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Optimization of *in vitro* pollen germination and viability testing of some Australian selections of date palm (*Phoenix dactylifera* L.) and their xenic and metaxenic effects on the tissue culture–derived female cultivar "Barhee"

Ahmed Al-Najm[1,2] · Steve Brauer[3] · Richard Trethowan[1] · Andrew Merchant[4] · Nabil Ahmad[1]

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Abstract

Information regarding efficient pollen viability testing methods and the most favourable pollen storage conditions for internationally important date palm cultivars is limited. No such information has been reported on Australian male selections. The *in vitro* pollen germination of three elite Australian male selections, "Big pod", "Campbell" and "Tanunda", was estimated using a modified Brewbaker and Kwack (BK) medium with combinations of various chemicals. Germination was maximized when 75 mg/l H_3BO_4, 400 mg/l Ca (NO3)2.4H_2O, 100 mg/l KNO_3, 50 mg/l $MgSO_4$.7H_2O and 15% (w/v) sucrose were used in a medium at pH 5.4. The viability of pollen of these three genotypes following storage in a refrigerator at 4°C was tested every 3 mo for 12 mo. Significant pollen viability was maintained after 9 mo of storage and the highest germinability (60.3%) was observed for the genotype "Tanunda". Six tests for determining viability, including TTC (2,3,5-triphenyltetrazolium chloride), IKI (iodine potassium iodide), lactophenol cotton blue (LPCB), acetocarmine, fluorescein diacetate (FDA) and Alexander's differential stain were evaluated and correlations between these pollen viability tests and *in vitro* germination were determined. A highly positive correlation between *in vitro* germination and the FDA viability test was observed. The metaxenic effect induced by pollinating the cultivar "Barhee" with three sources of pollen was investigated by assessing changes in the levels of malic acid, citric acid, fructose, glucose, sucrose, L-proline and *myo*-inositol during fruit development. The pollen source had a significant influence on the concentrations of these chemicals (metaxenic effect). Pollen of the genotype "Tanunda" significantly increased fruit size, fresh weight and seed size in comparison with other pollen sources.

Keywords: Pollen viability; · Date palm; · Stigma receptivity

Introduction

Date palm (*Phoenix dactylifera* L.) cultivation is of primary importance to the agricultural economies of several countries in the arid regions of West Asia and North Africa. The wide distribution of this dioecious species has led to the release of nearly 3000 cultivars, which produce close to 7.5 million tonnes of dates annually (FAOSTAT 2016). As a dioecious species, male and female flowers are borne on separate trees. The flowers are unisexual, adopting both pistillate and staminate characteristics. Artificial pollination is required to promote fruit set to a satisfactory standard (Zaid and Arias-Jimenez 2002). Characteristics of the pollen grain influence the size, shape, weight and time of maturation of fruit (DeMason and Sekhar 1988; Salomón-Torres *et al*. 2021). Inflorescence emergence in female date palm prior to the

✉ Nabil Ahmad
nabil.ahmad@sydney.edu.au; nabeeldeeb@yahoo.com

[1] Plant Breeding Institute, School of Life and Environmental Sciences, Faculty of Science, University of Sydney, 107 Cobbitty Road, Cobbitty, NSW 2570, Australia

[2] The Date Palm Research Center, University of Basrah, Basrah, Iraq

[3] Riverland Date Garden, 376 Gurra Road, Gurra Gurra, SA 5343, Australia

[4] Centre for Carbon, Water and Food, School of Life and Environmental Sciences, Faculty of Science, University of Sydney, 380 Werombi Road, Camden, NSW 2570, Australia

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opening of male spathes is common and results in a scarcity of pollen. This phenomenon forces farmers to access and employ pollen from untested and unknown sources. Such genetic material is commonly characterized by low fertility and tissue incompatibility. A common remedy to alleviate this problem is storage of pollen obtained during the pollination period (2–3 mo) or inter-seasonal storage for use in subsequent seasons (Mortazavi *et al*. 2008). Pollen storage is also a critical component of conservation and crop improvement programs (Lora *et al*. 2006). The most important determinants of maintenance of viability during pollen storage are temperature and relative humidity (RH).

Viability and vigour of pollen obtained from a range of storage conditions and genetic backgrounds can be assessed *in vitro via* germination and the assessment of tube growth. The exact amount of viable pollen can reliably be tested by germination (Bolat and Pirlak 1999; Rodriguez-Riano and Dafni 2000). However, different species require different media to promote germination (Heslop-Harrison 1987), further complicating this assessment. If the medium is optimized, then pollen germination will be maximized (Boavida and McCormick 2007). The Brewbaker and Kwack (BK) medium (Brewbaker and Kwack 1963), further modified by Furr and Enriquez (1966), became the base medium for date palm pollen germination (Zaid and Arias-Jimenez 2002).

The viability of stored date palm pollen was first documented by Albert (1930) who showed that the percentage germination of pollen stored at 3.3°C was better than at room temperature (approximately 20°C) in the same year. Using a modified Brewbaker and Kwack (1964) medium, Furr and Enriquez (1966) obtained high *in vitro* germination percentages. However, low temperature was an important factor in long-term pollen storage as demonstrated in several plant species (Lee *et al*. 1985; Maryam *et al*. 2015; Maryam *et al*. 2017; Mesnoua *et al*. 2018).

The genetic purity of date palm cultivars is usually maintained by traditional propagation from offshoots. However, with the development of tissue culture (TC) techniques, it was possible to greatly expand production of elite cultivars and to facilitate the large-scale transportation of plant materials over greater distances. *In vitro* propagation through TC produces plants that are phenotypically and genotypically similar to the mother plant (explant source). However, among TC-derived date palms, several off-types were detected in several studies (*e.g.*, Cohen *et al*. 2004) including variegated forms, variable leaf structure, reduced overall growth, sterility and abnormal flowers. Other reported changes include excessive carpels (Al-Wasel 2000; Djerbi 2000) and seedless parthenocarpic fruits (McCubbin *et al*. 2004). These somaclonal variants resulted from genetic and/or epigenetic changes through the tissue culture process (Kaeppler *et al*. 2000). A normal female date palm flower has three carpels, but only one develops into a normal fruit and the others degenerate after pollination. Pollination failure leads to the formation of triple parthenocarpic fruits of no economic value (Reuveni 1986; Zaid and De Wet 2002). The occurrence of these multicarpel fruits in TC-derived date palms can be detected only during the fruiting stage (*i.e.*, 5 to 7 yr after planting), causing severe economic losses to farmers. One of the reasons for abnormal fruit development might be pollination failure caused by inter-clonal pollen incompatibility. Djerbi (2000) observed that some date palm cultivars (cv.) produced higher yields when pollinated with specific males.

This study therefore optimized *in vitro* pollen germination and viability testing methods in several date palm genotypes and determined the influence of pollen source on the physical and biochemical fruit characteristics of the well-known date palm cv. “Barhee”.

