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SHORT COMMUNICATION

Pollen Storage of Hevea

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An investigation on the storage characteristics of Hevea *pollen with the aim of facilitating crosses of clones that do not flower synchronously was undertaken. Staminal columns, as compared to whole flowers, were found to be better storage materials. These underwent a certain degree of drying (to a minimum of 7% - 11% moisture content) before they were stored in liquid nitrogen. With this method the pollen was viable for five months even though the* in vitro *germination rate was declining.*

Pollen stored up to one-and-a-half months in liquid nitrogen was shown to retain its ability for fertilisation when 6% success was obtained in the laboratory assessment. Hand-pollination in the field however, bore three fruits out of the 108 flowers pollinated with one-month-old stored pollen Only one fruit was obtained in another crossing of 330 flowers

There are two main flowering seasons in a year in *Hevea* Even though the season only lasts for a couple of months each time, flowering is not synchronous in some clones. This non-synchronisation poses problems to the plant breeders in their hand-pollination programmes of selected parental clones. Hence to overcome this obstacle pollen needs to be stored, at least within the same season, while still maintaining its viability until it is required for the hand-pollination.

Literature on pollen storage is extensive including a few reviews[1-3]. For most species success on the extension of pollen viability was mainly through storage at lower temperatures ranging from 4°C in Avocado[4] to -196°C in many crops *e g.* potatoes[5] and maize[6]. If storage is to be successful at the lower temperatures, the moisture content of the pollen has also to be maintained within certain limits[7]. Storage at cryogenic temperature (liquid nitrogen) is also an ideal strategy for long-term preservation[8].

Pollen has also been shown to be successfully stored under different controlled relative humidities (RH) at room temperature using various concentrations of sulphuric acid[2]. Storage of the apple and pear pollen at lower RH (10%) increased the longetivity of the pollen. In others, it is the combination of temperature and RH that has created an optimum environment in preserving the germination capabilities of the pollen. In yet another method of storage, pollen was freeze-dried in sealed ampoules which therefore enabled pollen to be transported easily in an uncontrolled environment[9].

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In *Hevea*, the first pollen storage study was carried out by Dijkman[10] in 1938. He showed that pollen could successfully be stored for 19 days at 6°C above sulphuric acid solutions of 27% - 35% (equivalent to 70% - 80% RH). Workers in Sri Lanka showed that *Hevea* pollen survived 5 days storage under 75% RH at 5°C[11]. In this study results of various experiments conducted to obtain a suitable medium to store *Hevea* pollen are reported. The stored pollen was assessed for viability using the *in vitro* germination and fluoresceine diacetate staining. Hand-pollination in the laboratory and in the field was used to test for pollen fertility.

MATERIALS AND METHODS

Pollen Collection

Unopened mature male flowers were collected in the morning. The petals were removed leaving the staminal columns with the anthers intact. Samples of the pollen were immediately tested for moisture content and viability. Anther moisture content was determined from the difference of the fresh weight and constant dry weight, of which the latter was obtained by drying anthers at 85°C for 4 h.

Pollen Dehydration and Storage

Moisture levels have to be adjusted for successful storage at low temperatures. Various methods of dehydration were carried out on whole male flowers and staminal columns containing anthers only (*i.e.* with petals removed). These were dehydrated over (a) silica gel, (b) saturated solutions of potassium acetate and (c) a mixture of equal volumes of ammonium nitrate and sodium nitrate which gave relative humidities (RH) of 16%, 22.5% and 50% at 25°C respectively[12]. The moisture content was determined every 2 h until the equilibrium moisture content (EMC) was achieved.

Prior to storage, the pollen was tested for its viability using the methods mentioned below. The dried materials were then placed in cryovials and stored in liquid nitrogen (-196°C) storage tanks until required.

Assessment of Pollen Viability

Before carrying out viability tests, pollen that had either been stored or dehydrated was first rehydrated in a moist chamber at room temperature for one hour.

