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Original research article

Investigation of Diverse Cryopreservation Techniques for Long Term Storage of Coffee Leaf Rust *Hemileia vastatrix*

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ABSTRACT

Coffee leaf rust (CLR) is the most devastating disease in coffee (*coffea* sp.) and caused by the rust fungus *Hemileia vastatrix*. Currently, there are several studies on developing disease resistance varieties. Thus, long-term live storage of *H. vastatrix* is necessary. *Hemileia vastatrix* is a biotroph pathogen, which requires nutrients from living host cells and cannot be cultivated *in vitro*. However, to continue culturing CLR in living plants is not only labor intensive, but also not cost effective. Therefore, three cryopreservation techniques and two storage temperatures were investigated on three potential CLR isolates – DC204, DC404 and DC604 – collected from Chiang Rai province, Thailand. Although, all cryopreservation techniques failed to provide long term storage of *H. vastatrix, in situ* cryopreservation at -20 ºC showed potential for short term storage of this pathogen. Thus, this technique could be used as an alternative to *in vivo* culturing for maintaining pathogenic isolates in the laboratory.

Keywords: Coffee leaf rust; Cryopreservation; *Hemileia vastatrix;* Viability

1. Introduction

Coffee is produced in more than 80 countries. In 2016, worldwide production of coffee was nine million tons [1]. Sixty-five percent of the worlds' coffee production is arabica coffee (*Coffea arabica*), while the rest is robusta coffee (*C. canephora*). Aromatic types of arabica coffee beans provide fine-flavored coffee, which can be reminiscent of several aromas, such as, flower, fruit, honey, chocolate, caramel or toasted bread. Due to superior quality and

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testing, arabica coffee sales are more highly priced compared with those of robusta coffee. However, arabica coffee is highly susceptible to major diseases especially coffee leaf rust [2].

Coffee leaf rust (CLR) is the most devastating disease for arabica coffee cultivation worldwide. This disease is caused by *Hemileia vastatrix*, and mainly affects old leaves. The disease causes leaves to fall off resulting in a decrease of the photosynthesis rate in the infected plant. Thus, the plant cannot accumulate energy or store the appropriate resources for fruit production, which results in major yield losses [3]. The loss of yield from this fungus is about 40 % and it is possible to increase to 70% in severe cases (when young leave buds or fruit get infected) [4,5]. This has a huge impact on coffee production especially in the case of *C. arabica* [5,6]. Moreover, CLR outbreaks not only affect current yields, but also will most likely affect subsequent harvests.

Start Codon Targeted (SCoT) polymorphism, a recently developed molecular marker system, is gaining popularity in the genetic study of plants and fungi. The system is especially useful in wild populations, due to its higher polymorphism and better marker resolvability compared with several other marker systems [7]. The SCoT system is based on the short conserved region in genome surrounding the ATG translation initiation codon. The SCoT marker uses single primers designed from conserved regions, which means it shares similarities with RAPD or ISSR markers. However, SCoT is an effective supplement to the original widely used marker systems (RAPD and ISSR). Therefore, SCoT marker can be widely applied to various study fields including genetic diversity of agricultural and horticultural crops, wild plant species and fungi, studies in population structure or genetic relationships and revealing the geographical origins of cultivars [7,8,910]. To date, the marker has been used successfully in both plants and fungi, including rice (*Oryza sativa*) [11], mango [10], peanut (*Arachis hypogaea* L) [8], ramie (*Boehmeria nivea* L. Gaudich) [12], longan [13], citrus [14], mushroom (*Pleurotus eryngii* var. *tuoliensis*) [15], and ectomycorrhizal fungi on Scots pine [16].

