Sex Plant Reprod (1991) 4:182–187

Sexual Plant Reproduction

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Germination of *Pistacia vera* L. pollen in liquid medium

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Summary. The osmotic effect of Polyethylene glycol (PEG) has been shown to be sufficient to induce the germination of *Pistacia vera* L. pollen in liquid medium. The prehydration of the pollen in a saturated atmosphere for approximately 10 h was necessary to obtain maximum in vitro germination. Imbibition of the pollen in water resulted in the rapid leakage of solutes into the medium. These solutes consisted of approximately 50% carbohydrates, of which sucrose (0.65 μmol/mg), glucose (0.77 μmol/mg) and fructose (0.78 μmol/mg) were the major sugars; the remaining 50% comprised proteins with the following major molecular weights 63 kDa, 60 kDa, 59 kDa, 40 kDa, 36 kDa, 35.5 kDa, 31 kDa, other organic matter and minerals.

Key words: *Pistacia vera* L. – In vitro germination – Pollen – Osmotic effect

Introduction

Pollen grains of *Pistacia vera* L. have been considered to be difficult to germinate in vitro (Crane et al. 1974; Crane and Iwakiri 1981; Vithanage and Alexander 1985). This is in spite of the bicellular nature of its pollen (Copeland 1955; A. Golan-Goldhirsh unpublished data), which usually ensures ready germination (Knox et al. 1986). Recently it has been shown that controlled hydration of these pollen prior to incubation in germination medium increased percent germination (Polito and Luza 1988). The germination of pollen in vitro is greatly influenced by the relative humidity at which the pollen is pretreated (Gilissen 1977). The assessment of pollen quality by its in vitro germination is a useful method for determining the acceptability of pollen for artificial pollination (Stanley and Linskens 1974) as well as in biochemical studies on pollen viability. However, negative results must be interpreted cautiously as a lack of pollen germination may reflect either inviability or a deficiency in some requirement for pollen germination (i.e. hydration state, medium composition, temperature, etc.). Pollen of some species can germinate in water (Duffield 1954), while other species require more complex media for germination (Stanley and Linskens 1974). The importance of water and the effect of the osmotic potential of the medium on pollen germination has been reported extensively (Heslop-Harrison 1979; Dumas et al. 1988; Hoekstra 1986, and others). Nevertheless, there is still a lack of understanding of the early events in pollen hydration and germination (Crowe et al. 1989). In the present article we report the effect of water and medium osmotic potential on the germination and structure of *Pistacia vera* L. pollen in liquid medium.

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Materials and methods

Pollen of *Pistacia vera* L. cv 'Chico' were collected during the season of 1988 and sieved through a 90-μm sieve net. The pollen were kept at 4° C in containers plugged with cotton wool. The water content of the pollen was approximately 5% throughout the storage period.

Pollen germination in vitro

Pollen germination in vitro was done after hydration in a water-saturated atmosphere in closed containers or by direct imbibition in distilled water at room temperature. The liquid medium for germination contained 1.3 mmol/l calcium nitrate ($Ca(NO_3)_2 \cdot 4H_2O$), 0.81 mmol/l magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1 mmol/l potassium nitrate (KNO_3), 1.6 mmol/l boric acid (H_3BO_3) and 0.5 mmol/l sucrose (Harris et al. 1987). Sucrose or polyethylene glycol (PEG) were added to this medium as indicated. Pollen grains (40 mg) were added to 5 ml medium in a 100-ml Erlenmeyer flask and allowed to germinate for 12 h at 26° C on a rotatory shaker at 100 rpm. An aliquot (20 μl) of the suspension was taken for determining pollen germination using an automatic pipette fitted with a 200-μl capacity tip, the distal 2.5 mm of which had been cut off. This prevented breakage of the pollen tubes (Harris et al. 1987). Pollen tube length was measured on a Image Processing System (Kontron M15) attached to a Zeiss IM microscope via a video camera (Sit 66). Pollen grains were stained with Alexander stain for light microscopy (Alexander 1969). Scanning electron mi-