In vitro *germination*. Pollen was germinated in the standard Brewbaker-Kwack[13] solution but containing a higher concentration of sucrose (20%). The hanging drop method first introduced by van Tiegham[14] was adopted. Germination rate was determined after 24 h and four replicates were carried out counting all the pollen grains present which normally amounted to more than 100 pollen grains.

Fluoresceine diacetate staining. Pollen was stained with fluoresceine diacetate (FDA) and viewed under fluorescence microscopy[15].

Assessment of Pollen Fertility

Laboratory assessment. Floral inflorescences containing only female flowers (male flowers removed) were brought to the laboratory and the peduncles were kept in water. Hand-pollination was carried out using the stored staminal columns. Owing to their shrivelled condition of the staminal columns even after rehydration, about 2-3 of them were used for each pollination. These were left on the stigma for 48 h, protected by a small plug of cotton wool.

The flowers with the petals removed were fixed in Carnoy's solution for 24 h at least, before processing for fluorescence microscopy following Martin's method[16] of observing pollen tube growth in the floral style *(Figure 1)*. Only when three of the ovules from an ovary have been penetrated by pollen tubes was the pollen considered fertile. At least 50 pollinations were carried out for each storage conditions.

Field assessment. Stored pollen was also hand-pollinated in the field using the protocol above, except that one staminal column was placed on each stigma. This conforms to the normal method of hand-pollination carried out by the breeders. Fruit formation was scored four months after hand-pollination by which time all unfertilised flowers would have abscinded. Sometimes, where more than one staminal columns were used, fruit formation was scored 8 weeks after hand-pollination.

RESULTS AND DISCUSSION

Pollen Viability Assays

Comparing the two categories of viability assays carried out *i.e.* staining and pollen germination, the former was found to be fast and less problematic. Percentage viability obtained by staining was always higher than that by *in vitro* germination for both fresh and stored pollen *(Figure 2)*. The differences between these two assays ranged from 4% to 40% for stored pollen and as large as 65% for the fresh pollen.