The costs of fungicides to control CLR are extremely high and not environmentally friendly. Thus, the most effective way to protect coffee from CLR is through the use of resistant varieties. Therefore, breeding for rust-resistant varieties is indeed important and is becoming a priority in all coffee cultivating areas. Success in breeding for rust resistance in arabica coffee depends on two major factors; having a good source of resistance and dependable criteria of disease evaluation [17,18]. Currently, the International Coffee Collection (CATIE) under Tropical Agricultural Research and Higher Education center in Brazil has over 1900 cultivars of coffee in their collections. However, the one major constraint for the breeding program is lack of reliable criteria of disease evaluation. Since *H. vastatrix* is a biotroph-type pathogen, it is requires nutrients from living host cells and cannot be cultivated *in vitro*. However, to continue culturing CLR in living plant is not only labor intensive but also expensive. Therefore, long-term storage of *H. vastatrix* is necessary, especially for coffee breeding programs. In our study, three approaches of cryopreservation of this pathogen were investigated. Finding an appropriate cryopreservation method will benefit the CLR disease resistant breeding program in the future.

2. Materials and Methods

2.1 Collecting of *Hemileia vastatrix*

Hemileia vastatrix were randomly collected from four arabica coffee cultivation areas in Chiang Rai, Thailand as follows: DC 101 at 19°46'37.2"N

99°33'09.3"E, DC 202 at 19°48'59.1"N 99°34'04.6"E, DC 404 at 19°50'02.0"N 99°34'27.6"E and DC 604 at 19°52'12.3"N 99°29'01.8"E. Infected leaves that showed high density of rust pustules were placed in zip lock bags. Scanning electron microscopy (SEM) was used to observe urediniospores at 500X magnification. Subsequently, viability tests were performed on the urediniospores at the laboratory in Mae Fah Luang University as follows; aliquots of five mg of urediniospores were placed in 5 ml of sterilized water in 15 ml falcon tubes and mixed by vortexing for 10 seconds. One ml of spore suspension was spread uniformly on 2 % agar in petri plates under sterile conditions (4 replicates). Agar plates were sealed with parafilm and incubated for 48 hr at 23 ºC in the dark. Four pieces of agar (1 cm*1 cm) were randomly cut from each plate and then placed on microscopic slides. Germinating and non-germinating spores on each piece of agar were observed and counted under the microscope at 10X magnification.

2.2 Assessment of CLR genetic diversity

DNA of *H. vastatrix* was extracted in the laboratory as previously described [19]. Fifteen mg of urediniopores were ground in 200 µl of extraction buffer (3% SDS (w/v), 0.5 mM EDTA, 1 M NaCl and 0.1 M Tris-HCl, pH 8.0). Then 200 µl of 1:1 chloroform:phenol were added and mixed thoroughly by hand. The tubes were incubated at 65 ºC for 5 min, and then let cool at room temperature. The tubes were centrifuged at 10000 g at 4 ºC for 5 minutes. The supernatants were then transferred to clean tubes without disturbing the interface. An equal volume of cold 100 % isopropanol was added, mixed thoroughly and incubated at -20 ºC over night to enhance precipitation of total DNA. The tubes were spun again at 10000 g at 4 ºC for 10 minutes. Five hundred µl of cold 75% ethanol were gently overlaid over the pellet and the tubes were spun again at 10000 g at 4 ºC for 15 minutes. The DNA pellet was air dried and dissolved in 30 µl of sterile dd H_20. DNA was quantified by using nanodrop.

The 11 SCoT primers that were tested for their ability to produce clear and reproducible profiles are shown in table 1. The PCR mix consisted of a total volume of 20 µl containing 2 µl of 10X Taq polymerase buffer, 0.25 mM dNTPs, 0.5 mM of each primer, 1 U of *Taq* DNA polymerase, and 20 ng of the template DNA. The PCR reaction was performed using the following thermal cycling protocol: 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min followed by a final extension step at 72°C for 10 min. The SCoT products were resolved through electrophoresis on 1.8% agarose gels.

Table 1. List of SCoT markers and their sequences that were used in this study [11]

Primer name	SCoT primer sequence (5′-3′)	% GC
ScoT11	AAGCAATGGCTACCACCA	50
ScoT12	ACGACATGGCGACCAACG	61
ScoT13	ACGACATGGCGACCATCG	61
ScoT14	ACGACATGGCGACCACGC	67
ScoT15	ACGACATGGCGACCGCGA	67
ScoT16	ACCATGGCTACCACCGAC	56
ScoT17	ACCATGGCTACCACCGAG	61
ScoT18	ACCATGGCTACCACCGCC	67
ScoT19	ACCATGGCTACCACCGGC	67
ScoT20	ACCATGGCTACCACCGCG	67
ScoT21	ACGACATGGCGACCCACA	61

