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Potential use of seeds for long-term cryopreservation of oomycete cultures

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Oomycetes are difficult to preserve in culture using ordinary slow freezing on agar disks. Here we tested a vitrification method as an alternative approach for long-term preservation of oomycete strains. Selected seeds were used as a culture substrate before preservation at ultra-low temperature. Rape or sesame seeds were inoculated with oomycete strains, then vitrified and cryopreserved in liquid nitrogen. Thirteen out of 20 strains tested were successfully revived from the cryopreserved infested seeds with a viability of >50%. Viability was better on sesame seeds than on rape seeds. The possibility of cryopreservation using seeds was also investigated for the ordinary slow freezing method. Though viability was not stable among the conditions tested and varied among isolates, the oomycete strains examined were well preserved and showed higher viability when seeds were used rather than agar-culture disks. Also, when infested seeds were used, viability was higher for the slow freezing method than for the vitrification method in most cases. These results suggest that seeds after infestation can be a useful substrate for long-term cryopreservation of oomycetes.

Key words: infested seeds, slow freezing, viability, vitrification

INTRODUCTION

Culture collections are expected to maintain the reproducibility and biological features of their stored microorganisms. Slow freezing has been recognized as one of the most reliable methods currently available for achieving long-term stable storage of microorganisms, including oomycetes such as *Pythium* and *Phytophthora*. Historically, however, oomycetes preserved by the slow freezing method have been shown to exhibit poor viability (e.g. Smith & Ryan, 2012). Viability in cultures preserved by slow freezing has been improved by optimization of several conditions such as the composition of the cryoprotective agents, the rate of cooling, and the rewarming protocol. However, the success of cryopreservation varies widely among oomycete strains (Smith & Onions, 1994; Nishii & Nakagiri, 1991; Houseknecht *et al.*, 2012). At the NARO Genebank Microorganisms Section (MAFF), over 1,200 oomycete strains have been preserved by the slow freezing method using protocols designed for true fungi. The current method for oomycete preservation involves preparation of agar-culture disks for each strain, which are placed in a cryotube with a mixture of 10% (v/v) aqueous glycerol and 5% trehalose, and frozen using a controlled rate of cooling, before transfer and storage in liquid nitrogen vapor (around −170℃). However, even with the use of this technique, many strains are not stored successfully. Improved techniques are therefore needed to ensure long-term preservation of oomycete cultures.

Vitrification is one technique for long-term preservation of various cells and organisms, especially plant cells (Benson, 1994) and human gametes (Mukaida, 2009). This technique involves rapid cooling of samples, rather than a controlled rate of cooling. With the aim of applying this vitrification technique to oomycetes, a previous study has examined the effectiveness of applying rape seeds as a culture substrate, followed by vitrification (Uzuhashi, 2018). As a result, 27 out of 50 strains were, preserved successfully by this technique, and thus rape seeds were considered a desirable substrate for the vitrification method. However, viability varied widely among isolates, even among separate trials of the same strain. In the present study, to establish a more suitable preservation method for oomycetes, we additionally investigated the effectiveness of sesame seeds as a substrate for vitrification. We also demonstrated the effectiveness of seeds for achiev-

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ing better viability after preservation by the ordinary slow freezing method.

EFFECT OF USING SEEDS AS SUBSTRATE IN VITRIFICATION METHOD

First, we investigated the effectiveness of sesame seeds as a new substrate for the vitrification method, in addition to rape seeds. Twenty oomycete strains in our collection, comprising over 15 species, which have frequently shown very low viability (0–25%) by the slow freezing method, were used (Table 1). Each strain was pre-cultured on potato dextrose agar (PDA: Becton, Dickinson and Company, Sparks, MD, USA) at room temperature (ca. 25℃). Rape seeds (Kaneko Seeds Co., Ltd., Maebashi, Japan) and commercially available heat-treated sesame seeds were sterilized by autoclaving twice at 121℃ for 15 min for use as substrates for cryopreservation. Oomycete strains were cultured with the sterilized rape or sesame seeds placed on PDA or corn fish meal agar (CFMA, prepared in accordance with Tojo *et al*., 2012), and incubated at room temperature until use (ca. 2–4 weeks). The infested seeds harvested from PDA or CFMA were then immersed in a loading solution (LS) containing 18% glycerol (w/v) and 30% sucrose (w/v) for 15–20 min. The loaded seeds were then immersed in PVS2 containing 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% dimethyl sulfoxide (w/v) and 15% sucrose (w/v) (Sakai *et al*., 1990). After 20 min, the treated seeds were placed on a cryoplate (Taiyo Nippon Sanso Higashikanto Corp., Hitachi, Japan), and directly plunged into liquid nitrogen in a Dewar flask (around −196℃). After a few minutes, the infested seeds on the cryoplate in liquid nitrogen were quickly rewarmed in 18% (w/v) sucrose solution at around 30℃, transferred to sterilized filter paper for drying, then placed on new PDA plates. Viability was evaluated as the number of seeds showing hyphal regrowth on the plate.