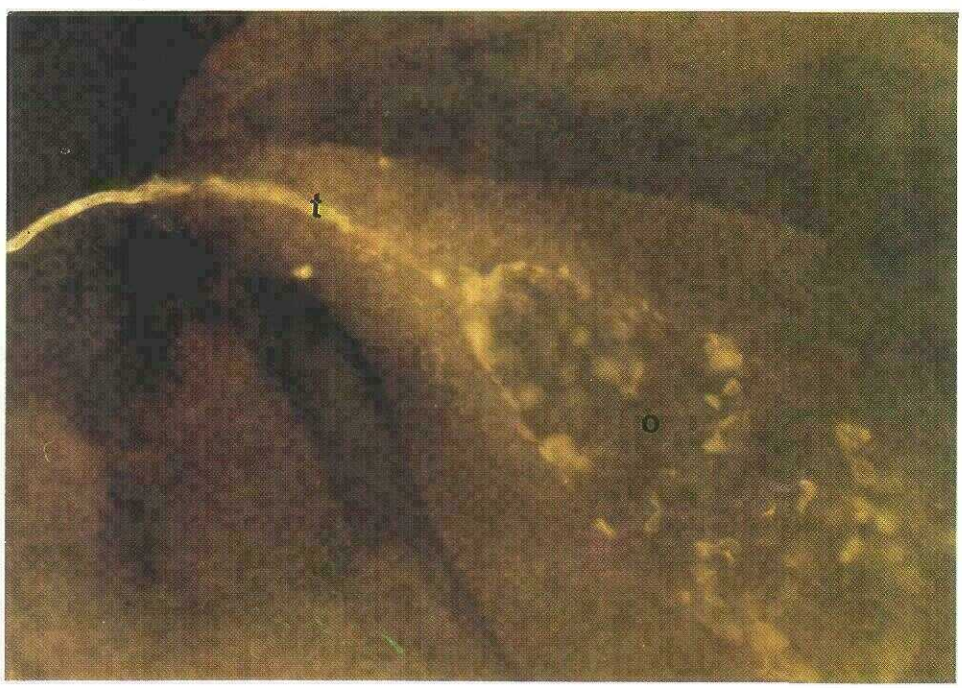
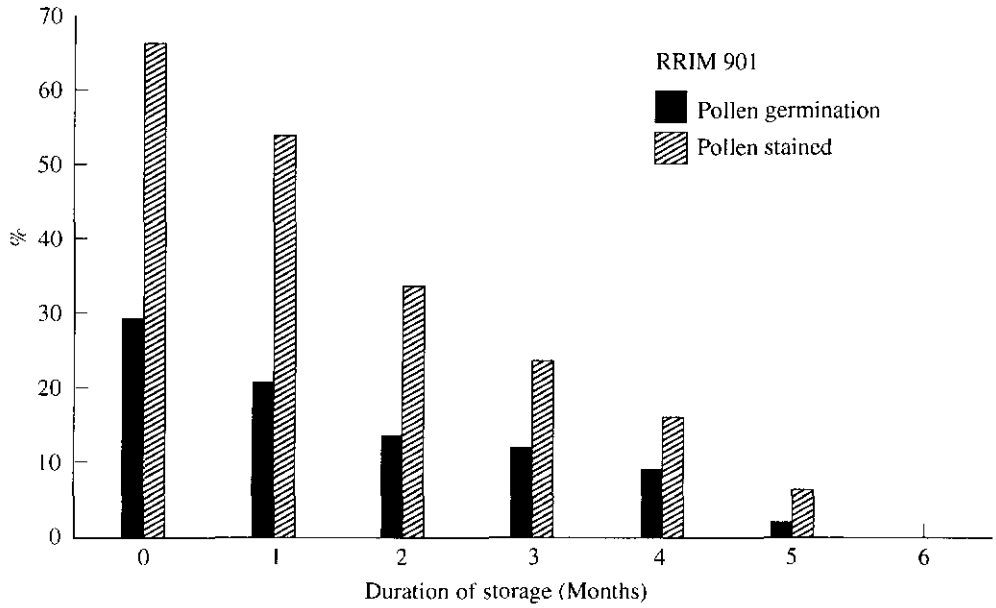


Figure 1. Fluorescence microscopy of a pollen tube (t) penetrating a single ovule (o).