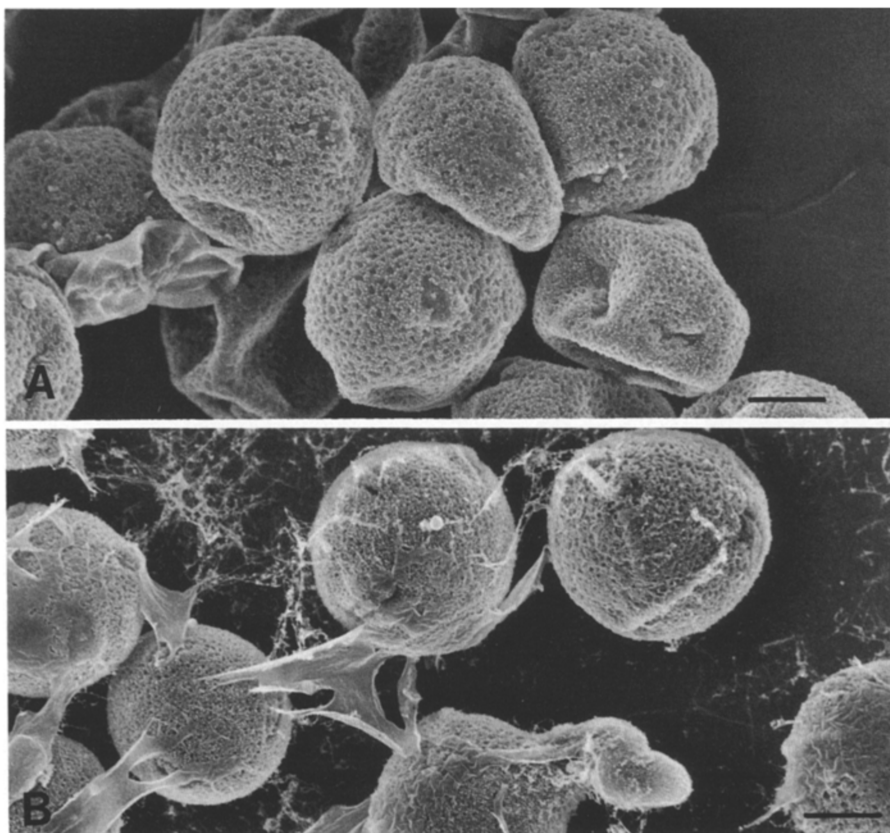

Fig. 1 A, B. Scanning electron micrograph of *P. vera* L. pollen. **A** Dry pollen, **B** water-imbibed pollen. *Bar* = 10 μm

crographs of gold-coated specimens after freeze drying were taken on a Hitachi S 900 microscope.

SDS-Polyacrylamide gel electrophoresis

SDS-PAGE was carried out on 10% polyacrylamide gel according to Laemmli (1970) and stained with coomassie brilliant blue.

Analytical procedures

Total C, N and S were determined with a Carlo Erba nitrogen analyser model 1500 (Carlo Erba Strumentazione, Milan, Italy). K, Ca, Mg, P and B were determined with an inductively coupled plasma emission spectrometer (Perkin Elmer, model ICP/5500, Ueberlingen, Germany). Nitrate was measured with a nitrate electrode (Orion, model 93-07) and sulfate was determined, semi-quantitatively with a reagent kit for photometric analysis (Merck, Microquant 14789). Vitamin C was determined fluorometrically using a centrifugal analyser equipped with fluorescence immunoassay attachment (Vuilleumier and Keck 1989). Sugars were determined by HPLC with a BIO-RAD HPX-87C column (Richmond, Calif.). The osmolalities of the media were determined by a vapour pressure osmometer (Wescor, 1500 B). Electrical conductivity was measured with a conductometer (E 518 Metrohm, Herisau); this was converted to osmolality assuming that $1\ mS \cdot cm^{-1} = 14.2\ mOs \cdot kg^{-1}$.