The percentage viability values of the isolates examined are shown in Table 1. Viability varied among conditions for many isolates; for example, *Pythium conidiophorum* MAFF 425320 showed 95.2–100% regrowth from sesame seeds cultured on CFMA, but only 10.0–39.1% regrowth from rape seeds cultured on PDA. Viability was also sometimes unstable among replicates under the same conditions; for example that of *Pythium* sp. MAFF 242215 on sesame seeds cultured on PDA was 90.9% on one occasion but only 0–4.8% at other times, and that of *P. aphanidermatum* MAFF 305857 on rape seeds cultured on CFMA was 92.0% on one occasion but 0% on 2 other occasions. For the two strains, MAFF 242215 and MAFF 305857, the number of days in pre-culture might have been responsible for the differences, i.e., longer culture resulted in higher viability in general. However, in some case viability was sharply declined at the longest pre-culture period possibly due to the overgrowth and aging of mycelia (see footnote ** in Table 1). Although 8 of the 20 strains examined (40%) achieved a viability of >90% for at least one condition, the viability of 13 strains (65%) was >50% under at least one condition. Because only selected strains in our collection that had previously shown very low viability using the slow freezing method were examined in this study, vitrification of oomycetes cultured on seeds might be useful for cryopreservation, especially if the optimum conditions for vitrification can be determined for each strain. On the other hand, among 15 strains for which a few repetitions were tested, an average viability of >50% for at least one condition was achieved for only 8 strains (53%) (Table 1). In order to introduce this technique to obtain more stable results for establishment of a culture collection, further examinations will be needed. Here, we focused only on viability to evaluate the effectiveness of vitrification. To clarify the reasons for the variability in the results, observations of the appearance or behavior of oomycete cells on the treated seeds would be important. Among the different substrates, the highest viability values were achieved using sesame seeds for 15 strains (75%). When compared to rape seeds, sesame seeds were sometimes destroyed by the fungal infection, suggesting that oomycetes might become established on sesame seeds more readily due to easier penetration by mycelia. Our present results indicate that vitrification using infested seeds, especially sesame seeds, is likely to improve the cryopreservation of oomycetes.

EFFECT OF USING SEEDS AS SUBSTRATE IN SLOW FREEZING METHOD

In the second investigation, the possibility of applying infested seeds for the ordinary slow freezing method was examined. Five strains of 5 oomycete species (MAFF 242215, 242227, 305906, 305857 and 425320) were randomly chosen from those in the

