Preservation of Conidia of Broccoli Downy Mildew Fungus with Cryogenic Protectants by Freezing at -80°C

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Abstract

Conidia of the downy mildew fungus (*Peronospora parasitica*) which is an obligate parasite were well preserved by freezing at -80°C using suitable cryogenic protectants. The germination rate of the conidia which were suspended in distilled water and frozen at -20°C or -80°C was very low compared with the untreated control. However, the germination rates of the conidia suspended in 5% dimethyl sulfoxide (DMSO) and 5% skim milk were 74.8% and 32.8% of the control, respectively, even after freezing at -20°C . On the other hand, in the preparations directly frozen at -80°C , the germination rate of the conidia decreased markedly in comparison with that after freezing at -20°C . Nevertheless, in the preparations of conidia frozen by the two-step freezing method at -20°C for 24 hr followed by -80°C for 24 hr, the germination rate remained the same as that in the preparations frozen only at -20°C . During the storage at -20°C , the conidia suspended in 5% DMSO+5% skim milk lost their germination ability after one month. The conidia suspensions which were supplemented with 10% DMSO+5% skim milk or 10% DMSO+10% skim milk and frozen by the two-step method at -20°C and at -80°C , showed a higher germination rate at least up to a period of 12 months. Moreover, these preparations exhibited a high pathogenicity to the broccoli seedlings. Based on these results, it was suggested that the conidia of the broccoli downy mildew fungus treated with protective substances such as 10% DMSO+5% skim milk or 10% DMSO+10% skim milk which were pre-frozen at -20°C for 24 hr followed by -80°C could be well preserved for a long period of time.

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Key words: *Peronospora parasitica*, broccoli, preservation, protectant, freezing.

INTRODUCTION

As obligate parasites such as downy mildew and powdery mildew fungi can not be cultured on artificial media, it is very difficult to handle many races of these fungi at the same time for phylogenetic studies. Therefore, obligate parasites, in general, have been maintained on their hosts or preserved by freezing in liquid nitrogen using protective substances such as glycerol and dimethyl sulfoxide (DMSO)⁶⁾. Sporangia of the hop downy mildew fungus, *Pseudoperonospora humuli*, and those of the vine downy mildew fungus, *Plasmopara viticola*¹⁰⁾, and conidia of the tobacco downy mildew fungus, *Peronospora tabacina*⁴⁾, were found to be infectious for a period of 12 months during storage in liquid nitrogen. In the same way, the pathogenicity of the sporangia of the cucumber downy mildew fungus, *Pseudoperonospora cubensis*, and vine downy mildew fungus was maintained for 200 days by freezing at -20°C ²⁾. As many attempts to culture downy mildew fungi on artificial media^{3,7,8)} have been unsuccessful, most of the obligate parasites are maintained on their plant hosts^{1,9,11)}. Although phytopathogenic fungi have been effectively preserved in liquid nitrogen, the facilities required for such preservation are expensive. Thus, we examined the possibility of preserving conidia of the broccoli downy mildew fungus, *Peronospora*

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parasitica, in electronically controlled freezers at -20°C or -80°C , using several cryogenic protectants instead of liquid nitrogen.

MATERIALS AND METHODS

Fungal isolate. The broccoli downy mildew fungus, *Peronospora parasitica*, was isolated from a broccoli plant in the field of Ano, Mie in 1990. The isolate was maintained and propagated on broccoli seedlings.

Preparation of conidia suspensions. Cotyledons of 7-day-old broccoli seedlings were inoculated with conidia of the broccoli downy mildew fungus and incubated in growth cabinets under 5,000 lux at 20°C . Many conidia were formed on the undersurface of the leaves 7 days after inoculation. Conidia were collected by washing the infected leaves with sterilized distilled water (D.W.). The conidia suspensions were mixed with the solutions of the protective substances to reach the final concentration shown in the Tables. Conidia were suspended in concentrations of more than 10^4 per 1 ml enough to be fully pathogenic to the broccoli seedlings (unpublished observation). Then, 400 μl of the conidia suspensions was put into 2 ml plastic screw-capped ampules (1 cm diam., 5 cm long), and preserved at -20°C or -80°C .

