Effect of culture medium and time storage on pollen germination and viability of *Hevea* clones

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ABSTRACT: The preservation of pollen rubber clones RRIT 251, PB 235, RO/C/9 23/419 and RO/A/7 25/114 in liquid nitrogen was investigated based on pollen viability during storage and using pollen staining and culturing in artificial media. Whole, male flowers at the blooming stage were stored in liquid nitrogen for 180 days. Pollen viability was assessed after storage every 15 days using staining and culturing in 20% sucrose (control), Brewbaker and Kwack (BK) and modified Brewbaker and Kwack (BK1). Pollen viability of RRIT 251 had a decreasing trend from 15 to 180 days after storage and was less than for RO/C/9 23/419, PB 235 and RO/A/7 25/114, respectively. There was no difference in pollen germination at 24 and 48 h after incubation. The vigor of the different rubber pollen clones was different with the different media-BK encouraged RRIT 251 while BK1 stimulated PB 235, RO/C/9 23/419 and RO/A/7 25/114. Pollen storage in liquid nitrogen for 30 days resulted in a higher viability which was sufficient to address non-synchronous flowering.
Keywords: rubber; liquid nitrogen; pollen viability

Introduction

Rubber (*Hevea brasiliensis* Muell-Arg) is a main crop in Southern Thailand and is now expanding to the Northeast and the North. There are more than 50 rubber clones but for a long time, only the RRIM 600 and RRIT 251 clones have been popular with rubber farmers (Rubber Research Institute, 2010). Rubber varieties are normally classified by the harvesting benefit of the natural latex or the timber, or both. Breeders have been attempting to hybridize rubber clones with favorable harvesting characteristics and also resistance to diseases like *Phytophthora palmivora* and *Corynespora* leaf spot. However, non-synchronous flowering and low natural pollination make breeding between clones of *Hevea* difficult. Previous studies have investigated pollen germination in a large number clones by testing on an artificial medium, with the results indicating low germination particularly at room temperature. This has caused complications for breeders attempting rubber hybridization. On the other hand, pollen may be stored for germplasm conservation, to create hybrids between plants that flower at different times or places, or for later use in hybridization programmes, and the quality must be monitored (Rubber Research Institute, 2010; Hamzah et al., 2002). However, pollen storage in liquid nitrogen (-196 ^{o}C) is an ideal strategy for long-term preservation for hybridization (Yeang, 2007). The drying of the whole flowers at 7-11% moisture content before storage in liquid nitrogen is used in the progressive storage method (Hamzah and Leene, 1996). These studies used the pollen of RRIM 600 and RRIT 251, where it was found that dehydrated pollen at 11% resulted in high pollen staining and pollen germination was more than 7% (Kaewbunjong and Tongkaemkaew, 2015).

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Pollen viability is important in agriculture and for plant breeders because pollen must be viable at the time of pollination for seed (or fruit) set to occur. Pollen viability and survival are influenced by both the environment and genotype (Dafni and Firmage, 1999). Staining is the fastest method and is generally used. However, it generally overestimates the pollen viability, because unviable pollen grains can be stained due to their enzyme or starch content or even due to other substances (Heslop-Hrrison, 1992). The *in vitro* method is the most convenient and is able to estimate the reserve substances and the membrane conditions as well as the reserve conversion for pollen germination. This method is influenced by several factors, such as the species, the culture medium, the temperature and time of incubation, the flower development stage when collected and the storage conditions. The most common medium utilized for *in vitro* pollen germination is made of sugar and boric acid to which other nutrients can be added with the objective of providing osmotic equilibrium between the pollen and germination medium, as well as providing an energy source to aid the pollen development process. The boron in the culture medium stimulates pollen tube growth. Boron interacts with sugar, producing a sugar-borate complex which can act more rapidly on the cell membranes (Franzon et al., 2005). The calcium in the culture medium plays a similarly prominent role in pollen germination and pollen-tube growth. The pollen of most species germinates and grows like a tube when placed in a solution of calcium, boron and an osmotic using the tissue culture technique. Germination and tube growth are robust under experimentally defined conditions for most species, rendering *in vitro*-based studies of relevance to the *in vivo* situation. Finally, the most widely used media are modifications of the optimal medium from Brewbaker and Kwack (1963). Subsequently, an adequate germination medium is set up by comparing pollen performance *in vitro* on different media with that of pollen germinating *in vitro*. Hence, this study aimed to determine the culture medium *in vitro* on the pollen viability of *Hevea* 4 clones as well as observing the possibilities of storage in the liquid nitrogen.

Material and Methods

1. Plant materials and pollen storage

Plant material was used from the RRIT 251, PB 235, RO/C/9 23/419 and RO/A/7 25/114 clones. Important characteristics of the rubber clones selected were that RRIT 251 has high natural latex production while PB 235 has medium natural latex and high wood production. Both varieties are the top priority for promotion by Office of Rubber Replanting Aid Fund (ORRAF). RO/C/9 23/419 and RO/A/7 25/114 represent conventional breeding germplasm of the Rubber Research Institute (RRI). The plants were grown at the RRI, Surat Thani province, Thailand. The study utilized a completely randomized design and pollen of each rubber clone was tested for viability using pollen staining after storage, as well testing the pollen germination in different artificial media.

