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Cryopreservation of Pine Wood Nematode, *Bursaphelenchus xylophilus*

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Abstract ; Pine wood nematode isolates from Haman, Gyoungsangnam-do were cryopreserved with three different cryoprotectants (glycerol, ethylene glycol and dimethylsulfoxide), Nematode Growth medium (NGM) and M 9 buffer solution, respectively, and then survival rate, reproduction ability, and pathogenicity of stored nematodes were compared. Survival rates of juvenile was excellent in 15% glycerol solution as 72±5.3%. The survival ability of nematodes at juvenile stage was more efficient than that of nematodes at adult stage. Pre-incubation treatment at 4°C before storing in liquid nitrogen didn't affect the survival ability of pine wood nematode. When the reproduction ability of nematodes was confirmed on *Botrytis cinerea* agar plates at 28°C incubator, there was no difference between cryopreserved and non-cryopreserved. Pathogenic ability of cryopreserved nematodes was also evaluated and confirmed by artificially inoculating them on 2-year old red pine seedlings.

Key words : *Pine wood nematode,* Bursaphelenchus xylophilus, *Cryopreservation*

Introduction

The benefits of indefinite storage of biological material have been recognized for several decades. Initially, maintaining a specimen or germline for long periods was the major reason for cryopreservation (Galway and Curran, 1995). Many parasitic and free-living nematodes have been cryopreserved with varying success, using cryoprotective agents (dimethylsulfoxide, ethanediol, glycerol) or partial dehydration approaches that are believed to induce a cryoprotectable state in nematodes (Curran *et al.*, 1992; Galway and Curran, 1995; James, 1981; Popiel and Vasquez, 1991; Sayre and Hwang, 1985; Triantaphyllou and McCabe, 1989). In the present work, the partial dehydration method that was successfully applied to entomopathogenic nematodes (Curran *et al.*, 1992; Popiel and Vasquez, 1991) was evaluated for cryopreserving the plant-parasitic lesion nematodes (Galway and Curran, 1995). Different organisms usually require different protocols for pretreatment and speed of cooling and thawing, and these protocols are established empirically. Successful cryopreservation in liquid nitrogen has been reported for free-living and mycophagous nematodes of the genera *PanagreUus, Turbatrix, Caenorhabditis,* and *Aphelenchoides* using dimethylsulfoxide (DMSO) as a cryoprotectant (Hwang, 1970). The same cryoprotectant was not as successful with juveniles of the plant-parasitic nematode *Ditylenchus dipsaci* and was unsatisfactory for second-stage juveniles (J2) of a *Meloidogyne* spp. (Sayre and Hwang, 1985). A solution of glycerol with certain salts is routinely used for cryopreservation of *Caenorhabditis elegans* (Sulston and Hodgkin, 1988), but the method is not effective for preservation of *Meloidogyne* spp.. Ethylene glycol has been known as an efficient cryoprotectant at least since 1952 (Luyet and Keane, 1952).

More recently, it has become a key tool in recording and maintaining genetic diversity in a given population. Cryopreservation has become a reliable and effective means of maintaining lines and is essentially a readily accessible, viable database. It can save time for the continuous culturing of organisms, eliminates the recurring problems of loss of lines through infection or cross-contamination, and ensures the availability and uniformity of material or lines for ongoing research (Galway and Curran, 1995). Thus, to develop a long term storage technique for the pine wood nematode, cryopreservation technique using several cryoprotectants was applied to the pine wood nematode at the different concentrations, and their survival rates, reproduction ability, and pathogenic ability after storage in the liquid nitrogen were

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evaluated and compared.

Materials and Methods

1. Isolation and culture

Pine wood nematode (PWN; *Bursaphelchus xylophilus*) was isolated from the infected wood tissue collected at Haman area, Gyoungsangnam-do, 2004. Isolated PWNs were transferred and reared on *Botrytis cinerea* cultured on potato dextrose agar at 28°C incubator. Twelve to fourteen days later, juvenile and adult nematodes were extracted from *B. cinerea* culture medium by Baermann funnel method (Chawla and Prasad, 1975; Viglierchio and Schmit, 1983). The extracted nematodes were washed three times throughly with sterile distilled water.

