation
3	Dowicide	23 ± 2	Weak and inhibited growth and development; no shoot formation
4	Gentamycin	57 ± 5	Weak and inhibited growth and development; no shoot formation
5	Nystatin	23 ± 2	Except for a delay, normal growth, development and plantlet formation
6	Quintozene		No growth
7	Penicillin G	25 ± 2	Normal callus growth; no plantlet formation
8	Sodium omadine	23 ± 2	Except for a delay, normal growth and development
9	Vancomycin		No growth
	Mixtures		
	a Benomyl Gentamycin Nystatin Penicillin G		No growth


b Benomyl
Gentamycin
Nystatin
Penicillin G
Sodium omadine
30 ± 5
A small callus formed within 3 weeks, but died 14 days later
c Amphotericin
Benomyl
Gentamycin
Nystatin
Penicillin G
Vancomycin
25 ± 3
A small callus formed within 3 weeks, but died 14 days later
d Amphotericin B
Benomyl
Quintozene
Penicillin G
Sodium omadine
No growth
e Amphotericin B
Benomyl
Nystatin
Penicillin G
Sodium omadine
No growth
f Amphotericin B
Benomyl
Sodium omadine
No growth

root development in *Asparagus officinalis* tissue cultures [28]. These facts render benomyl an especially attractive fungicide for use in tissue culture. However, caution is necessary in such use since benomyl had an inhibitory effect on shoot tip cultures of *Cymbidium* (Table 6) and *Colocasia esculenta* (Arditti and Gonzales, unpublished results). These deleterious effects may be due to metabolic and/or enzyme inhibition [16] as well as the induction of chromosome abberations [29].

A number of antibiotics have been used in tissue cultures with varying results. Bacitracin (50–100 ppm), griseofulvin (2–5 ppm), oxytetracycline (2.5 and 5 pm), and streptomycin (2.5 ppm or less) are not toxic to *Catharanthus roseus* tissue cultures [4]. Rifampicin (50 μg/ml) was shown to be effective against contaminating bacteria in cultured explants of *Helianthus tuberosus* without affecting differentiation of tracheary elements, rates of cell division, or DNA synthesis [17]. Low levels of kanamycin enhanced shoot differentiation in callus cultures of tobacco and carrots [15]. Therefore, our findings with shoot tips of *Cymbidium* (Table 5) and *Colocasia esculenta* (unpublished results) suggest that these plants are particularly sensitive to the fungicides and bactericides we screened, or that these compounds are more toxic to explants than bacitracin, griseofulvin, oxytetracycline, steptomycin, rifampicin, and kanamycin.

Our findings (Tables 4 and 5) suggest that (a) on occasion seeds may be so heavily laden with spores that nonphytotoxic concentrations of fungicides and/or bactericides cannot prevent contamination, or (b) some contaminants may be unaffected by the compounds used. Support for the latter comes from reports that benomyl (a) was not as effective as other compounds [14] in the control of *Alternaria* spp., *Botrytis* sp., *Cladosporium* spp., *Cochliobolus sativus, Penicillium* spp., *Rhizopus* sp. and *Streptomyces* spp., (b) did not eliminate pathogens [13] from pea seeds, (c) could not control [5] *Aphanomyces cochlioides* in sugar-beet seedling, and (d) controlled only temporarily the contamination of *Hibiscus esculentus, Vigna sinensis,* and *Dolichos lablab* seedlings on nutrient ager [9].

The need for, and importance of, agents which can combat contamination can be expected to increase as the use of tissue culture and in vitro seed germination becomes more widespread. Our findings suggest that useful compounds may be identified and appropriate combinations can be formulated for general use.

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