Materials and Methods

Pollen Source, Collection, Storage and Pollination Date palm pollen was obtained from date palm trees grown in Southern Australia, specifically from spathes of three Australian male date palm selections, namely, “Big pod”, “Campbell” and “Tanunda” as soon as they cracked (Fig. 1). To minimize variation among pollen spadices, five to six male spadices of each tree were mixed. Different pollen sources were stored in three glass tubes in a domestic refrigerator (4°C) for 12 mo. Pollen germinability was tested at 0, 3, 6, 9 and 12 mo from the time of collection. Each female spadix was pollinated by dusting 1 g of pollen on its flowers. All pollinated inflorescences were covered with perforated paper bags immediately after pollination. The paper bags were removed 2–3 wk after pollination to allow the fruits to develop normally.

Optimization of pH in the *In Vitro* Pollen Germination Medium A preliminary experiment was conducted over the pH range of 5.0 to 6.2 with three genotypes on a basic BK medium containing 15% sucrose to optimize pH.

Optimization of the Pollen Germination Medium The first experiment comprised of 22 selected media composition with one sucrose concentration (15% w/v) and one concentration of the following mineral components: five H_3BO_4 concentrations (25, 50, 75, 100 and 150 mg/l), six $Ca(NO_3)_2.4H_2O$ concentrations (50, 100, 200, 300, 400 and 500 mg/l), five KNO_3 concentrations (50, 100, 200, 300 and 400 mg/l) and six $MgSO_4.7H_2O$ concentrations (25, 50, 100, 200, 300 and 400 mg/l). A total of 22 different germination media were utilized.

The percentage pollen germination was subsequently calculated according to the formula:

Germination % = (germinated pollen/total number of pollen grains) × 100.

FIGURE 1 Traditional spathe collection, drying and pollen extraction in selected seedling genotypes. (*A*) Male inflorescence inside the enclosed spathe before cracking. (*B*) Cracked spathe exposing the enclosed spadix at the time of pollen collection. (*C*) Inflorescence drying on paper inside a dry room at 25–30°C. (*D*) Extracted pollen grains.

Scoring of Pollen Cultures A pollen grain was scored as germinated when the length of the pollen tube was equivalent to or greater than its diameter. Approximately 100 pollen grains were scored on each slide in three randomly selected microscopic fields. The total number of pollen grains in each field and number of germinated grains were recorded. The samples were examined at ×100 magnification using normal bright field optics and a Leica DMIL light microscope (Leica Microsystems Pty Ltd, Wetzlar, Germany) fitted with a Nikon Photo Coolpix 900 camera (Nikon Corporation, Tokyo, Japan) for imaging.

Pollen Viability/Stainability Tests Histochemical tests were used to assess pollen viability. For each stain and genotype combination, three replicate slides were prepared. Pollen germination was assessed on each slide and scored in three fields of view per slide using the same microscope and magnification as specified above. Pollen viability was assessed using the following staining methods.

Lactophenol Cotton Blue Pollen grains were dispersed in a drop of stain (Fluka analytical, Munich, Germany, Code: 101386211) on a microscope slide. A cover glass was placed on the preparation and the preparation was then warmed on a hot plate for 15 min and subsequently examined under a microscope. Non-stained pollen grains were scored as non-viable.

Acetocarmine The acetocarmine stain consisted of 2% w/v carmine (Sigma-Aldrich, St. Louis, MO, code: C1022) in 45% acetic acid. Slides were prepared as per the "Lactophenol Cotton Blue" section and non-stained pollen grains were scored as non-viable.

IKI (1%) An iodine potassium iodide (IKI) reagent was prepared by melting 1 g iodine in 100 ml aqueous 2% (w/v) potassium iodide. Pollen grains were dispersed as described above, and non-stained and lightly stained pollen grains were scored as non-viable.

Tetrazolium Chloride The tetrazolium procedure was based on Cook and Stanley (1960). 2,3,5-Triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO) was prepared at 0.5% (w/v) in a 12% (w/v) aqueous sucrose solution. Pollen grains were

dispersed in a drop of medium on a microscope slide and covered immediately with a cover slip to exclude oxygen. The slides were incubated at 60°C in a dark oven for 3 h, and then examined under a light microscope. Scoring was restricted to grains in the central area of the sample. Non-stained pollen grains were scored as non-viable.

Fluorochromatic Reaction (FCR) Test Fluorescing pollen grains were assessed after the pollen was dispersed in a drop of fluorescein diacetate (FDA) (Sigma-Aldrich, St. Louis, MO, code: 201642). Viability was determined by scoring the pollen grains under an ultraviolet (UV) source (excitation filter, 485 nm; barrier filter, 520 nm) provided by a mercury lamp. Esterase in viable pollen hydrolyzes the FDA and releases fluorescein that fluoresces under UV light. Pollen grains that do not fluoresce were scored as non-viable.

Alexander's Stain Alexander's staining solution was prepared (from chemicals made by Sigma-Aldrich, St. Louis, MO) according to Alexander (1969). Pollen samples were dispersed in a drop of the stain on a microscope slide and covered with a coverslip. The solution was warmed on a hot plate for 15 min and allowed to stain for 7–8 h. Incubation for 7–8 h at 60°C in a dark oven made the staining easier. Red or pink colour indicated viable pollen; green colour indicated non-viable pollen.

Stigma Receptivity Test Stigma receptivity was determined by observing pollen tube growth on stigmas and through the short style. Enzymatic activity on the stigmatic surface was also used to determine stigma receptivity. The enzymatic activity was assessed using a Peroxtesmo KO kit (Machery-Nagel, Duren, Germany) as reported by Dafni and Maués (1998).

The stigma receptivity of 20 fresh flowers was evaluated every hour from the time of spathe cracking to 24 h after anthesis by immersing a test paper (15 × 15 mm) in 1 ml of distilled water and subsequently adding a droplet of the solution onto the stigmatic surface. Blue colouration was then determined after several minutes. Flowers in three areas of each inflorescence (outer strands, middle strands and central strands) were tested and peak receptivity was derived from the combined results. All observed stigmas were examined under a stereomicroscope (×30) to ensure that the surface of the stigma was neither damaged nor contaminated with pollen grains as these can cause some "enzymatic activity irrespective of the stigma receptivity" (Kearns and Inouye 1993; as cited in Borna *et al*. 2016).

Enzymatic activity was also confirmed by applying a drop of hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, Code: H1009) (6%) to the stigma and observing the production of oxygen bubbles as an indicator of the peroxidase activity.

Pollen Tube Elongation in Stigmas and Carpels The flowers on the same inflorescence strand were pollinated with the same pollen source. The base of the strand was placed in a deep glass tube containing 10 ml of sterile deionized water. The pollinated female flowers were incubated at 28°C for a period of 48 h, and then single flowers were separated under a stereomicroscope (ZEISS Stemi 2000-c stereomicroscope, Oberkochen, Germany) and subsequently fixed in an ethanol:acetic acid (2:1) solution. The internal tissues of carpels and styles were visualized by cutting each carpel longitudinally, and through the short style. The dissected carpels were cleaned by soaking in 10 M NaOH for 24 h at room temperature followed by three thorough washes in deionized water. Dissected carpels were subsequently stained with 0.2% aniline blue (Sigma-Aldrich, St. Louis, MO, code: B8563) in 0.35% K_3PO_4 and examined under a Leica DMIL fluorescence microscope with a UV excitation filter (Leica Microsystems) and photographed with a Nikon Coolpix 990 camera mounted on the same microscope.