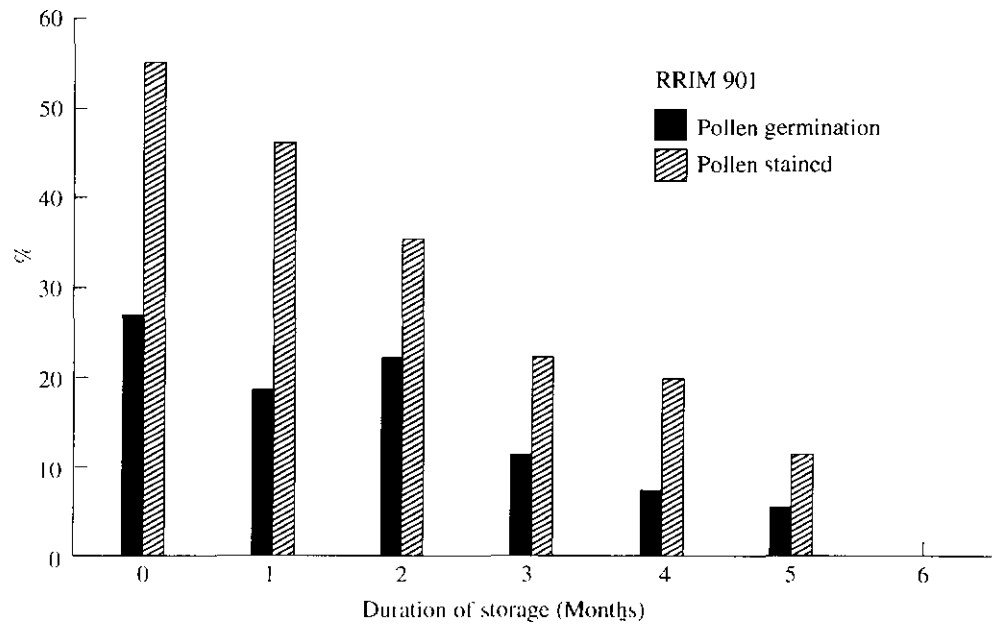


Figure 2. Comparison of viability assays (in vitro *germination and FDA staining) of RRIM 901 and RRIM 600 pollen during storage.*

The ability of the pollen to germinate in an artificial media is often assumed to correlate well with *in vivo* germination. The latter gives the most definitive answer as to whether the pollen is functional under natural conditions. However, it is best suited to only a small number of samples due to the tedious nature of the method. Hence, when devising methodolgy to optimise storage conditions in this study, the *in vitro* germination was still preferred for identification of samples with high germination potential.

Pollen Dehydration

The extent and rate of dehydration in the anther/pollen is controlled by many factors *e.g.* medium/salt used, gradient between RH of salt and moisture content of anther and also the pollen type[17]. *Figure 3A* showed that when silica gel (RH 16%) was the drying agent, it took about eight hours for the RRIM 901 staminal columns to approach equilibrium. Dehydration over saturated salt solution of higher humidities, RH 25% and 50%, however took longer, requiring about 24 h and 26 h, respectively *(Figures 3B and 3C)*. Whole flowers took longer time to reach equilibrium and the values of their EMC were also higher than those of the staminal columns containing anthers *(Table 1)*. Moisture content has a bearing on the storage performance of the pollen later on (see below). Even though the process of removing the petals to expose the pollen is tedious initially, it would still be easier than to remove them after storage, when the petals have dried up. Later experiments use only the staminal columns.

TABLE 1 EQUILIBRIUM MOISTURE CONTENT (EMC) OF FLORAL MATERIALS DRIED UNDER VARIOUS R H VALUES

RH (%)	Staminal columns	Whole flowers
16.0	1.8	3.0
25.5	2.1	4.4
50.0	3.7	6.6

Effect of Pollen Moisture Content on Pollen Storage in Liquid Nitrogen

For many pollen, pre-drying is essential before storage at temperatures below 0°C. This has been confirmed by X-ray analysis studies of ice being formed in pollen containing relatively high moisture content stored at low temperatures[18]. In clone RRIM 901, pollen (refers to staminal columns with anthers) with the lowest moisture content (11%) at the onset of the storage showed the ability to store for longer duration in liquid nitrogen *(Figure 4)*. Pollen with 24% moisture content on the other hand, was only viable for 3 months. Similar trend was also shown by clone RRIM 600. However, the percentage of pollen germination after a 5-month storage was relatively higher than that in RRIM 901. This could be a clonal characteristic or perhaps the lower pre-storage moisture content in RRIM 600 (7.8%) as compared to RRIM 901 (11%) which has contributed to this longetivity.

It should also be noted that the pre-storage moisture content of the pollen (refers to staminal columns with anthers) in the above exercise was higher than the values of the EMC obtained in the earlier experiments *(see Table 1)* . This was due to the pollen being dehydrated for only 8 h before storing. Referring to the graphs in *Figure 3*, the duration given was not long enough to reach equilibrium. On the other hand, an EMC value of 2.0 obtained by drying pollen over silica gel for 24 h gave zero germination rate (not shown here). This indicates that drying must not be excessive prior to storage in liquid nitrogen. A too low pre-storage moisture content will not maintain viability of pollen after liquid nitrogen storage.