2.3 Cryopreservation of CLR

The following three cryopreservation techniques were tested for their ability to preserve urediniospores of *H. vastatrix*: 1) preserving 10 mg of urediniospores in 1.5 ml microfuge tubes [20] and stored at -20 ºC and -80 ºC, 2) drying infected leaves by placing them between newspapers at room temperature for one week. The dry infected leaves were examined for sporulation spots, which were cut in pieces and placed in silica

gel and then stored at -20 ºC and -80 ºC and 3) *in situ*, whereby aliquots of 10 mg of dry urediniospores were stored into 1.5 ml of microfuge tubes along with 200 µl of glycerol and stored at -20 ºC. Viability tests were performed before storage and monthly intervals until no viability was found. The viable percentage of each month was calculated by the following equation

$$\frac{(\text{number of germinate/number of non-germinated spore}^{a*}) \times 100}{(\text{number of germinate/number of non-germinated spore}^{b**})}$$

where; a* is data from each month after storage and b** is data from viability test before storage.

2.4 Statistical analysis

The results were analyzed by factorial-CRD analysis of variance with two factors including storage technique and storage temperature (-20 ºC and -80 ºC) and viable percentage as the independent variable. All statistical analyses were performed using The Statistical Package for the Social Sciences (SPSS).

3. Results and Discussion

3.1 Genetic diversity of CLR

Four samples of CLR were collected from different locations of arabica plantations at Doi Chang, Chiang Rai and observed under SEM. All infected leaves had CLR pustules that showed clustering of urediniospores and no evidence of teliospores (Fig. 1.). Regrettably, DC 101 showed evidence of contamination with another fungus and was excluded from the study. Therefore, only three samples (DC202, DC404, and DC604) were further analyzed with SCoT markers. In this study, five out of eleven SCoT markers produced clear and reproducible profiles with DNA from CLR. The three CLR samples had different patterns with the five above-mentioned SCoT markers (Table 2.). These data suggested that the three CLR used in this study are very likely genetically different isolates indicating occurrence of more than one isolate in the same location. Further analysis would require obtaining pure cultures of CLR and applying more SCoT markers.

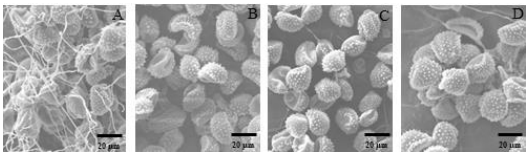

Fig. 1. Urediniospores of *H. vastatrix* under SEM A) DC 101; B) DC202; C) DC404 and D) DC604.

Table 2. The alleles generated by using five SCoT markers in three isolates of CLR

SCoT	CLR	Allele				
		1	2	3	4	5
SCoT13	DC202	-	+	-	-	-
	DC404	+	-	+	+	+
	DC604	+	+	+	-	-
SCoT14	DC202	-	-	+	-	-
	DC404	-	+	+	-	+
	DC604	+	+	+	+	+
SCoT15	DC202	-	+	-	+	+
	DC404	+	-	+	+	-
	DC604	+	-	+	+	+
SCoT19	DC202	-	+	+	+	+
	DC404	+	+	+	+	-
	DC604	+	-	-	+	-
SCoT21	DC202	+	-	+	+	+
	DC404	-	+	-	-	+
	DC604	+	-	-	+	-

3.2 Cryopreservation of CLR

Cryopreservation is a technique allowing for long term and stable storage of important microorganisms. Thus, it is often used to maintain fungal collections for several research purposes such as genetic diversity, genetic variability and population structure or breeding programs for disease resistance. Three preservation procedures and two storage temperatures (-20 ºC and -80 ºC) were studied for their effect on long

term storage of CLR. One of the procedures was using glycerol as cryoprotectant. Glycerol along with trehalose and DMSO have been reported to reduce the risks of cryoinjury [21]. On the other hand, liquid nitrogen, a commonly used cryopreservative, was not included in this study because of its high cost and risk, if safety systems are not in place.