Effect of Pollen Source on "Barhee" Fruit and Seed Characteristics The fruits were harvested at the Khalal and Rutab stages of development for evaluation and subsequently subjected to the following analyses:

- Fruit and seed size measurements (length and width) determined using a sample of five fruits produced by each pollen source. Three palms and three bunches per palm have been used for each pollen source.
- Whole fruit and seed weight.
- Fruit setting (%) determined as: (number of normal fruit / number of flowers) x 100.
- Organic metabolite distribution.

Tissue Extraction and Analysis of Soluble Metabolites Tissues were extracted using a methanol:chloroform:water extraction (12:5:3) solution that was subsequently deionized according to protocols outlined in Merchant *et al*. (2006). Sample extracts were analyzed by a gas chromatography triple quadruple mass spectrometer (Agilent Technologies, Santa Clara, CA) as per Merchant *et al*. (2006). Metabolites were separated using an Agilent 7890A gas chromatograph (Agilent Technologies) with a HP5 column (0.25 mm) (*i.e.*, 30 m, 0.25-mm film thickness; Agilent Technologies). A split injection was made at 300°C with an oven temperature of 60°C sustained for 2 min, then increased to 300°C at a rate of 10°C min^{-1} and then maintained for 10 min. The flow rate of the column was maintained at 1.5 mL min^{-1}. Integration of the peaks was achieved using Agilent Mass Hunter Workstation software (Agilent Technologies).

Calculations for Gas Chromatography–Mass Spectrometry The dry weight of each sample was calculated as:

(Final concentration × Final volume in vial µL/1000 × 700/volume of extract in vial µL × 1300/700)/dry weight in mg = concentration in µg/mg dry weight. This value was multiplied by 1000 to give the concentration in µg/g dry weight.

Analysis of Amino Acids The liquid chromatography–mass spectrometry (LC-MS) analysis of underivatized extract was conducted on a 1290 Infinity LC System (Agilent Technologies) coupled to a 6520 QTOF mass selective detector (Agilent Technologies). A 3.5-µL sample was injected into a Zorbax SB-C18 column (2.1 mm × 150 mm, 3.5 µL), and separation was achieved by gradient elution with water and methanol. The QTOF was tuned to operate at a low mass range of <1700 AMU and data acquisition was performed in scan mode (60–1000 m/z) with ionization in a positive ion mode. The LC-MS results were generated based on retention times relative to standards and their formula mass. Peaks were integrated, and their relative quantities calculated using Mass Hunter software (Agilent Technologies).

Calculations for Liquid Chromatography–Mass Spectrometry The dry weight of each sample was determined as:

(Final concentration × Dilution factor if any × Final volume in vial µL/1000 × 700/volume of extract in vial µL × 1300/700)/dry weight in mg = concentration in µg/mg dry weight. This value was then multiplied by 1000 to give µg/g dry weight.

Statistical Analysis ANOVA was performed on all data for separation of means using the ANOVA function in GenStat version 18 (Payne *et al.* 2013) and significance determined using the post-hoc Fisher's LSD test at P=0.05. Correlations between the individual staining/viability analyses and *in vitro* germination values were determined and linear regression analyses conducted using GenStat software version 18.

Results and Discussion

Optimizing the pH of the Germination Medium A preliminary experiment over pH range 5.0–6.2 with three genotypes tested on basic BK medium containing 15% sucrose showed that the highest germination occurred in the range pH 5.2–pH 6.2 (Fig. 2). All three pollen sources showed similar response patterns with an optimum pH 5.4.

Optimizing the Mineral Component of the Medium A preliminary test to identify the minimum dose required to produce an effect on pollen germination or pollen tube growth revealed that *in vitro* germination of freshly shed pollen on BK original medium (Brewbaker and Kwack 1963) or variants derived from it had no effect. However, subsequent experiments sought an optimized medium for *in vitro* testing of pollen viability in date palm (Table 1). The 22 selected media compositions (Table 1) produced significantly different results. Several concentrations of H_3BO_4, $Ca(NO_3)_2.4H_2O$, KNO_3 and $MgSO_4.7H_2O$ were ignored because they had no or very low effect on pollen germination (Table 1). Genetic variation in among male date palm genotypes and different media components clearly affected pollen germination.

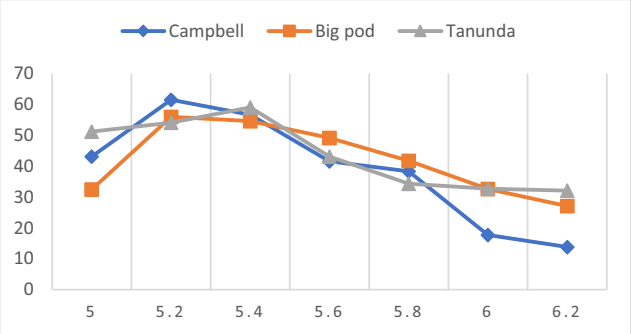


Figure 2 Effect of the medium pH on germination of pollen from Australian date palm selections "Big pod", "Campbell" and "Tanunda". LSD = 2.127. Each measurement is the average of 9 fields of view.

The medium optimized for the three male genotypes ("Big pod", "Campbell" and "Tanunda") might be applicable for optimizing germination in other date palm genotypes (Table 2). However, Weinbaum *et al.* (1984) reported that pollen germination capability differed between varieties of the same and different species. According to the current study, the optimized medium contained 15% (w/v) sucrose, 50 mg/l boric acid, 75 mg/l calcium nitrate, 400 mg/l potassium nitrate, 100 mg/l magnesium sulphate and 50 mg/l MOPS (MW209) added to the Brewbaker and Kwack basal medium. The boric acid concentration was also similar to the concentration reported by Mortazavi *et al.* (2010). However, the medium in the current study differed from those previously reported for sucrose (10% w/v) and calcium nitrate (150 mg/l). The optimal concentration of calcium in the culture medium can play a very important role in enhancing pollen germination; however, inhibitory effects can also occur if the concentration is not optimized (Brewbaker and Kwack 1963; Malik and Mehan 1975; Khan and Perveen 2006). This can sometimes cause calcium toxicity in the medium, as observed in cultivar "Abdolreza" by Kavand *et al.* (2014). Pollen tube elongation usually depends on exterior calcium stored in the pistil (Ge *et al.* 2007); thus, Ca^{2+} is responsible for regulating the growth of pollen tubes (Prajapati and Jain 2010). In the current study, significant differences in pollen germination were associated with boric acid and calcium nitrate treatments (Table 1). The concentrations of sucrose (15% w/v), 75 mg/l, H_3BO_4, 400 mg/l $Ca(NO_3)_2.4H_2O$, 100 mg/l KNO_3 and 50 mg/l $MgSO_4.7H_2O$ produced significantly better results than