Table 1 Viability (%) of all strains after vitrification

Species	MAFF	Days in pre-cultivation	Rape seeds (No. of seeds treated)		Sesame seeds (No. of seeds treated)	
			PDA	CFMA	PDA	CFMA
Pythium aphanidermatum	305857	14	25.0 (12)	0 (13)	0 (11)	0 (10)
		14	10.0 (10)	0 (13)	14.3 (14)	15.4 (13)
		21	21.7 (23)	54.2 (24)	65.0 (20)	55.0 (20)
		28	0 (24)	**92.0***(25)	0 (24)	72.0 (25)
Ave.			14.2**	36.5	19.8**	35.6
Pythium arrhenomanes	511548	14	**26.1***(23)	0 (20)	4.3 (23)	4.5 (22)
Pythium conidiophorum	425320	13	25.0 (12)	27.3 (11)	33.3 (12)	**100*** (11)
		13	10.0 (10)	45.5 (11)	7.7 (13)	**100*** (9)
		28	31.8 (22)	40.0 (25)	91.7 (24)	**100*** (23)
		28	39.1 (23)	80.0 (20)	95.0 (20)	95.2 (21)
Ave.			26.5	48.2	56.9	98.8
Pythium helicoides	242896	14	0 (23)	0 (22)	0 (23)	0 (22)
		24	18.8 (16)	**25.0***(16)	–	–
Ave.			9.4	12.5	0	0
Pythium myriotylum	242159	15	9.5 (21)	20.8 (24)	13.6 (22)	**100*** (23)
		20	13.0 (23)	17.4 (23)	55.0 (20)	40.9 (22)
		21	4.8 (21)	4.5 (22)	10.0 (20)	15.0 (20)
		28	8.3 (24)	0 (24)	0 (24)	73.9 (23)
Ave.			8.9	10.7**	19.7**	57.5
Pythium myriotylum	242227	15	14.3 (21)	0 (21)	36.0 (25)	50.0 (22)
		19	12.5 (24)	4.5 (22)	57.1 (21)	44.0 (25)
		20	4.5 (22)	16.7 (24)	**59.1***(22)	54.2 (24)
Ave.			10.4	7.1	50.7	49.4
Pythium myriotylum	242263	14	0 (12)	0 (12)	**50.0***(12)	8.3 (12)
		14	0 (12)	0 (14)	46.7 (15)	25.0 (12)
Ave.			0	0	48.3	16.7
Pythium oedochilum	712270	14	4.8 (21)	0 (18)	0 (25)	0 (19)
		15	4.5 (22)	9.1 (22)	0 (20)	0 (24)
		16	0 (22)	0 (22)	22.7 (22)	21.7 (23)
		18	4.5 (22)	0 (22)	0 (14)	**33.3***(18)
		21	17.4 (23)	13.6 (22)	–	–
Ave.			6.2	4.5	5.7	13.8
Pythium torulosum	425405	10	71.4 (21)	80.8 (26)	26.3 (19)	19.0 (21)
		17	77.3 (22)	75.0 (20)	**95.2***(21)	85.7 (21)
Ave.			74.4	77.9	60.8	52.4
Pythium ultimum	725014	21	18.2 (22)	22.7 (22)	73.7 (19)	**100*** (21)
Pythium sp.	242215	14	10.5 (19)	16.7 (18)	0 (22)	36.8 (19)
		18	–	–	0 (20)	28.6 (21)
		19	8.0 (25)	8.3 (24)	**90.9***(22)	75.0 (24)
		20	14.3 (21)	34.8 (23)	4.8 (21)	0 (23)
Ave.			10.9	19.9	23.9**	35.1**
Pythium sp.	242216	20	0 (21)	0 (21)	**33.3***(21)	27.3 (22)
Pythium sp.	241119	10	36.4 (22)	31.8 (22)	**40.7***(27)	28.6 (21)
Phytopythium litorale	425402	14	58.3 (12)	0 (12)	0 (12)	16.7 (12)
		14	**81.8***(11)	8.3 (12)	35.7 (14)	0 (14)
Ave.			70.1	4.2	17.9	8.3

* Bold: the highest viability value of each strain among different seed types, **Viability values are declined at the pre-culture period. "–": not tested

Table 1 continued

Species	MAFF	Days in pre-cultivation	Rape seeds (No. of seeds treated) PDA	Rape seeds (No. of seeds treated) CFMA	Sesame seeds (No. of seeds treated) PDA	Sesame seeds (No. of seeds treated) CFMA
Globisporangium sp.	305906	12	0 (22)	27.3 (22)	72.7 (22)	56.5 (23)
		21	9.1 (22)	19.0 (21)	13.6 (22)	16.7 (24)
		24	0 (23)	34.6 (26)	**73.9*** (23)	42.9 (21)
		28	0 (25)	25.0 (24)	70.4 (27)	25.0 (24)
Ave.			2.3	26.5	57.7	35.3
Globisporangium sp.	731159	10	0 (29)	12.9 (31)	**30.0*** (20)	5.0 (20)
Phytophthora cinnamomi	238145	14	4.8 (21)	0 (23)	31.8 (22)	13.6 (22)
		16	18.2 (22)	63.6 (22)	**91.7*** (24)	33.3 (21)
Ave.			11.5	31.8	61.7	23.5
Phytophthora cryptogea	242880	14	35.0 (20)	52.2 (23)	4.3 (23)	52.4 (21)
		18	–	–	**80.0*** (20)	25.0 (20)
Ave.			35.0	52.2	42.2	38.7
Phytophthora palmivora	245856	15	4.3 (23)	0 (30)	**13.0*** (23)	0 (26)
		15	4.0 (25)	0 (24)	0 (22)	0 (27)
Ave.			4.2	0	6.5	0
Achlya flagellata	305742	15	0 (22)	4.3 (23)	0 (24)	0 (23)
		20	22.7 (22)	**95.5*** (22)	0 (20)	66.7 (21)
Ave.			11.4	49.9	0	33.3