The trends in the *in vitro* germination of stored pollen also appeared to be somewhat

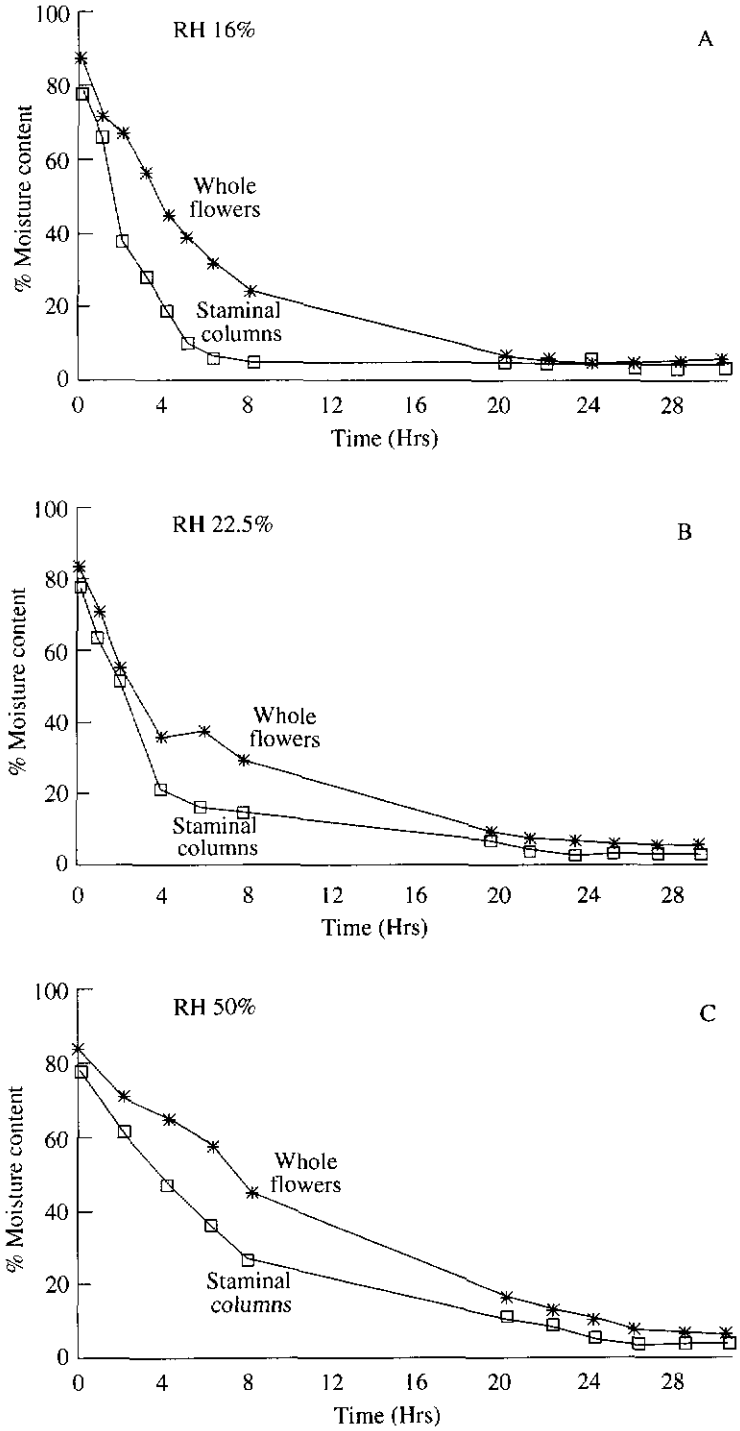


Figure 3. Dehydration of RRIM 901 whole flowers and staminal columns over silica gel (A), saturated solutions of potassium acetate (B) and a mixture of equal volumes of ammonium nitrate and sodium nitrate (C).