Table 1 Effect of boric acid, calcium chloride, potassium nitrate and magnesium sulphate on germination of pollen from three Australian date palm genotypes. All media were supplemented with 15% sucrose and adjusted to pH 5.4

Genotype	H_3BO_4		$Ca(NO_3)_2$		KNO_3		$MgSO_4$	
	Conc. (mg/l)	Germination (%)	Conc. (mg/l)	Germination (%)	Conc. (mg/l)	Germination (%)	Conc. (mg/l)	Germination (%)
Campbell	25	20.96 k	50	23.47 i	50	14.44 ef	25	8.43 n
	50	32.92 ij	100	35.12 f	100	52.73 a	50	63.59 a
	75	46.4 d	200	13.65 l	200	34.55 cd	100	33.16 f
	100	20.65 k	300	37.08 e	300	28.46 d	200	29.03 hi
	150	33.84 i	400	56.52 c	400	18.86 de	300	11.37 m
			500	9.04 n			400	9.41 n
Big pod	25	31.97 j	50	18.73 k	50	15.17 e	25	12.85 l
	50	49.06 c	100	20.42 jk	100	49.44 b	50	60.56 b
	75	69.76 a	200	10.2 n	200	36.5 cd	100	29.67 h
	100	37.61 g	300	20.73 j	300	18.61 de	200	38.26 e
	150	40.15 f	400	60.95 a	400	14.88 ef	300	26.08 i
			500	19.41 jk			400	14.92 k
Tanunda	25	36.17 gh	50	27.41 h	50	38.06 c	25	10.92 mn
	50	42.94 e	100	28.85 g	100	45.96 bc	50	50.63 c
	75	50.33 b	200	20.1 jk	200	30.88 cd	100	43.47 d
	100	34.23 h	300	48.67 d	300	24.93 de	200	31.99 fg
	150	34.01 hi	400	59.12 b	400	26.47 de	300	21.25 j
			500	12.98l m			400	31.26 g

Values with different *letters* within *columns* are significantly different ($P < 0.05$)

all other treatments and this treatment was used for all subsequent pollen viability testing. Pollen germination and pollen tube length varied among genotypes and the differences were statistically significant. Genotype "Tanunda" had higher respective pollen germination and pollen tube length (77.75%, 104.33 mm) than "Campbell" (63.28%, 95.44 mm) (Table 2; Fig. 3).

Pollen Viability and Stainability Tests Figure 4 shows the germination of *P. dactylifera* pollen grains subjected to six histochemical tests recommended for the evaluation of pollen viability. In the acetocarmine stain, red colouring was indicative of viable pollen grains, whereas the non-viable pollen grains were colourless, especially at low levels of illumination. However, the tests that relied on differences in depth of staining (lactophenol cotton blue, IKI and 2,3,5-triphenyltetrazolium) required more detailed observation and interpretation to achieve consistent results. The results of all staining tests are shown in Table 3. Genotype × staining test interaction was significant for pollen viability percentage in all five tests except FCR. Differences among genotypes were recorded for the FCR test, which was relatively closely related to *in vitro* germination results (Table 3). Each of the other five tests indicated approximately 80% viability regardless of the *in vitro* germination percentages and therefore appeared to be of little value for the assessment of pollen viability in *P. dactylifera*. Lack of agreement between *in vitro* pollen germination and viability tests using various staining procedures has often been reported and that is supported by the current study. Viability percentages were higher (due to false positives) in the acetocarmine staining test compared to germination on culture media (Shaheen 2004), whereas Khatun and Flowers (1995) found that both 2,3,5-triphenyltetrazolium and lactophenol cotton blue were essentially useless in rice, as observed for date palm in the current study.

Table 2 Pollen germination of selected genotypes using optimized germination medium

Genotype	*In vitro* germination	Pollen tube length (μm)
Campbell	63.28 a	95.44 a
Big pod	69.31 b	99.00 b
Tanunda	77.75 c	104.33 c

Values with different *letters* within the same *column* are significantly different ($P < 0.05$)

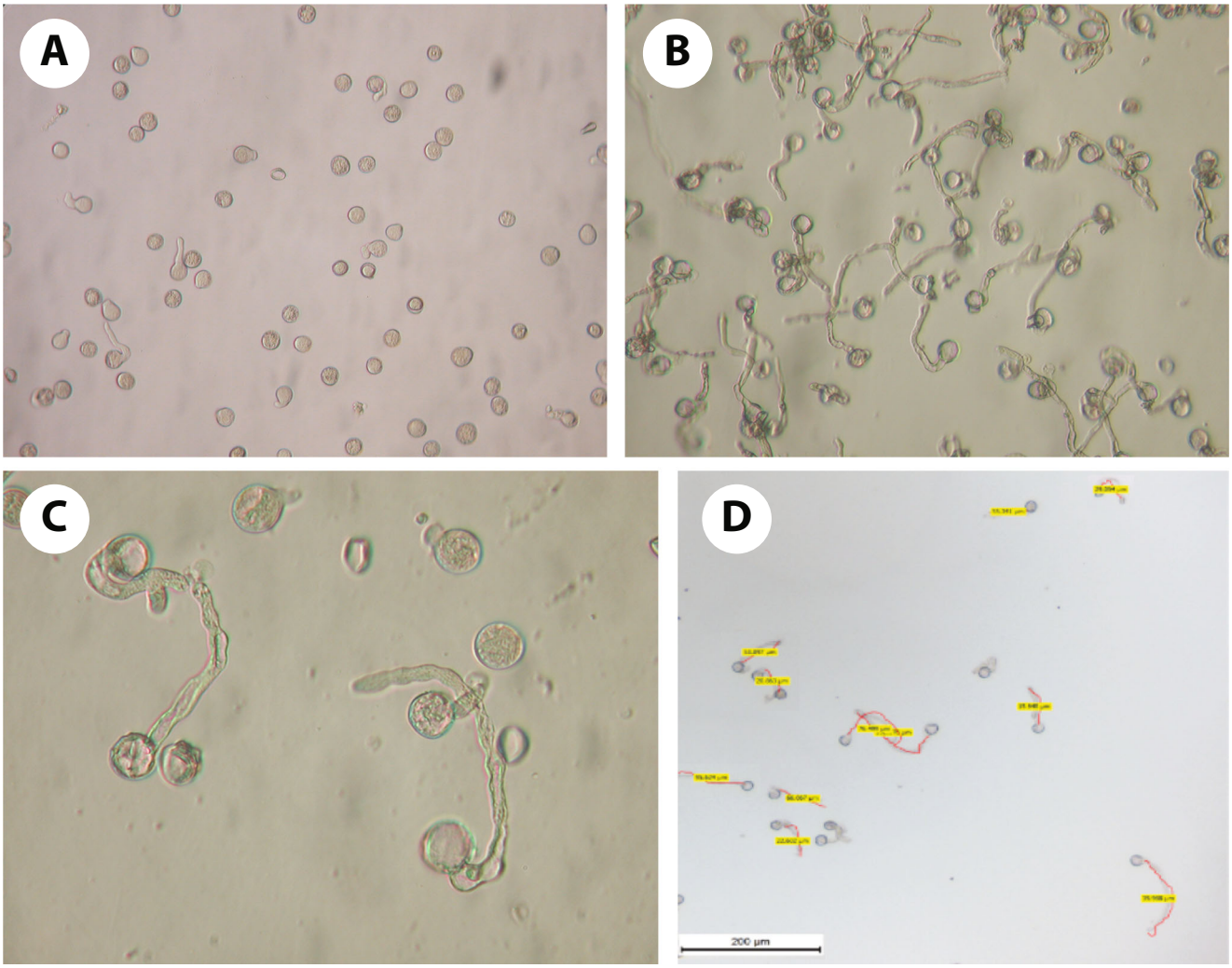


Figure 3 *In vitro* pollen germination and pollen tube growth of Australian male date palm selections on BK medium. (*A*) Pollen germination on basic BK medium. (*B*) Pollen germination on modified and optimized BK medium. (*C*) Magnified view of the germinated pollen grains. (*D*) Pollen tube length measurements.