In the cases of preservation using powdery substances, the conidia preparations were treated according to the method of Tetsuka and Katsuya¹⁰⁾. Leaves with conidia were cut into 5 mm² segments and dredged with agar, skim milk or polyvinyl alcohol powder. These preparations were pre-frozen at -20°C for 24 hr and preserved at -80°C . After freezing and thawing, the leaf segments were transferred to centrifuge tubes containing about 1 ml D.W. Then, they were stirred with a mixer and centrifuged at 3,000 rpm for 3 min to remove the protective substances. Pellets of conidia were resuspended in D.W. and used for the germination tests.

Conditions of freezing and thawing. Conidia suspensions were placed in a freezer at -20°C or -80°C . The preparations of conidia were thawed for 30–40 min at room temperature (slow thawing) or in water at 40°C (rapid thawing). The procedure adopted for the freezing of the conidia suspensions at -20°C followed by -80°C was designated as the two-step freezing method used in this study.

Estimation of survival of conidia. To determine whether conidia were alive, the presence of the germ tube was used as a criterion. The thawed preparations were placed on 1.5% water agar and incubated at 20°C in the dark overnight. More than one hundred conidia in each of the preserved preparations were examined for the germination tests. It was assumed that conidia with a germ tube were alive. Unfrozen preparations suspended in D.W. were used in all the tests as controls. The values of the germination rate of the conidia in the frozen preparations were expressed as a percentage of the unfrozen original control unless otherwise stated. Bursting or deformation of the conidia after freezing and thawing was diagnosed based on the apparent shape of the conidia under an optical microscope.

Pathogenicity test. Pathogenicity of the conidia which had been preserved by freezing to the broccoli seedlings was checked by the development of the symptoms after inoculation. Eight (16 cotyledons) 7-day-old broccoli seedlings was inoculated with eighty μl of the thawed preparations (5 μl per one cotyledon) and incubated in growth cabinets under 5,000 lux at 20°C for 7 days.

RESULTS

Germination rate and damage sustained by conidia after the freezing and thawing treatment

The incidence of bursting or deformation of the conidia in the preparations which were suspended in D.W. and frozen at -20°C or -80°C for one hr increased in comparison with the untreated control after thawing. Especially, by freezing at -80°C the bursting or deformation of the conidia were often induced. The percentages of germination of the conidia in these preparations decreased to 5.5% and 0.0%, respectively, compared with the untreated control.

Table 1. Effect of various protective substances on the germination ability of conidia immediately after freezing^{a)}

Protective substances	Germination ability (%)
5% DMSO	74.8 ^{b)}
5% adonitol	50.5
5% glycerol	48.2
5% sucrose	44.7
5% glucose	44.6
5% sorbitol	42.9
5% skim milk	32.8
1% peptone	19.8
1% lysine	13.2
1% sodium glutamate	11.9
1% blue dextran	11.8
1% polypeptone	9.4
1% tryptone	7.9
1% ficoll	5.6
5% polyvinyl alcohol	4.2
1% albumin	1.7
1% polyvinylpyrrolidone K-30	1.3
D.W.	2.4

a) The preparations of conidia were frozen at -20°C for one hr and tested subsequently for their germination ability after thawing in water at 40°C .

b) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of four experiments.

Effect of thawing temperatures

After freezing at -20°C for one hr, there was virtually no difference in the germination rate between rapid and slow thawing in the preparations of conidia suspended in D.W. or supplemented with 5% glycerol + 5% skim milk (data not shown). Consequently, the preparations for the following examinations were thawed in water at 40°C .

Effect of various protective substances in the germination rate of conidia during the freezing and thawing treatment

The germination rate of the conidia in the preparations frozen at -20°C in D.W. for one hr decreased to 2.4% of the value of the unfrozen original preparation. After the same procedure, the germination rates of the conidia in the preparations supplemented with each 5% of DMSO, adonitol, glycerol, glucose, sucrose, sorbitol or skim milk were 74.8%, 50.5%, 48.2%, 44.7%, 44.6%, 42.9% and 32.8%, respectively. These values were considerably higher than that of the D.W. preparation. Also, the addition of 1% of peptone, lysine, sodium glutamate, blue dextran, polypeptone, tryptone, ficoll or 5% polyvinyl alcohol to the preparations produced a slight protective effect. The effect of substances such as 1% albumin or 1% polyvinylpyrrolidone K-30 was negligible (Table 1).