In general, storage technique, storage temperature, as well as, technique and temperature interaction were statistically significant. Interestingly, storage temperature played an important role in maintaining spore viability for all three storage techniques. The CLR stored at -80 ºC had significantly lower spore viability when compared with the one stored at -20 ºC (Fig. 2.). This is possibly due to the formation of a high amount of ice crystals during storage at -80 ºC. The ice could cause physical damage by rupturing the cell membranes of microorganisms. Spore viability of dry urediniospores derived from dried leaves was in the range of 10.32 to 18.06 % one month after storage in both storage temperatures (Fig. 2.). Several factors can cause cryoinjury of dry urediniospores including dissolved gases, electrolyte concentration, intracellular crystallization resulting in loss of water from macromolecules, and cell shrinkage [22]. Meanwhile, glycerol, which functions as a cryoprotectant, could protect from cryoinjury of CLR for only one month. Spore viability from urediniospores that were stored in glycerol was not maintained spore at two months after storage (Fig. 2.).

Taken together, the data suggest that rust fungi were preserved better in the dry storage condition. Though research has shown skim milk-glycerol (8.5% + 10%) and DMSO (15%) are excellent cryoprotectants for 30 phytopathogenic fungi, this is not the case for rust fungi, which require dry conditions [23]. Thus, these two approaches are not recommended for cryopreservation of CLR.

On the other hand, *in situ* cryopreservation provided the longest storage period for CLR. This is more likely because no disruptions occur on urediniospores before storage. Typically,

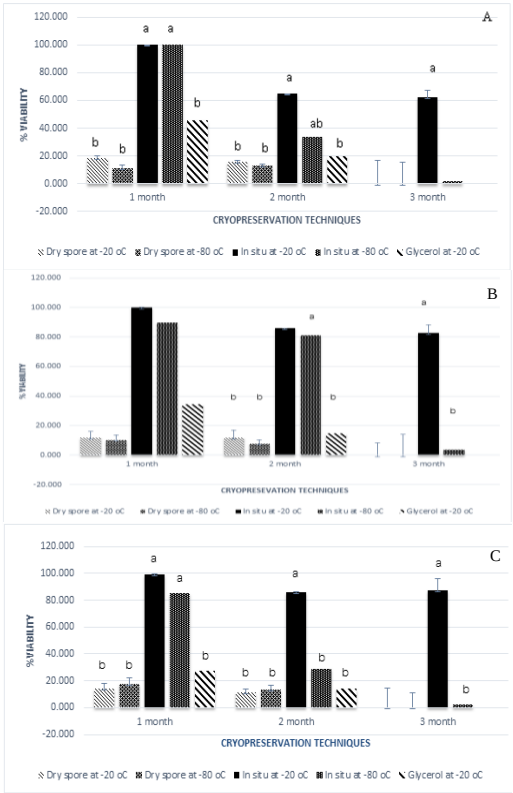

Fig. 2. Percent viability of A) DC204 B) DC 404 C) DC604 from one to three months after storage**.**

urediniospores contain fine spines over their entire surface (Fig. 1.), which are disrupted when razor blades are used to separate urediniospores from infected leaves. Using *in situ* cryopreservation does not cause physical damage of cell membranes before storage; thus, the observed decrease in spore viability is a result of long term storage. As shown in Fig. 2., spore viability from urediniospores derived from *in situ* cryopreservation at -20 ºC was 62.41 to 87.46 % at three months after storage (Fig. 2.). This is similar to other findings, whereby, *in situ* cryopreservation was also the only method that had potential for long

term cryopreservation for 10 isolates of another rust fungus, *Puccinia spegazzinii* [24]. These data suggest that *in situ* cryopreservation at -20 ^{o}C is appropriate for maintaining spore viability for at least three months**.**

4. Conclusion

Despite the successful of in situ cryopreservation to maintain spore viability, this approach could not be used for long term storage of *H. vastatrix.* However, in situ cryopreservation that store in -20 ^{o}C show potential for short term storage of this pathogen. Thus, this cryopreservation approaches, can be beneficial in maintaining isolate of *H. vastatrix* in laboratory by alternate this technique with *In vivo* culture.

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