2. Cryopreservation

The nematodes were transferred in each cryotube containing one of various concentrations of cryoprotectant or other solution, i.e., glycerol (5, 10, 15, 20%), ethylene glycol (5, 10, 15, 20%), dimethylsulfoxide (0.5, 1.0, 1.5, 2.0%), M 9 buffer solution (25, 50, 100%) and nematode growth medium (50, 100%). Cryotubes were pre-incubated for 3, 6, 12, 24 hours at 4°C, and subsequently cryopreserved in liquid nitrogen. Approximately 1×10^3 nematodes per 300 μl cryoprotectant were incubated into for each treatment. In the mixtures of juvenile, mature male and female nematodes, mature male were determined when they have spicule in their tail part; mature female were determined when they don't have spicule in their tail part and their body is longer than that of mature male; the others were considered as juvenile.

3. Thawing and survival rate evaluation

Survival rates of stored nematodes were evaluated by determining the percentage of motile nematodes after thawing (the mean of 3 replicates of 60 observations for each treatment). Nematodes were determined as dead if their body was straight and they did not move, even after mechanical prodding. Thawing was proceeded in 50~60°C water bath rapidly.

4. Reproduction ability and pathogenicity test

Cryopreserved nematodes were reared (50 and 100 nematodes/10 ul) on *B. cinerea* agar plates and the populations were evaluated after two weeks. Pathogenicity test was conducted by inoculating 3×10^3 nematodes for each part at both upper and lower parts of 2-year old red pine seedlings, and maintaining in the growth chamber at 28°C with 70% relative humidity.

Results and Discussion

1. Effects of cryoprotectants

Generally, the storage in glycerol showed relatively high

Table 1. Survival rates of *Bursaphelenchus xylophilus* after storage in liquid nitrogen for 1 week.

Cryoprotectant (%)		Survival rates (%)					
		Female		Male			
Glycerol	5	22.0±5.7	bcd**	26.3±7.3	bcd	57.6±0.5	bc
	10	24.0±4.6	bcd	27.0±2.2	bcd	59.8±4.3	bc
	15	36.2±3.4	a	41.6±1.9	a	72.0±5.3	a
	20	17.1±4.0	bcd	31.8±4.9	bcd	47.9±3.4	d
Ethylene glycol	5	4.3±3.7	efghi	8.4±3.0	efghijklm	16.3±2.8	ghijk
	10	2.6±4.4	efghi	6.8±0.3	efghijklm	12.0±2.3	hijklmn
	15	2.4±4.1	efghi	4.3±3.7	efghijklm	9.4±2.0	jklmno
	20	2.1±3.6	efghi	4.3±3.7	efghijklm	11.2±1.5	jklmno
DMSO	0.5	2.6±4.4	efghi	7.6±0.8	efghijklm	22.5±1.5	ef
	1	2.4±4.1	efghi	6.7±0.4	efghijklm	17.4±5.0	fghi
	1.5	2.0±3.4	efghi	6.4±0.2	efghijklm	12.6±4.5	hijklmn
	2	2.0±3.4	efghi	4.4±3.8	efghijklm	8.9±4.6	jklmno
M 9 buffer	25	2.1±3.6	efghi	2.8±4.8	efghijklm	8.0±2.3	jklmno
	50	0.0		2.2±3.8	efghijklm	10.8±1.9	jklmno
	100	0.0		0.0		5.7±2.0	jklmno
NGM	50	0.0		0.0		0.0	
	100	0.0		0.0		0.0	

Each value indicate means ± standard deviation of three replicates of 60 observations for each treatment. DMSO: Dimethylsulfoxide, NGM: Nematode Growth Medium.

**The same letters in each column are not significantly different (p=0.05) by Duncan multiple range test.

survival rates when compared with other cryoprotectants or solutions. The 15% glycerol solution showed the best results for female, male, and juvenile nematodes. The highest survival rate of stored nematode was shown as 72% at the juvenile stage. Ethylene glycol and DMSO did not showed any significant differences, but the nematodes could not recovered from the storage in NGM. The survival ability of nematodes at juvenile stage was more efficient than that of adult stage (Table 1).

2. Effects of pre-incubation

Different periods of pre-incubation at 4°C before cryopreservation didn't affect the survival ability of pine wood nematodes after the storage in liquid nitrogen. The survival rate of nematodes without pre-incubation was rather higher than other treatments, and the rates were decreased as the pre-incubation periods are increased. The survival rates at the juvenile stage were always higher than either male or female stage within the same pre-incubation period (Figure 1).

3. Reproduction ability and pathogenicity

After cryopreservation, the reproduction ability was still maintained when the nematodes were reared on *B. cinerea* agar plates at 28°C incubator, and there was no difference in the reproduction ability between cryopreserved and non-cryopreserved nematodes (Figure 2).