Effect of various concentrations of protective substances on the germination rate of conidia during the freezing and thawing treatment

The effect of higher concentrations of substances which showed a protective effect was examined after freezing at -20°C for one hr and thawing. As shown in Table 2, there was little difference in the germination rate of the conidia between 5% and 10% DMSO, the values being 62.4% and 58.4% of that unfrozen of the control, respectively. However, the germination rate of the value of the conidia in the preparations supplemented with 20% DMSO was 38.0%, a value lower than that when 5% or 10% DMSO was employed. Similarly, the percentages of germinated conidia decreased with the increase of the concentrations of glycerol. In the cases of glucose and adonitol, there were almost no differences in the protective effect among the concentrations of 5%, 10% and 20%. The addition of 20% sucrose was more effective than that of 5% or 10% sucrose. Consequently, the preparations of conidia supplemented with

Table 2. Effect of several concentrations of protective substances on the germination ability of conidia immediately after freezing^{a)}

Protective substances	Germination ability (%)			
	Concentrations of suspensions			
	0%	5%	10%	20%
DMSO		62.4 ^{b)}	58.4	38.0
glycerol		31.5	16.5	10.0
glucose		24.1	22.2	18.4
adonitol		23.4	21.1	19.0
sucrose		16.0	20.9	27.3
D.W.	1.3			

a) The preparations of conidia were frozen at -20°C for one hr and tested subsequently for their germination ability after thawing in water at 40°C .

b) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of three experiments.

Table 3. Effect of the combination of protective substances on the germination ability of conidia immediately after freezing^{a)}

Protective substances	Germination ability (%)
5% DMSO	72.8 ^{b)}
5% DMSO+5% glucose	72.9
5% DMSO+5% sucrose	72.7
5% DMSO+5% glycerol	71.7
5% DMSO+5% adonitol	63.8
5% DMSO+5% skim milk	61.9
5% DMSO+1% peptone	44.2
5% DMSO+1% sodium glutamate	31.3
5% DMSO+1% lysine	26.6
D.W.	1.1

a) The preparations of conidia were frozen at -20°C for one hr and tested subsequently for their germination ability after thawing in water at 40°C .

b) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of three experiments.

5% or 10% DMSO exhibited higher germination rates than those of other protective substances.

Effect of simultaneous addition of protective substances with DMSO on the germination rate of conidia during the freezing and thawing treatment

After freezing at -20°C for one hr and subsequent thawing, the germination rates of the conidia in the preparations supplemented with 5% glucose, 5% sucrose or 5% glycerol in addition to 5% DMSO were 72.9%, 72.7% and 71.7% of the value of the unfrozen original control, respectively (Table 3). These results showed that the combination of those substances with 5% DMSO induced the same rate of germination compared with the single addition of 5% DMSO (72.8%). Also, in the preparations of conidia supplemented with 5% DMSO+5% adonitol or 5% DMSO+5% skim milk, the germination rates of conidia were apparently higher than that of the D.W. preparation, the values being 63.8% and 61.9% of that of the unfrozen control, respectively. The values in the preparations with the combination of 1% peptone, 1% sodium glutamate or 1% lysine with 5% DMSO were low. Thus, a synergistic effect of the combination of various substances with DMSO could not be observed in any of the experiments.

Preservation of conidia by freezing

The preparations of conidia suspended in several protective substances were preserved at -20°C . During one month of storage, the germination rate of the conidia in the preparations containing 5%

Table 4. Effect of protective substances on the germination ability of conidia preserved at -20°C

Protective substances	Germination ability (%)			
	Period of storage ^{a)}			
	1 hr	1 month	3 months	6 months
5% DMSO	90.3 ^{b)}	3.2	0.9	0.0
5% DMSO+5% skim milk	83.5	52.8	1.0	0.0
5% DMSO+5% glucose	81.2	38.8	0.0	
5% DMSO+1% sodium glutamate	65.0	4.7	0.0	

a) Period of storage after freezing at -20°C .

b) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of two experiments.