* Bold: the highest viability value of each strain among different seed types, **Viability values are declined at the pre-culture period. "−": not tested

first experiment described above (Table 1). The strains were cultured simply on PDA or CFMA, or with sterilized rape or sesame seeds on PDA or CFMA. Two to four weeks later mycelial disks, and rape or sesame seeds, harvested from the PDA or CFMA were placed separately into sterile 2-m*l* screw-cap cryotubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) containing 1 m*l* of a mixture of 10% (v/v) aqueous glycerol and 5% trehalose as a cryoprotectant. The cryotubes were then placed in the freezing chamber of a programmable freezer (TNP-87S-DX, Nihon Freezer, Tokyo, Japan) and cooled to 10℃ at a rate of 3℃ per min, then from 10℃ to −50℃ at a rate of 1℃ per min, and finally from −50℃ to −80℃ at a rate of 5℃ per min. After reaching −80℃, the cryotubes were transferred to the −80℃ deep freezer and kept at that temperature for more than 24 h, then transferred rapidly into liquid nitrogen vapor (around −170℃). After storage for a few weeks, the cryotubes containing the agar disks or infested seeds were thawed in a water bath at 50℃ for 3 min, and then the contents were placed or scattered on PDA plates. Viability was evaluated as the number of disks or seeds showing hyphal regrowth on the plate.

The results obtained with the slow freezing method using selected strains with 2 or 3 replications are shown in Table 2. In two strains, *P. aphanidermatum* MAFF 305857 and *P. conidiophorum* MAFF 425320, the average viability exceeded 60% when infested seeds were used under all conditions. Although viability exceeded 60% when mycelial agar disks were used as well, except for MAFF 305857 on CFMA disks, viability was higher when infested seeds were used for these two strains. For *P. myriotylum* MAFF 242227, viability seemed to be related to the culture media, i.e., the average viability was higher when the substrates were cultured on PDA. In other two strains, *Pythium* sp. MAFF 242215 and *Globisporangium* sp. MAFF 305906, an average viability of >60% was achieved only by using infested sesame seeds on PDA. For all of the strains tested, the average viabilities obtained using infested seeds were almost the same or even higher than those obtained using mycelial agar disks (Table 2), i.e., all strains were more successfully preserved on infested seeds than on mycelial agar disks. When viability was compared between rape seeds and sesame seeds on the same culture medium (PDA or CFMA), sesame seeds provided better viability in all strains. One exception was observed for MAFF 242227 *P. myriotylum* on PDA, although the viability on rape and sesame seeds was almost same, i.e. 69.6% and 64.6% respectively (Table 2). Sesame

Table 2 Viability (%) of 5 strains by slow freezing method and vitrification method