2. Pollen storage

Male flowers of the rubber clones were picked from the peduncle and kept in liquid nitrogen (-196 °C) and immediately transported to the laboratory at RRI, Songkhla province, Thailand. The whole male flowers were referred to as pollen and kept in long storage using liquid nitrogen. Samples were taken every 15 days of storage until 180 days and were tested using pollen

staining and also for germination (Kaewbunjong and Tongkaemkaew, 2015).

3. Assessment of pollen viability

Pollen dehydration: The effect of dehydration on viability was assessed. The moisture content in whole male flowers was reduced using silica gel at 25 °C for 2 hours. After that, the pollen was stained and *in vitro* germination in an artificial medium was determined.

Pollen staining: Five male flowers from four replications (20 male flowers) were used for staining. The anthers were separated and added to a drop of 1,2,3-triphenyl tetrazolium chloride (TTC) stain placed on a microscope slide and subsequently smeared with a small glass tube and observed under a compound microscope. Pollen staining was incubated for 1 hour before viewing under a fluorescence microscope.

Artificial medium for testing pollen vigor: The pollen germination was tested using three formulas of medium culture: 1) 20% sucrose as the control; 2) 20% sucrose, 100 pm H_3BO_3, 300 ppm $Ca(NO_3)_2.4H_2O$, 200 ppm $MgSO_4.7H_2O$, 100 ppm H_3BO_3 known as Brewbaker and Kwack (BK); and 3) a modification of BK with 20% sucrose, 100 ppm H_3BO_3, 400 ppm $Ca(NO_3)_2.4H_2O$, 400 ppm $MgSO_4.7H_2O$ known as Brewbaker and Kwack 1 (BK1).

Pollen culture *in vitro* condition: Five male flowers/replicate using four replicates were counted for the pollen grains present. Pollen was removed from the anther by smearing with a small glass tube. The artificial medium was placed on a concave culture slide (size 1 ml). The pollen was germinated at room temperate (22-25 °C) in the dark before being observed under a compound microscope. Pollen germination was determined after 24 and 48 hours, incubation based on the pollen tube being equal to or greater than the pollen grain diameter (Heslop-Hrrison, 1992).

Results and Discussion

1. Pollen viability

Pollen staining of the four rubber clones declined up to the last day of storage. There was no significant difference in the percentage of pollen stained from 15 to 165 days. Staining of non-aborted pollen at 180 days of RRIT 251 and PB 235 was significantly greater compared to RO/C/9 23/419 and RO/A/7 25/114 (**Table 1**).

2. Pollen germination

The pollen germination of the four rubber clones in the BK medium from 15 to 180 days after storage was compared following 24 and 48 h incubation. The pollen germination was higher 48 h after incubation than at 24 h after incubation. The PB 235 clone was slower to germinate and had a lower germination percentage than the other clones from 15 to 180 days after storage (16.70% to 2.85%), followed by RRIT 251 (17.85% to 2.82%), RO/C/9 23/419 (19.90% to 2.79%) and RO/A/7 25/114 (19.46% to 2.47%), respectively (**Figure 1**).

The percentage of pollen viability *in vitro* responded to the different medium formulas. The germination of the four pollen clones was higher after 30 days storage. BK and BK1 tended to encourage greater pollen germination after 15 days to 180 days storage than the control. The RRIT 251 clone had a germination percentage of 28.51% and 27.42% at 30 and 45 day after storage (DAS), respectively, using BK, whereas the control medium resulted in 26.18% at 15 DAS. In addition, BK stimulated pollen germination from 30 to 135 DAS, while BK1 supported germination from 145 to 180 DAS. For the RO/C/9 23/419 clone, BK and BK1 tended to stimulate pollen

germination at 15 DAS (26.32% and 26.84%, respectively). Both BK media stimulated pollen germination throughout the storage period. For the PB 235 clone, BK1 stimulated pollen germination throughout the whole storage period. However, at 30 DAS, there was higher pollen germination using BK (28.72%) and at 15 DAS using BK1 (27.93%). For the RO/A/7 25/114 clone, BK1 encouraged pollen germination throughout the whole storage period. Pollen germination was higher in the three formulas of artificial medium at 30 DAS, with 26.02% in BK, 25.83% in the control and 25.02% in BK1 (**Figure 2**).