Table 5. Effect of freezing time and temperature on the germination ability of conidia immediately after freezing

Protective substances	Germination ability (%)				
	two hr freezing			48 hr freezing	
	-20°a)	$-20 \rightarrow -80^{\circ}\text{b)}$	-80°a)	-20°c)	$-20 \rightarrow -80^{\circ}\text{d)}$
5% DMSO	33.2 ^{e)}	4.1	0.2	22.1	16.1
5% DMSO+5% skim milk	33.6	6.1	0.2	14.8	25.1
5% DMSO+5% glucose	33.1	2.3	0.7	12.9	5.5

a) The conidia suspensions were frozen at -20°C or -80°C for two hr.b) The conidia suspensions were frozen at -20°C for one hr followed by -80°C for one hr.c) The conidia suspensions were frozen at -20°C for 48 hr.d) The conidia suspensions were frozen at -20°C for 24 hr followed by -80°C for 24 hr.

e) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of three experiments.

DMSO+5% skim milk was very high (52.8%), though almost all the conidia lost their germination ability after three months of preservation (Table 4). The addition of 5% DMSO+5% glucose also resulted in a considerable protective effect one month after freezing. Nevertheless, the germination rate of the conidia in the preparations containing 5% DMSO or 5% DMSO+1% sodium glutamate decreased even after one month of storage at -20°C .

As mentioned previously, since we failed to preserve the conidia of the broccoli downy mildew fungus at -20°C for a long period of time, we improved the method in order to obtain high germination rates of the conidia immediately after freezing at -80°C .

First, the effect of the temperature was compared among freezing treatments at -20°C or -80°C for two hr, and at -20°C for one hr followed by -80°C for one hr (two hr two-step freezing treatment). As shown in Table 5, by applying the two-step freezing and thawing method, the germination rates of the conidia treated with 5% DMSO, 5% DMSO+5% skim milk or 5% DMSO+5% glucose decreased markedly, being 4.1%, 6.1% and 2.3% of the value of the unfrozen control, respectively. The preparations which were frozen at -80°C for two hr and thawed showed much lower values of 0.2%, 0.2% and 0.7%, respectively. These freezing treatments did not make it possible to preserve the materials at -80°C for a long period of time because the germination rate of the conidia decreased markedly compared with the freezing treatment at -20°C for two hr (Table 5). Furthermore, we prolonged the freezing time (48 hr). Based on these results, in the preparations subjected to freezing at -20°C for 24 hr followed by -80°C for 24 hr (48 hr two-step freezing treatment) and thawing, the germination rate of the conidia treated with 5% DMSO+5% skim milk reached a value that was 25.1% of that of the unfrozen control, for above the 14.8% value of the freezing treatment at -20°C for 48 hr. In the conidia suspensions treated with 5% DMSO or 5% DMSO+5% glucose, although the germination rates decreased to 16.1% and 5.5% of the value of the unfrozen control by the 48 hr two-step freezing treatment, respectively, these values were higher than those recorded in the two hr two-step freezing

Table 6. Effect of protective substances on the germination ability and pathogenicity of conidia preserved at $-80^{\circ}\text{C}^{\text{a)}$

Protective substances	Germination ability (%) and pathogenicity					
	Period of storage ^{b)}					
	1 day	3 months	6 months	12 months		
5% DMSO	14.1 ^{c)} + + ^{d)}	2.1 —	2.0 —			
5% DMSO+5% skim milk	7.5 + + +	12.9 +	10.2 +	10.0 + + +		
5% DMSO+10% skim milk	38.3 + +	11.3 +	12.7 + +	3.9 + +		
10% DMSO+5% skim milk	53.7 + +	66.4 + + +	55.5 + +	47.7 + + +		
10% DMSO+10% skim milk	40.9 + +	79.1 + + +	75.8 +	46.9 + + +		
D.W.	0.0 —	0.0 —				

a) The preparations of conidia were frozen at -20°C for one day followed by -80°C .

b) Period of storage after freezing at -80°C .

c) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of two experiments.

d) Pathogenicity to broccoli seedlings : + + + (high) ~ — (absent).