The correlation between pollen viability and *in vitro* pollen germination was also determined, and this varied depending on the stain used. FDA, in particular, was significantly correlated with germination % (Table 4), but correlations with other tests were generally low. These results are identical to those reported by Parfitt and Ganeshan (1989), who concluded that pollen stain tests (including TTC) were not reliable nor consistent and generally not indicative of *in vitro* pollen germination. However, this study showed that FDA can be used for testing pollen viability in date palms.

Effect of Pollen Storage Temperature and Duration on Pollen Viability Short-term storage of pollen grains for different periods had a significant effect on pollen viability (Table 5). All three genotypes showed similar trends after 12 mo of refrigeration at 3–4°C and the refrigerated pollen had lower viability than fresh pollen. However, pollen viability was about 50% after storage for 12 mo and these levels were similar to those reported by Al Taher and Asif (1982).

Stigma Receptivity Stigma receptivity was determined chemically by testing enzymatic activity using hydrogen peroxide (Fig. 5*A*) and Peroxtesmo KO (Fig. 5*B*, *C*) tests. Maximum stigma receptivity was observed during the first 24 h of anthesis. Stigmatic tissue receptivity was also confirmed by observation of pollen tube growth within the pistil.

Male/Female Compatibility and Physical and Biochemical Characteristics of the Fruit Pollinations of cv. "Barhee" with each of the three Australian male selections were performed (Barhee × Campbell; Barhee × Tanunda; Barhee × Big pod) using pure pollen to test male/female compatibility (Fig. 6).

The effects of pollen source (or pollinator) on fruit setting, fruit size and the fresh weight of the fruit and seed were significant (Table 6; Fig. 7). Significant differences were

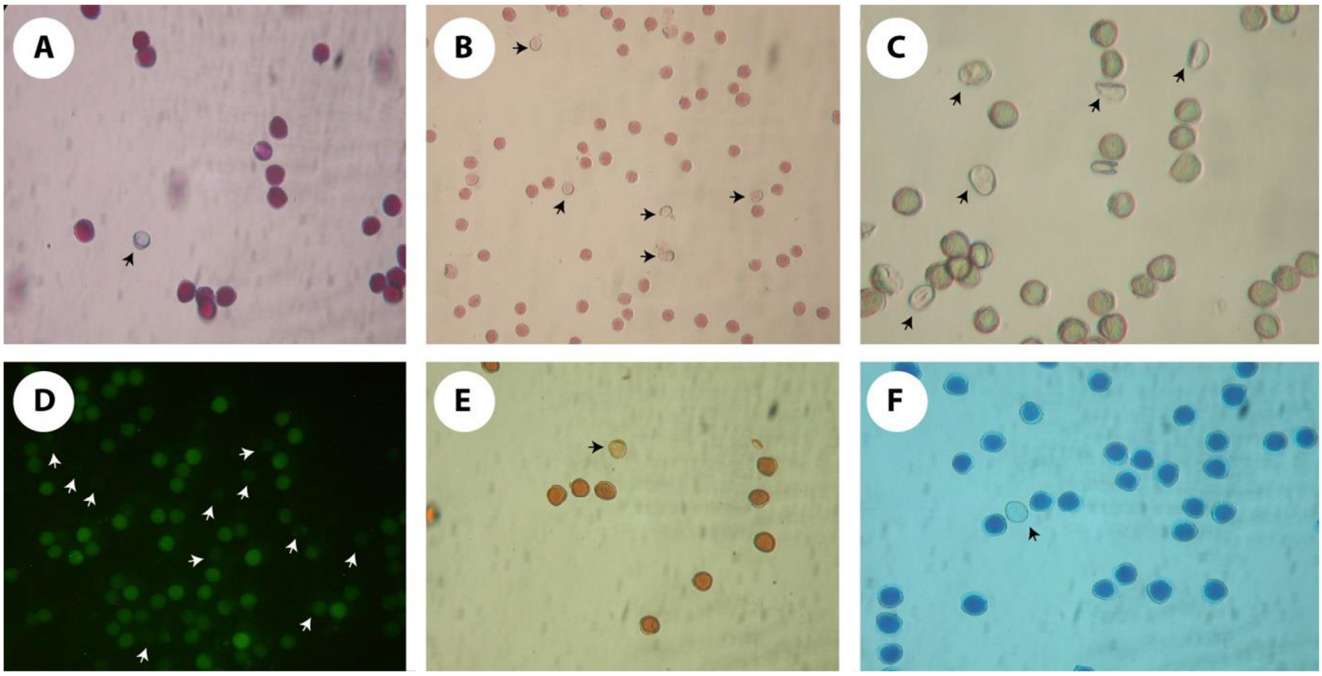


Figure 4 Pollen viability in *P. dactylifera* genotype "Tanunda". Non-viable grains are indicated by *arrows*. (*A*) Alexander's staining test, (*B*) acetocarmine test, (*C*) tetrazolium reaction test, (*D*) FCR test, (*E*) IKI test and (*F*) lactophenol cotton blue test.

observed among fruits produced by pollen from each of the three male genotypes. Fruits produced from pollen of "Tanunda" were larger and heavier than those produced from "Big pod" and "Campbell" pollen. The lowest mean seed weight was obtained from "Big pod" pollen. The pollinator "Tanunda" also gave the highest flesh/seed ratio (13.5). Similar effects using other crosses were reported by Khalifa *et al*. (1980), Nixon (1935), Osman *et al*. (1974), Ream (1976), Shafaat and Shabana (1980) and Swingle (1928).