These studies showed that long periods of preservation could not maintain pollen viability. A suitable period of pollen storage for hybridization in the same season was 30 days (4 weeks). However, storage of pollen for 3-7 weeks was sufficient to address the non-synchronous flowering issue among clones (Hamzah, et al, 1999). The study also found the levels of pollen viability of RRIT 251, PB 235, RO/C/9 23/419 and RO/A/7 25/114 at 15 to 30 DAS were useful for hybridization. This was similar to the research reported by Kaewbunjong and Tongkaemkaew (2015), who found that the rubber clones RRIT 251 and RRIT 600 had higher levels of pollen staining and germination at 15 DAS. Subsequently, the percentages of both viability tests constantly declined until the last day of storage (180 days). The results of pollen storage and testing of pollen vigor using culture medium are beneficial to breeders. However, additional investigation should be carried out to test fruit setting using hand pollination in the field. Hamzah and Leene (1996) reported that the percentage of pollen germination in laboratory assessment was quite high for pollen stored in liquid nitrogen; but the success rate for fruit setting was only 3% using hand pollination in the field. This indicated the stored pollen was indeed viable and fertile (12.6%). Although the success of fruit set was low, the target of crossing clones which do not flower synchronously was achieved. Therefore, a much shorter duration of pollen storage is sufficient to alleviate the problem of non-synchronous flowering in *Hevea*. The scoring of pollen cryo-preservation over several years is a useful application for germplasm conservation (Hamzah and Leene, 1996).

Table 1 Pollen staining of rubber clones after storage.

Days of storage	Pollen stained (%) of rubber clones					
	RRIT 251	PB 235	RO/C/9 23/419	RO/A/7 25/114	F-test	C.V. (%)
15	68.86	66.64	63.63	68.05	ns	10.69
30	65.85	63.75	57.25	64.81	ns	8.60
45	37.68	44.98	39.49	47.51	ns	15.80
60	33.76	40.53	37.44	42.67	ns	12.17
75	32.64	36.23	34.27	39.97	ns	13.37
90	28.10	26.95	28.69	28.93	ns	10.90
105	27.13	24.78	26.18	26.48	ns	10.85
120	26.24	23.78	24.73	25.53	ns	8.86
135	24.47	22.62	23.38	23.92	ns	5.80
150	23.43	22.23	22.55	22.50	ns	7.97
165	19.25	19.14	18.88	18.33	ns	9.91
180	15.94a	14.67ab	12.23bc	11.57c	**	12.23

Values with the same lowercase letter in a row are not significantly different at P≤0.05 using LSD test. ns = not significant.

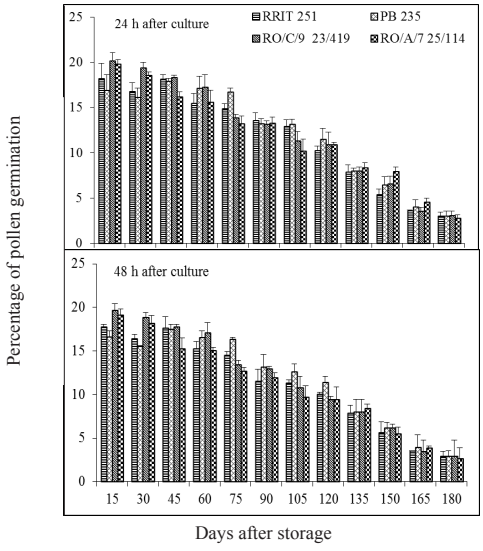

Figure 1 Pollen germination of four rubber clones in BK medium after culture for 24 and 48 h.

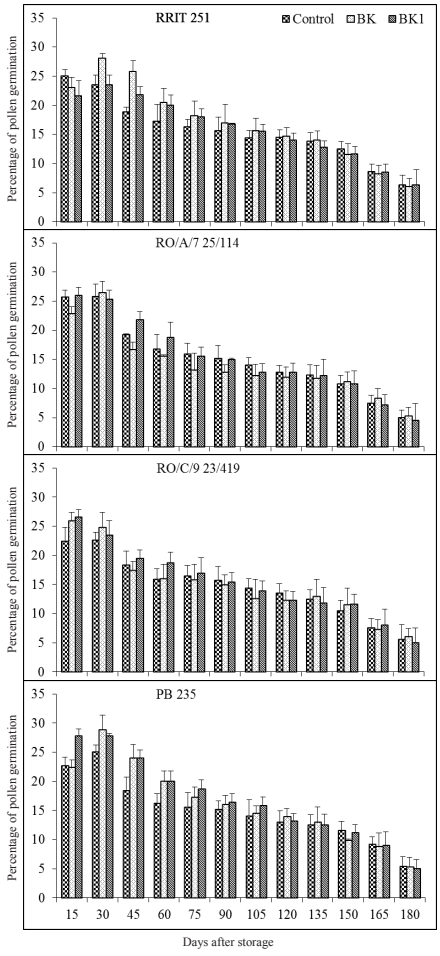

Figure 2 Pollen germination of four rubber clones in three artificial media.

Conclusions

The preservation of *Hevea* pollen in liquid nitrogen allowed for long storage periods, but the pollen viability continually declined. Thirty days of storage were sufficient to overcome non-synchronous flowering issues within the same season (3 months in Thailand). Pollen germination testing during storage indicated pollen vigor. In particular, the addition of boric acid, calcium nitrate and magnesium sulfate to the medium culture stimulated pollen germination following longer periods of storage. These results showed that the storage of *Hevea* pollen could be useful for hand-pollination among clones.

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