Table 7. Effect of the application of powdery substances on the germination ability of conidia preserved at $-80^{\circ}\text{C}^{\text{a)}$

Protective substances	Germination ability (%)			
	Period of storage ^{b)}			
	1 day	1 month	3 months	6 months
Powder ^{c)}				
agar	10.8 ^{d)}	7.7	1.7	0.0
skim milk	1.2	2.3	15.2	1.4
polyvinyl alcohol	2.8	0.0	5.3	1.1
Suspension ^{e)}				
10% DMSO+10% skim milk	28.3	36.8	40.8	26.5
D.W.	1.2	0.0	0.6	0.0

a) The preparations of conidia were frozen at -20°C for one day followed by -80°C .

b) Period of storage after freezing at -80°C .

c) Leaves with conidia were put into ampules containing each protective substance.

d) Numbers represent the percentage of germination of conidia in frozen preparations compared with that of the unfrozen control. The values are means of two experiments.

e) Suspended conidia were collected from a part of the leaves which were tested for the effect of the powdery substances.

treatment (Table 5). These results indicated that the 48 hr two-step freezing treatment make it possible to maintain a high germination rate of conidia. Consequently, the pre-freezing treatment at -20°C for 24 hr was employed to preserve the preparations of conidia at -80°C for a long period of time.

During the storage at -80°C , the germination ability of the conidia suspended in 5% DMSO was lost almost within three months. The conidia in the preparations supplemented with 5% DMSO+5% skim milk or 5% DMSO+10% skim milk showed lower germination rates although they were pathogenic to broccoli seedlings after 12 months of storage. On the other hand, the addition of 10% DMSO+5% skim milk or 10% DMSO+10% skim milk induced a remarkable protective effect, and the germination rates of these preparations were 47.7% and 46.9% of the value of the unfrozen control, respectively, suggesting that these values did not decrease appreciably even 12 months after the preservation. The pathogenicity to the broccoli seedlings also remained high (Table 6).

Moreover, we preserved the conidia of the broccoli downy mildew fungus at -80°C by the method of Tetsuka and Katsuya¹⁰⁾ who used agar, skim milk or polyvinyl alcohol powder for the preservation of the hop and vine downy mildew fungi in liquid nitrogen. After six months of storage, the germination rates of the conidia dredged with agar, skim milk or polyvinyl alcohol powder were 0.0%, 1.4% and

1.1%, respectively, values which were significantly low as compared with those of preparations with 10% DMSO+10% skim milk (Table 7).

DISCUSSION

It appeared that the conidia experienced damage in their germination ability by freezing when they were not treated with protectants. In particular, the damage of conidia sustained by freezing at -80°C was more serious than at -20°C , and resulted in the loss of germination ability. However, by the 48 hr two-step freezing treatment at -20°C followed by -80°C the germination ability of the conidia remained as high as that by freezing at -20°C for 48 hr. Moreover, the germination ability of the conidia preserved by the 48 hr two-step freezing treatment sometimes increased compared with freezing at -20°C . It appeared that protectants with a high permeability (DMSO and glycerol) and saccharides (adonitol, sucrose, glucose and sorbitol) were effective as protective substances for the preservation of the conidia by freezing (Table 1). Nevertheless, high concentrations of DMSO, glycerol, glucose and adonitol inhibited the germination of the conidia. Basically, the addition of 5% DMSO or 10% DMSO played an important role in the maintenance of the high germination ability of the conidia (Table 2). The effect of the speed of thawing of the conidia was less important in relation to germination.