The pollinator clearly affected fruit setting (Table 6). Pollination with "Tanunda" minimized partial fruit set failure

Table 3 Viability of *P. dactylifera* pollen assessed by an optimized *in vitro* procedure and staining by six histochemical methods. Mean percentages of viable pollen are shown for three genotypes

Genotype	*In vitro* germination	FDA	TZ	IKI	Acetocarmine	Lactophenol cotton blue	Alexander's stain
Campbell	63.28 c	61.97 b	78.70 a	79.53 b	79.10 a	80.97 b	78.20 c
Big pod	69.31 b	71.77 a	81.41 a	81.00 ab	79.70 a	80.84 b	79.74 b
Tanunda	77.75 a	73.50 a	82.61 a	82.78 a	81.20 a	82.70 a	81.57 a

FDA, fluorescein diacetate; *TZ*, 2,3,5-triphenyltetrazolium chloride; *IKI*, iodine potassium iodide. Values with different *letters* within *columns* are significantly different ($P < 0.05$)

Table 4 Pearson correlation and regression analysis between *in vitro* pollen germination of the Australian selection "Big pod" and two viability (tetrazolium and FCR) and four stainability tests

	Pollen viability and stainability test						
	In vitro germination	I-KI	Acetocarmine	Lactophenol cotton blue	Tetrazolium test	FCR	Alexander's stain
Pollen viability (%) *	70.1 ± 2.10	93.7 ± 1.65	92.8 ± 1.55	94.9 ± 1.24	80.9 ± 0.79	69.4 ± 1.96	93.8 ± 1.64
Pearson's r		0.303	0.085	0.186	0.698	0.923	0.040
Regression equation		Y=77.2+0.24x	Y=88.5+0.06x	Y=87.3+0.11x	Y=62.7+0.26x	Y=10+0.85x	Y=91.6+0.03x

*Percentages of *in vitro* germination, viability and stainability ± standard error

Table 5 Effect of cold storage at 4°C on pollen germination of three male genotypes of date palm (*P. dactylifera*)

Genotype	*In vitro* pollen germination (%) after different times of storage				
	Fresh pollen	Three months	Six months	Nine months	Twelve months
Campbell	63.3	60.0	55.4	51.1	48.6
Big pod	69.3	65.3	59.3	54.1	50.4
Tanunda	77.8	71.6	65.1	60.3	56.1

LSD values: genotype, 1.226; storage time, 1.583; interaction, 2.742

and the number of parthenocarpic fruit (Fig. 7) compared to other pollen sources.

Partial fruit set in date palm can occur under hot arid conditions and this is one of the main problems limiting date palm productivity (Awad and Al-Qurashi 2012). A significant interaction between season and pollinator was reported in cv. "Nabbut-Ali" and identified as the reason for partial fruit set (Awad and Al-Qurashi 2012). Moreover, differences in pollen germinability and pollen tube length among different pollen sources also affected fruit setting and yield of female parents.

Variability in the percentage of normal fruit setting on cv. "Barhee" with different pollen sources was also affected by variation in inter-clonal incompatibility between the pollen source and female cultivar. Incompatibility depends on a recognition response between specific incompatibility proteins carried by the pollen grains and the matching proteins produced in the stigmas or styles (Heslop-Harrison 1975). This recognition results in variable degrees of acceptance, ultimately resulting in the production of variable numbers of seedless parthenocarpic fruits.

In the trees studied, the occurrence of abnormal multicarpel fruit was very high in most of the TC-derived palms (Fig. 8). Similar observations were reported by Al-Khalifah (2006) and Cohen *et al.* (2004), who compared fruit derived from TC-derived and off-shoot-derived plants from cv. "Barhee". Structural abnormalities as well as flower abnormalities might correlate to the poor fruit setting obtained from controlled pollination experiments. For example, the styles of distorted flowers were longer and the stigmas twisted, pointing in various directions, and attached with a narrow carpel head (Fig. 8H) compared to normal stigmas shown in Fig. 5. These abnormalities possibly caused failures in pollen tube growth and thus lack of fertilization. Similar results were reported by other researchers (Cohen *et al.* 2004; Shair *et al.* 2016). However, the current study indicated not only flower abnormalities contributing to low fruit setting, but also that the pollen source has a direct effect on fruit setting success and eventually yield.

Distribution of Organic Metabolites The three male parents had different effects on the physical and chemical characteristics of fruit produced on cv. "Barhee" (Tables 7 and 8). These differences were possibly responsible for some of the manifestations of metaxenia observed in this study (Tables 7 and 8) and reported by others (Nixon 1935; Aldelaim and Ali 1969; Denney 1992). Swingle (1928) was the first to suggest that metaxenia can occur because of endogenous hormones produced by the embryo or endosperm. Significant variation in malic acid, citric acid, fructose, glucose, sucrose, L-proline, *myo*-inositol and other chemicals in the different pollen sources in this current study could be used to identify the most suitable pollen for pollinating "Barhee". Pollen source had a significant influence on levels of these substances in fruit produced on "Tanunda" (Tables 7 and 8). Fruit produced by "Big pod" pollen obtained the highest levels compared to "Campbell" pollen at the Khalal stage. Pollination source also

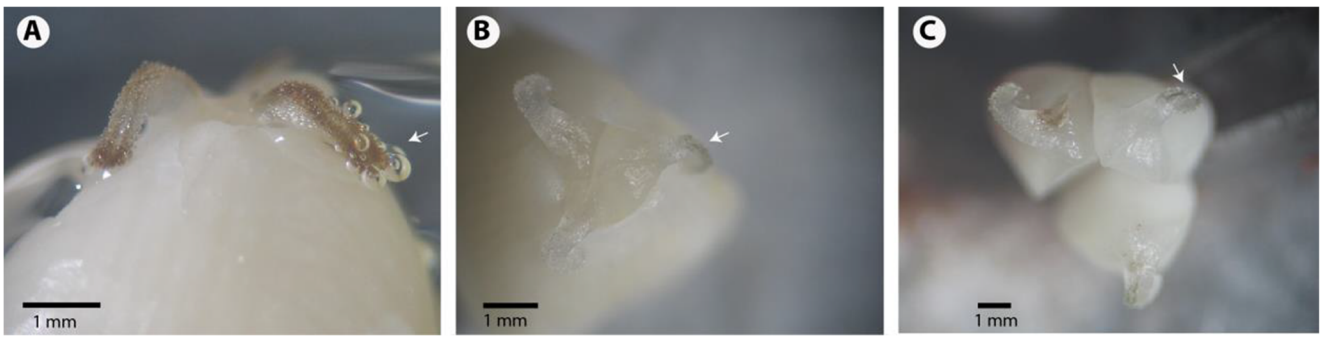


FIGURE 5 Stigma receptivity in *P. dactylifera* cv. "Barhee". (*A*) Hydrogen peroxide test showing bubbles; (*B*) esterase enzymatic indicator; (*C*) enzymatic reaction test showing a receptive stigma. *Scale bar* = 1.0 mm.

Figure 6 Male selections used for pollination. Tree morphology and spadix of each type are shown.

Table 6 Effect of pollen source on the physical characteristics of fruits and seeds produced on date palm cv. "Barhee"

Pollen source	Fruit length (mm)	Fruit diameter (mm)	Fruit weight (g)*	Seed length (mm)	Seed width (mm)	Seed weight (g)	Fruit setting (%)**
Big pod	38.98 ab	26.18 c	13.89 b	23.84 c	9.96 c	1.08 c	18
Campbell	37.60 b	27.16 b	14.53 b	25.98 b	10.11 b	1.16 b	13
Tanunda	40.20 a	29.54 a	18.57 a	28.04 a	10.44 a	1.28 a	22

Values with different *letters* within *columns* are significantly different ($P < 0.05$).