Species	MAFF	Days in pre-cultivation	Slow freezing (No. of seeds treated)						Vitrification (No. of seeds treated)			
			Agar disks		Rape seeds		Sesame seeds		Rape seeds		Sesame seeds	
			PDA	CFMA	PDA	CFMA	PDA	CFMA	PDA	CFMA	PDA	CFMA
Pythium aphanidermatum	305857	21	40.0 (10)	0 (10)	**81.8** (22)	**78.3** (23)	**100** (24)	**100** (21)	21.7 (23)	54.2 (24)	**65.0** (20)	55.0 (20)
		28	**100** (10)	30.0 (10)	**80.0** (25)	**100** (24)	**64.0** (25)	**100** (28)	0 (24)	**92.0** (25)	0 (24)	**72.0** (25)
Ave.			**70.0**	15.0	**80.9**	**89.2**	**82.0**	**100**	10.9	**73.1**	32.5	**63.5**
Pythium conidiophorum	425320	21	**100** (10)	30.0 (10)	**90.9** (22)	59.1 (22)	**100** (25)	**100** (23)	30.4 (23)	59.1 (22)	0	0
		28	0 (10)	**60.0** (10)	0 (23)	45.0 (20)	22.7 (22)	**73.9** (23)	31.8 (22)	40.0 (25)	**91.7** (24)	**100** (23)
		28	**80.0** (10)	**100** (10)	**100** (28)	**90.0** (20)	**100** (26)	**100** (20)	39.1 (23)	**80.0** (20)	**95.0** (20)	**95.2** (21)
Ave.			**60.0**	**63.3**	**63.6**	**64.7**	**74.2**	**91.3**	33.8	59.7	**62.2**	**65.1**
Pythium myriotylum	242227	20	**60.0** (10)	10.0 (10)	**79.2** (24)	21.4 (28)	**62.5** (24)	38.1 (21)	12.5 (24)	4.5 (22)	57.1 (21)	44.0 (25)
		21	**80.0** (10)	30.0 (10)	**60.0** (25)	20.8 (24)	**66.7** (24)	19.4 (31)	4.5 (22)	16.7 (24)	59.1 (22)	54.2 (24)
Ave.			**70.0**	20.0	**69.6**	21.1	**64.6**	28.8	8.5	10.6	58.1	49.1
Pythium sp.	242215	20	10.0 (10)	**100** (10)	24.0 (25)	25.9 (27)	**95.8** (24)	57.7 (26)	8.0 (25)	8.3 (24)	**90.9** (22)	**75.0** (24)
		21	0 (10)	0 (10)	9.1 (22)	13.0 (23)	43.5 (23)	31.0 (29)	14.3 (21)	34.8 (23)	4.8 (21)	0 (23)
Ave.			5.0	50.0	16.6	19.5	**69.7**	44.4	11.2	21.6	47.9	37.5
Globisporangium sp.	305906	12	0 (10)	0 (10)	0 (23)	30.4 (23)	44.0 (25)	13.0 (23)	0 (22)	27.3 (22)	**72.7** (22)	56.5 (23)
		24	0 (10)	10.0 (10)	**62.5** (24)	26.1 (23)	**60.9** (23)	31.8 (22)	0 (23)	34.6 (26)	**73.9** (23)	42.9 (21)
		28	0 (10)	**80.0** (10)	8.3 (24)	**62.5** (24)	**79.2** (24)	**88.0** (25)	0 (25)	25.0 (24)	**70.4** (27)	25.0 (24)
Ave.			0	30.0	23.6	39.7	**61.4**	44.3	0	29.0	**72.3**	41.5

Bold: the over 60% viability value

seeds showed similar effectiveness in the vitrification method. On the other hand, viability was higher for the slow freezing method than for the vitrification method in most cases (Table 2). Based on these results, it can be suggested that slow freezing using infested seeds is the most suitable method for preservation of oomycetes.

CONCLUSION

Vitrification using infested seeds has been examined to establish a new method for oomycetes that can replace slow freezing using a programmable freezer. As in a previous study (Uzuhashi, 2018), more than half of the strains we examined here showed a viability of >50% using the vitrification method under at least one condition, although the viability varied widely among both the strains and the conditions employed. If a vitrification method can be established as an alternative approach for long-term preservation of oomycetes, use of an expensive programmable freezer for precise adjustment of the freezing rate may become unnecessary, thus reducing cost and labor, especially for culture collections. On the other hand, our present results suggest that the viability obtained using slow freezing can be improved by changing the substrate from mycelial disks to infested seeds. This suggests that damage to the fungal cells caused by freezing might be reduced as a result of oomycete penetration into the seeds. Additionally, the viability obtained using the slow freezing method was almost the same or even higher than that of vitrification for the tested strains. On the other hand, some strains exhibited differences in viability depending on the culture media and types of seeds. Interestingly, cryopreservation with both types of seeds tended to result in higher viabilities than without seeds. Therefore, when infested seeds, especially sesame seeds and mycelial agar disks are preserved together in the same tube, more stable viability would be expected in a single preservation trial. In any event, the present study has demonstrated the potential use of seeds for long-term preservation of oomycete species.

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卵菌類培養株の長期保存における種子利用の可能性

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卵菌類は，糸状菌では一般的な含菌寒天片を基質とした緩慢凍結法による超低温保存が困難な微生物として知られている．そこで，その代替方法として期待されるガラス化法による超低温保存の効果を，緩慢凍結法での生残率が特に低い 20 菌株を用いて検討した．各供試菌株は，滅菌処理したナタネ種子およびゴマ種子とともに培養し，菌株が感染・定着した種子を保存基質として用いた．これらの種子をガラス化法により処理し，液体窒素で急速冷却し，保存した結果，13 菌株で 50%以上の生残率が得られ，特にゴマ種子においてより高い生残率が認められた．この結果から，感染種子の基質としての有効性が期待されたため，これら感染種子を用いた緩慢凍結法による超低温保存についても検討した．その結果，生残率は種子の種類や反復間で不安定であり，菌株間でも異なっていたが，従来の含菌寒天片を用いた場合と比較して，同程度あるいはより高い値を示した．これらの結果から，卵菌類の超低温保存における，感染種子の基質としての有効性が示された．