During the preservation by freezing at -20°C , the germination rates of the conidia suspended in 5% DMSO which were the highest immediately after freezing decreased significantly after one month of storage. Also, by the addition of 5% skim milk or 5% glucose to 5% DMSO, the germination rates of the conidia remained high only for one month. Consequently, the storage of the conidia at -20°C was not very effective for a long period of time (Table 4). However, it was suggested that the addition of DMSO+skim milk or DMSO+glucose could possibly improve the preservation of the conidia at ultra-low temperatures for a long period of time.

The germination ability of the conidia which were suspended in DMSO and skim milk and preserved at -80°C after pre-freezing at -20°C remained high for a long period of 12 months. The pathogenicity against the broccoli seedlings was also maintained at a high rate. In particular, since the addition of 10% DMSO+5% skim milk or 10% DMSO+10% skim milk induced a highly protective effect on both the germination and pathogenicity of the conidia (Table 6), it was suggested that these protectants may be suitable for the preservation of fungi by freezing.

On the other hand, Tetsuka and Katsuya¹⁰⁾ described a method for the storage of hop downy mildew fungus, *Pseudoperonospora humuli*, and vine downy mildew fungus, *Plasmopara viticola*. In their procedures, the leaflets with conidia were dredged with agar, skim milk or polyvinyl alcohol powder, and preserved in liquid nitrogen. In our experiments, although we preserved the conidia dredged with powdery substances at -80°C instead of using liquid nitrogen, the germination rates of the preparations using these powdery substances decreased markedly during the preservation (Table 7). The storage of conidia suspensions using available protective substances such as 10% DMSO+5% skim milk, 10% DMSO+10% skim milk was superior to the method using powdery substances in the case of storage at -80°C , and was suitable for the conidia of the broccoli downy mildew fungus (*P. parasitica*). On the other hand, in the cases of the conidia suspended in D.W. or 5% skim milk +0.75% sodium glutamate that were treated by freeze-drying or liquid-drying⁶⁾, no germination was observed immediately after these treatments (data not shown). Although Dahmen *et al.*⁸⁾ also reported the use of protectants such as skim milk, glycerol and DMSO, *etc.* for the preservation of phytopathogenic oomycetes fungi such as *Phytophthora*, *Plasmopara* and *Bremia*, they did not apply this method to *Peronospora*. We developed a method for the preservation of the conidia of the broccoli downy mildew fungus treated with 10% DMSO+5% skim milk or 10% DMSO+10% skim milk, which were pre-frozen at -20°C for 24 hr and preserved at -80°C . Thus, it is anticipated that our method may make possible easy and economical preservation of broccoli downy mildew fungus for a long period of time, and could be applied to other fungi with similar physiological characteristics such as *P. parasitica*.

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和 文 摘 要

佐藤 衛・福本文良：-80℃の凍結によるブロッコリーベと病菌分生胞子の保存

純寄生菌であるブロッコリーベと病菌(*Peronospora parasitica*)の分生胞子を蒸留水に懸濁し、-20℃で凍結した場合、発芽率は無処理に比較して著しく低かった。しかし、5% dimethyl sulfoxide (DMSO) および5% skim milk に分生胞子を懸濁した場合には、分生胞子の発芽率はそれぞれ74.8%、32.8%と高く維持された。また、胞子懸濁液を-80℃で直接凍結した場合、発芽率は著しく低下したが、-20℃で24時間凍結後-80℃に移すと、分生胞子の発芽率は-20℃でのみ凍結した懸濁液と同程度に維持された。-20℃で保存した場合、5% DMSO+5% skim milk に懸濁した分生胞子は、わずか1か月で発芽力を失った。しかしながら、10% DMSO+5% skim milk、10% DMSO+10% skim milk を分散媒とした分生胞子の懸濁液を-20℃で凍結した後に-80℃で保存した場合、分生胞子の発芽力は少なくとも12か月間高く維持された。これらの分生胞子では、ブロッコリーの子葉に対しても高い病原性が認められた。これらのことから、ブロッコリーベと病菌の分生胞子は、10% DMSO+5% skim milk または10% DMSO+10% skim milk の分散媒に懸濁したものを-20℃で予備凍結後-80℃で凍結保存することによって長期保存が可能であることが示唆された。