Results

Pollen hydration

A scanning electron micrograph of water-imbibed *P. vera* L. pollen is shown in Fig. 1 A, B. In the dry state the pollen appeared shrunken with a convoluted surface (Fig. 1 a). After being subjected to water for approximately 10 min, the pollen swelled and became spherical (Fig. 1 b). No wall rupture was seen, but there was a leakage of solutes to the medium (Fig. 2). It appeared as if the water-extractable matter came out the wall matrix. The vesicles (v, Fig. 2) on the wall surface resisted the water wash. During the three washes of the pollen with water (ca. 10 min each time) there was a rapid leakage of solutes, with approximately 32% of the pollen dry matter leaking out in the first wash (Fig. 3). In the following two washes only small amounts of dry matter came out of the pollen (Fig. 3). The leakage of solutes from pollen suspended in germination medium was difficult to measure because of the high concentration of solutes in the medium itself. Preliminary data (not shown) indicated that in germination medium both the rate of leakage and the quantity of solutes leaked were lower than in distilled water. The composition of the water-washable solutes from the pollen in distilled water is shown in Table 1. Approximately 50% of these solutes were carbohydrates, of which sucrose (0.65 μmol/mg), glucose (0.77 μmol/mg) and fructose (0.78 μmol/mg) were the major sugars. The nitrogenous portion of the dry matter contained a protein fraction that was composed of a large number of proteins (Fig. 4). The major polypeptides had molecular weights of 63 kDa, 60 kDa, 59 kDa, 40 kDa, 38 kDa, 36 kDa, 35.5 kDa and 31 kDa (Fig. 4). Nitrate could not be detected with the nitrate electrode, and sulfur was mainly in the form of sulfates forming approximately 1% of the dry matter (Table 1).

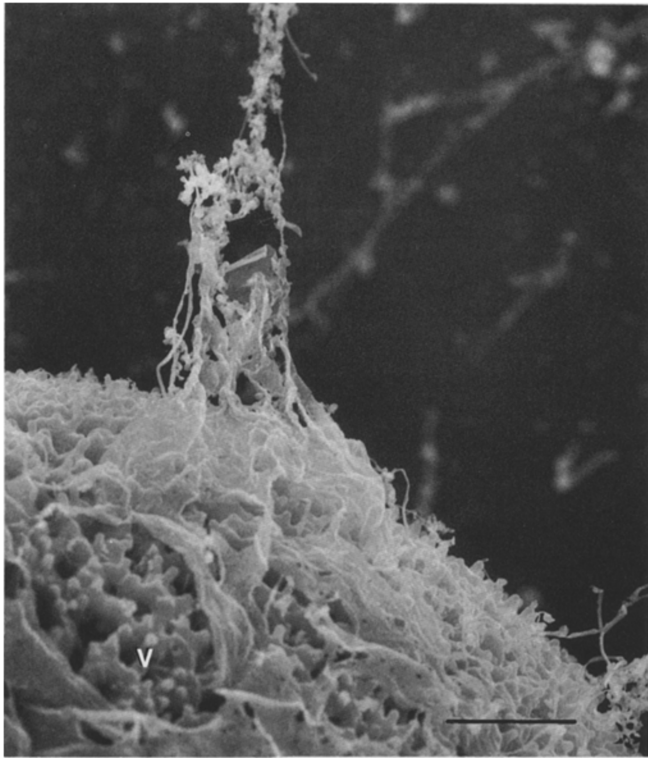

Fig. 2. Scanning electron micrograph of *P. vera* L. pollen surface after washing with distilled water. *v*-vesicles. *Bar* = 2 μm

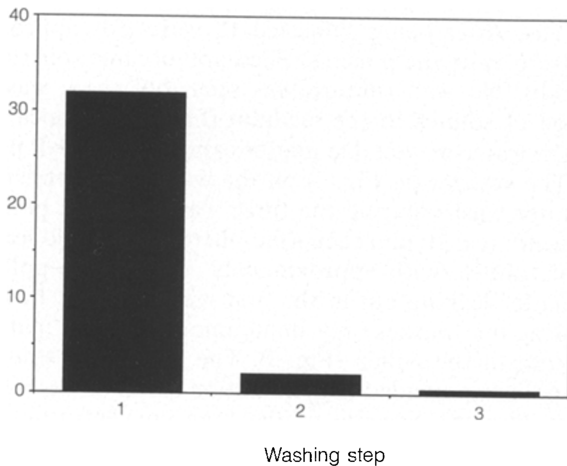

Fig. 3. Water washing of *P. vera* L. pollen, in three steps of approximately 10 min each

Table 1. The composition of water-washable solutes from *Pistacia vera* L. pollen

Component	Dry weight (%)
Nitrogen	3.82 ± 0.03
Carbon	39.76 ± 0.4
Sulphur[a]	0.31 ± 0.01
Ash:	6.94 ± 0.04
Potassium	3.85
Calcium	0.11
Magnesium	0.11
Phosphorus	0.92
Boron	0.002
Vitamin C	0.021
Sugars:	50.3 ± 3.0
Sucrose (μmol/mg)	0.65 ± 0.06
Glucose (μmol/mg)	0.77 ± 0.08
Fructose (μmol/mg)	0.78 ± 0.06