*Including the seed

**Mean of three inflorescences pollinated by freshly harvested pollen

affected total sugar percentages, although this was not statistically significant. The highest average total sugar (74.72%) was recorded in "Barhee" pollinated with "Tanunda". The lowest (51.33%) was recorded in "Barhee" pollinated with "Big pod". El-Kassas *et al.* (1996), Marzouk *et al.* (2002a), Marzouk *et al.* (2002b) and Ghaffar and Iqbal (2003) also reported that male source had a significant effect on total sugar content of the resulting fruit. Similar effects were observed for other compounds, including malic acid, citric acid, fructose, glucose, L-proline and *myo*-inositol. For instance, the highest

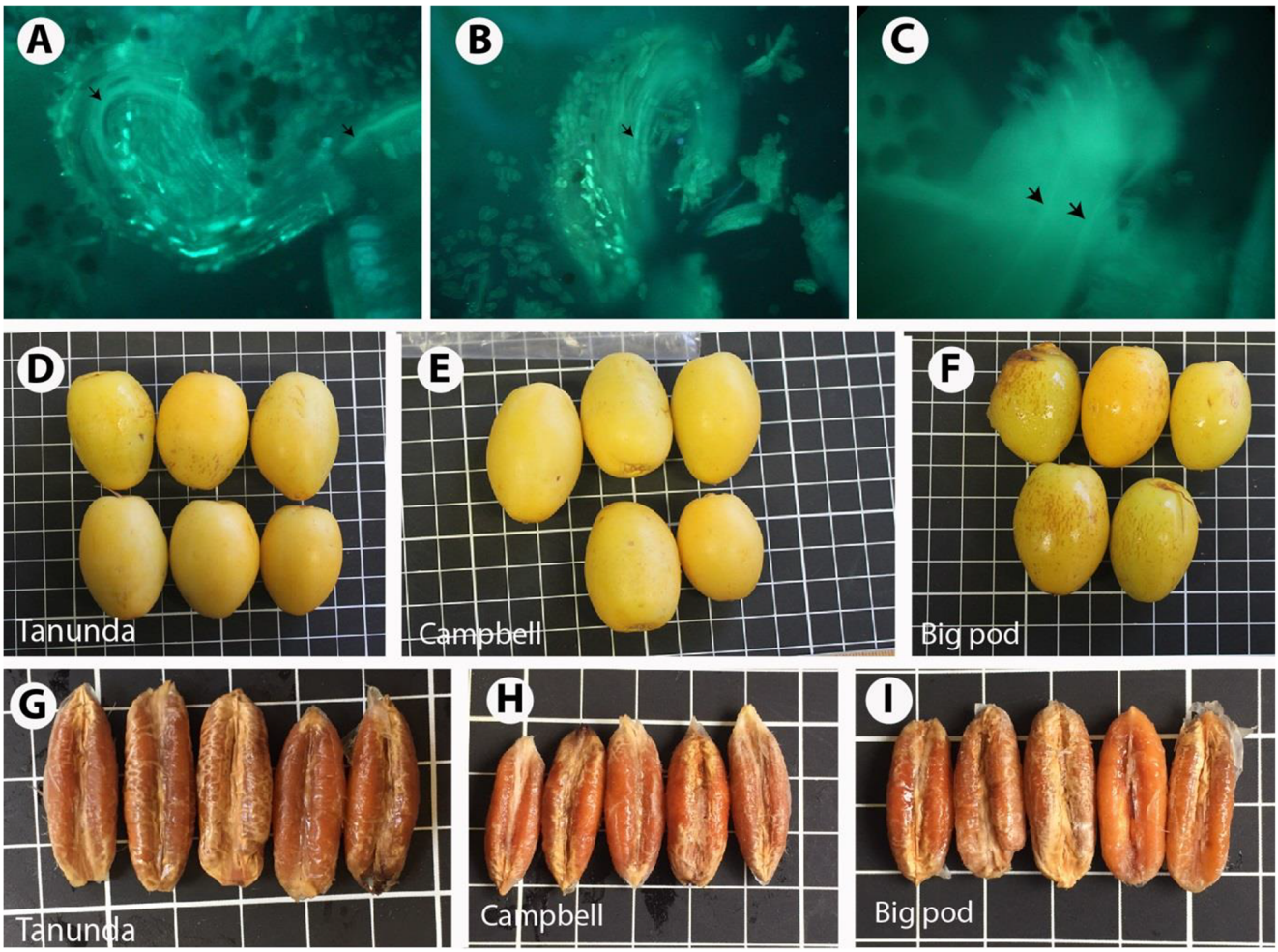
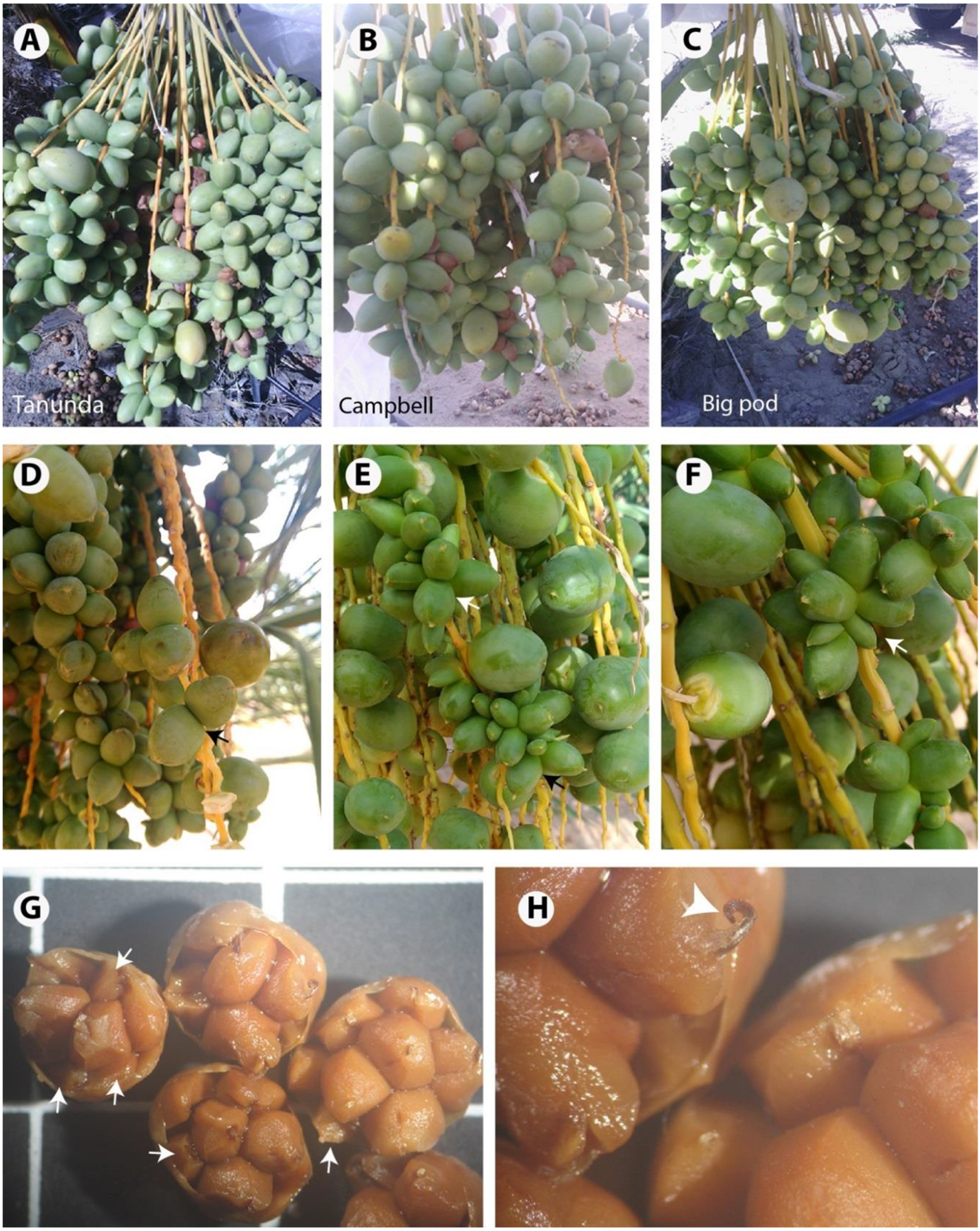