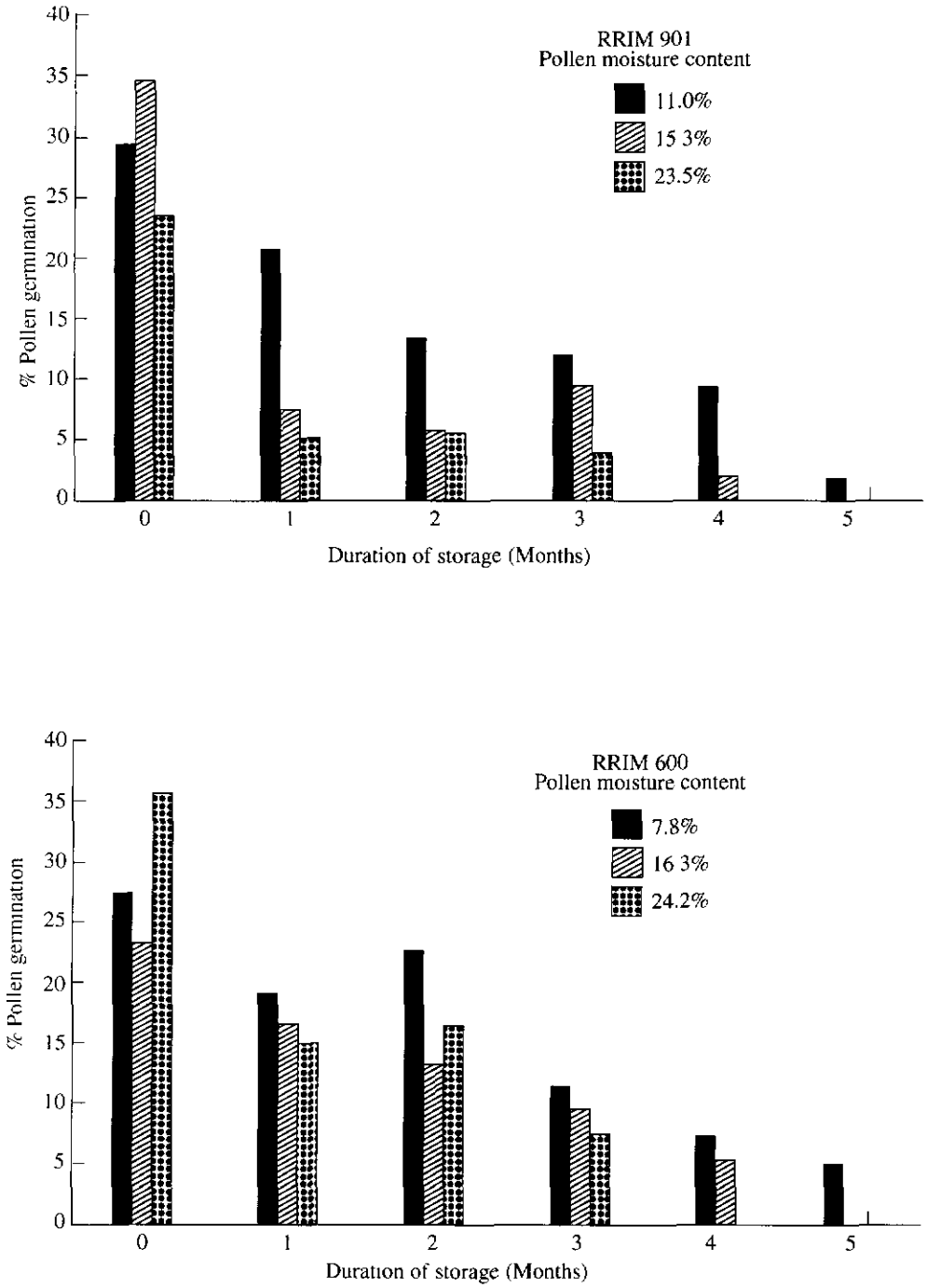


Figure 4 Effect of pollen moisture content (prior to liquid N_2 storage) on the duration of storage in two clones