Cryopreserved nematodes have kept the pathogenicity when these were inoculated on 2-year old red pine seedlings. The needles of inoculated pine seedlings were wilted 10 days after inoculation, and they finally died at 40 to 50 days after inoculation (Figure 3).

This cryopreservation method seems to be more cost-effective and reliable in maintaining mass cultures of *B. xylophilus* than subculturing on *B. cinerea* cultures.

However, the survival rate of stored nematodes was

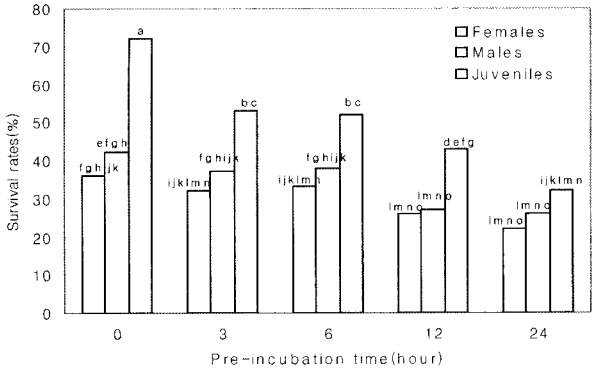

Figure 1. Effects of pre-incubation periods on the survival rates of nematodes after cryopreservation in liquid nitrogen. The same letters on the bar are not significantly different (p=0.05) by Duncan multiple range test.

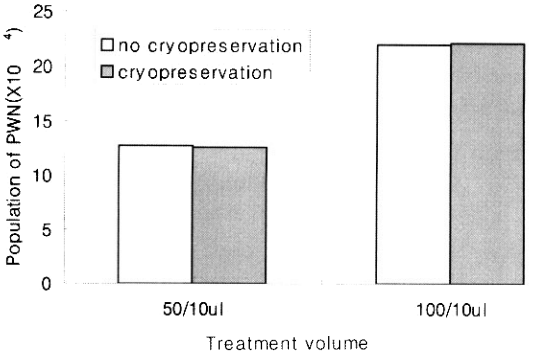

Figure 2. Comparison of the reproduction ability between cryopreserved and non-cryopreserved nematodes. LSD0.01=1158.55 for treatment volume 50/10 μl. LSD0.05=414.85 for treatment volume 100/10 μl.

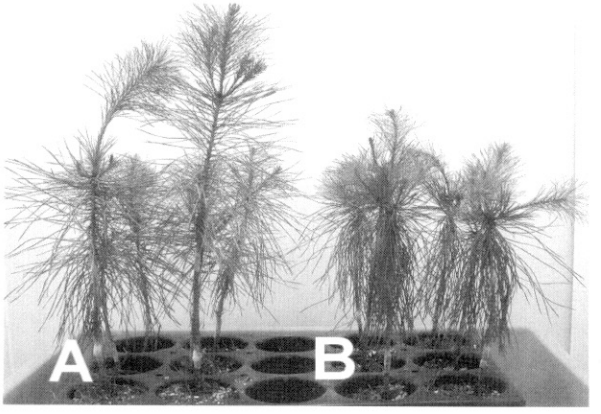

Figure 3. Comparison of 2-year old red pine seedlings. (A) Un-inoculated seedlings, (B) Inoculated seedlings with cryopreserved nematode. The photo was taken 32 days after inoculation.

shown to be relatively low in comparison with the results from other plant-associated nematode experiments (Bridge and Ham, 1985; Curran *et al.*, 1992; Galway and Curran, 1995; Popiel and Vasquez, 1991; Triantaphyllou and McCabe, 1989). To increase the survival ability of nematodes, quick handling of all materials are basically required in each step of storage processes (Popiel and Vasquez, 1991). The concentration of cryoprotectant and incubation time could be changed to optimize the survival rate of all species and isolates of nematodes (Curran *et al.*, 1992). But, in this study, the pre-incubation time did not affect the survival rates. Curran *et al.* (1992) also reported that the higher survival rate was also noted when round-bottomed cryotubes were used rather than conical-bottomed, and cryoprotectant aliquots of <50 μl were frozen. In addition, in further experiments, the appropriate populations of nematodes stored in each cryotube and the use of effective cryoprotectants are needed and it is also necessary to combine efficiently these two factors. (Popiel and Vasquez,

1991; Galway and Curran, 1995).

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