Figure 7 Male/female compatibility test, metaxenic and xenic effects of the pollinator on the physical characteristics of cv. "Barhee". (*A–C*) Fluorescence microscopic images showing pollen-style compatibility indicated by pollen germination on stigmatic surfaces and pollen tube growth through the styles (*arrows*). (*D–F*) Fruit setting on "Barhee" inflorescences showing higher settings in those inflorescences pollinated with "Big pod" and "Tanunda" compared to that pollinated by "Campbell". (*G–I*) Seed physical characteristics in as affected by the pollinator.



average fructose content was observed in fruit produced on "Big pod" (399.43 mg/ml) and the lowest on "Tanunda" (213.59 mg/ml). Similarly, the highest average of glucose content was observed in fruit from "Big pod" pollen

◀ **Figure 8** Effect of pollen source on fruit setting on TC-derived "Barhee" with a high incidence of flower abnormalities. (*A–C*) Pollen source effect on fruit setting success indicated by normally fertilized fruitlets. (*D–F*) Multi-carpel formation among triple parthenocarpic fruitlets showing fruitlets having two in *D*, three and multiple carpels in *D–F* (*arrows*). (*G*, *H*) Supernumerary carpels (*arrows*) in *G* and distortions of carpels and stigmas shown in *H* contribute to structural abnormalities and lower fruit setting. Cutting board *squares* in *G* are 1 × 1 cm.

(161.70) and the lowest from "Tanunda" (91.56). For glutamine, "Campbell" pollen gave the highest average (0.50) and "Tanunda" the lowest (0.22). Differences in the levels of these chemicals among date palm fruit attributed to different pollen sources might explain, in part, the observed metaxenia.

Conclusions

The FCR test was found to be an excellent guide to pollen viability and was positively correlated with *in vitro* germination. The optimal germination media comprised 15% (w/v) sucrose, 75 mg/l H_3BO_4, 400 mg/l $Ca(NO_3)_2.4H_2O$, 100 mg/l KNO_3 and 50 mg/l $MgSO_4.7H_2O$ at a pH of 4.3.

Table 7 Effect of pollen source on concentrations (mg/ml) of various organic metabolites in fruit

Metabolite	Pollen source			
	Big pod	Campbell	Tanunda	Fruit stage
Malic acid	1.95 b	3.72 a	1.10 c	Khalal
	1.39 c	1.55 b	4.43 a	Rutab
Mannose	6.78 b	9.80 a	5.09 c	Khalal
	9.87 a	7.69 c	9.34 b	Rutab
Citric acid	7.72 a	7.07 b	4.09 c	Khalal
	5.73 a	4.49 b	3.81 c	Rutab
Fructose	399.4 a	333.4 b	213.6 c	Khalal
	325.6 a	320.9 b	272.6 c	Rutab
Glucose	161.7 a	122.3 b	91.6 c	Khalal
	164.5 a	159.8 b	132.5 c	Rutab
Sucrose	51.3 c	61.8 b	74.7 a	Khalal
	32.2 c	65.7 b	165.5 a	Rutab
Myo-inositol	0.61 a	0.60 a	0.45 b	Khalal
	0.96 a	0.61 b	0.52 b	Rutab
L-Ascorbic acid	0.0 a	0.0 a	0.0 a	Khalal
	4.66 a	4.99 a	4.92 a	Rutab
D-Sorbitol	1.95 b	3.84 a	1.76 b	Khalal
	14.76 b	19.59 a	20.34 a	Rutab

Values with different *letters* within *rows* are significantly different ($P < 0.05$)

Table 8 Means (mg/ml) for various amino acids (LC-MS) in fruits resulting from use of different pollinators

Metabolite	Big pod	Campbell	Tanunda	Stage
Serine	0.32 a	0.0 c	0.07 b	Khalal
	0.0 b	0.05 a	0.06 a	Rutab
Glutamine	0.31 b	0.50 a	0.22 c	Khalal
	0.60 c	0.99 a	0.84 b	Rutab
Proline	0.0 a	0.10 a	0.10 a	Khalal
	0.0 c	0.34 a	0.09 b	Rutab
Isoleucine	0.01 a	0.009 a	0.01 a	Khalal
	0.04 a	0.02 b	0.02 b	Rutab
Leucine	0.01 ab	0.003 b	0.02 a	Khalal
	0.03 a	0.02 a	0.01 a	Rutab
Tyrosine	0.01 a	0.01 a	0.01 a	Khalal
	0.02 ab	0.03 a	0.01 b	Rutab
Phenylalanine	0.02 a	0.02 a	0.02 a	Khalal
	0.04 a	0.02 a	0.02 a	Rutab

Values with different *letters* within *rows* are significantly different ($P < 0.05$)

The storage conditions of pollen grains were important for situations when artificial pollination is required. Storage temperature and duration had a significant impact on short-term pollen viability. Storage of pollen at 4°C maintained adequate pollen viability for 9 mo. Stigma receptivity was highest within the first day after spathe cracking and the Peroxtesmo esterase and hydrogen peroxide (6%) tests were effective in establishing of stigma receptivity.

Three pollen sources showed variable responses in fruit characteristics. However, there is a need to identify the most suitable and compatible pollinator, especially if the pollen is amenable to long-term storage. These traits are important for commercial fruit production. Moreover, the three pollen sources had significant impacts on the physical and biochemical characteristics of the fruit produced on cv. "Barhee". Compatibility studies are required to determine the most compatible (male/female) combinations. This is essential for establishing or expanding highly productive date palm orchards. Large-scale multiplication (micropropagation) of the most effective male parent would maximize the yield of date palms in Australia and elsewhere.

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