TABLE 2 LABORATORY ASSESSMENT OF FERTILITY OF RRIM 901 POLLEN STORED IN LIQUID NITROGEN

	Storage (months)		
	1	1.5	4.5
Pre-storage pollen moisture content (%)	7.62	7.62	12.08
In vitro germination of fresh pollen (%)	24.33	24.33	29.17
In vitro germination of stored pollen prior to crossing (%)	20.01	18.51	3.85
Fertilisation (%)	8.3	6.12	0

unpredictable as there are many factors in the assay protocol that can contribute to this. For example, the pollen might be concentrated on the apex of the hanging drop due to surface tension and this may reduce the germination rate. Many authors[3,9] have also observed this unpredictability in the germination assays from low-temperature stored and freeze-dried materials.

In vivo Pollen Tube Growth and Fertility

Laboratory assessment of *in vivo* pollen tube growth is a more valid test for pollen viability and perhaps fertility, since pollen was made to grow in natural conditions but short of fruit development. The fact that three ovules were penetrated gave a strong indication that fertilisation has taken place. However, fruits do get aborted along the way to maturity due to many underlying factors.

Table 2 showed that the percentage of fertilised ovules (laboratory assessment) was still quite high for pollen stored up to 1.5 months in liquid nitrogen (6.12%); but dropped to zero after 4.5-months of storage. The poor result shown by the 4.5-month stored pollen was possibly contributed by the low pollen viability (3.8%) after storage. A high pre-storage moisture content (12%) could also probably play a part in storage of longer duration.

A 3% success in fruit set showed by 4-week-old stored PB 260 pollen *(Table 3)* hand-pollinated in the field indicated that the stored pollen was indeed viable and fertile. The

TABLE 3 POLLEN FERTILITY OF FOUR-WEEK-OLD STORED POLLEN OF VARIOUS CLONES

	Clones		
	RRIM 901	RRIM 600	PB 260
Pollen moisture content (%)	5.92	13.29	17.1
Viability of fresh pollen (%)	7.63	20.76	2.14
Viability of dried pollen (%)	16.16	0	3.91
Viability of stored pollen (%)	NA	NA	12.6
No. of ovules pollinated	91	107	108
Fruit set (%)	0	0	3.0

NA = not available

TABLE 4 POLLEN FERTILITY OF THREE-WEEK-OLD STORED POLLEN

	Clone 84/57*	Clone 75/86**
Pollen moisture content (%)	8.25	14.23
Viability of fresh pollen (%)	44.53	33.77
Viability of dried pollen (%)	12.58	15.8
Viability of stored pollen (%)	5.69	11.35
Fruit set (%)	0	0.3

*600 crosses
**330 crosses

percentage success obtained was similar to that obtained for crosses of normal, unstored pollen in the main flowering season[19]. Besides the relatively high viability of the stored pollen, the success could have partly been attributed to the use of more than one staminal column for this set of hand-pollination. In fact, Harihar and Yeang[19] have showed that by increasing the number of pollen grain deposited on the stigma resulted in a higher percentage of fruit set. In these crosses the rather high moisture content (17%) does not appear to affect the viabilty of the pollen after storage, perhaps not with shorter storage duration.

In yet another set of hand-pollination (in the field) where a higher number of crosses (330) were carried out using only a single staminal column, the success of fruit set was only 0.3% *(Table 4)*. This particular female clone flowers rather early and is also known to be a poor seeder. The result obtained is perhaps a true measure of the fertility of stored pollen as the procedure followed was that of the normal practice of the breeders. Although the success of fruit set was low, the target of crossing clones which do not flower synchronously has at least been achieved.

CONCLUSION

For now, success of fruit set using stored pollen appeared to be determined by the viability of the stored pollen itself, immaterial of the pollen pre-storage moisture content. But again, it must be taken into consideration that the fruit set success shown so far only takes effect on four-week-old stored pollen. Perhaps, with their high pre-storage moisture content (17% or 14%), pollen would not be viable if stored for a longer period. Therefore, further work has to be carried out to determine the critical moisture level, below which viability is still retained after storage in liquid nitrogen.

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