[a] As 1% sulphate

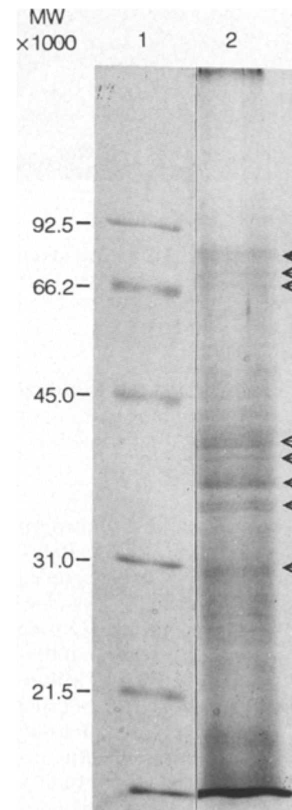

Fig. 4. SDS-PAGE of pistachio pollen water-washable fraction (*2*) and standard proteins (*1*). The *arrows* point to the major polypeptide bands

Osmotic effect

The *P. vera* L. pollen germinated poorly after imbibition in distilled water. However, after prehydration in a water-saturated atmosphere there was an increase in germination in both liquid nutrient media (Fig. 5). When the water content of the pollen was approximately 20% water, germination was highest in the sucrose-containing medium (Fig. 5).

The low osmotic potential required for pistachio pollen germination led to the hypothesis that an osmotic component is strongly involved in the initiation of pollen germination. The replacement of sucrose with PEG at the same osmolality (1000 mosmol/kg) resulted in about 10% pollen germination (Fig. 5). While germination in medium containing PEG was lower than in medium containing sucrose, it did indicate that an osmotic signal might be involved in pistachio pollen germination. PEG does not enter the cells; therefore, the low osmotic po-

tential of the medium led to shrinkage of the germinated pollen (Fig. 6a), in contrast to well swollen pollen in sucrose-containing medium (Fig. 6b). Pollen tube length in both media is shown in histograms (Fig. 7). The pattern of pollen tube length distribution was similar in both media. There was a large variability in tube length, 250±142 μm and 263±172 μm in sucrose and PEG, respectively. This indicated that pollen viability and maturity at dehiscence and dispersal were quite variable.

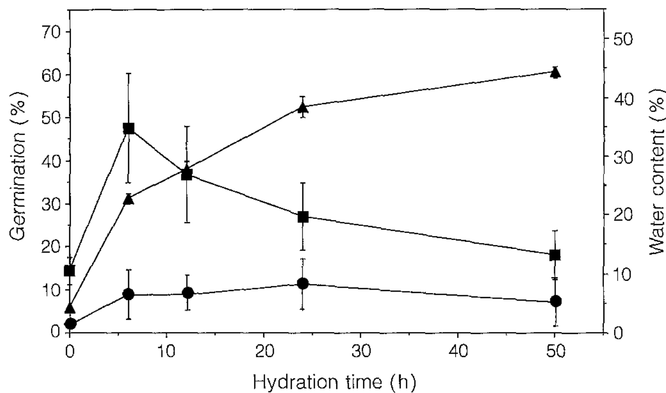

Fig. 5. Germination of pistachio pollen in media containing sucrose (■ ■) or PEG (● ●). The osmolality of each medium was approximately 1000 mosmol/kg. ▲ ▲ the water content of prehydrated pollen

Discussion

The test most commonly used to assess pollen viability is in vitro germination. The known difficulties in germinating pistachio pollen can be partially overcome by prehydration, as has been shown by Polito and Luza (1988) and in this work (Figs. 5 and 6). While artificial medium cannot fully simulate the complex pollen-stigma interaction in vivo, pollen tube development in vitro appeared to be normal (Fig. 6b) under the appropriate conditions. Furthermore, in a number of taxa the percentage of in vitro germination of stored pollen can be correlated with its ability to set fruits and seeds following in vivo pollination (Janssen and Hermsen 1980; Visser 1955).

In the experiments reported here, we have examined the early stages of pollen hydration and germination and shown that rapid hydration of the dry pollen did not result in much cell breakage, nor in germination. The membranes of these pollen grains were apparently not effective in preventing the movement of solutes out of the pollen (Fig. 3). Pollen germination in the sugar-containing medium was substantially increased by prehydration of the pollen in a humid atmosphere (Fig. 5). These results may be explained by the model proposed by Crowe et al. (1989): the membrane phospholipids in the dry organism (pollen) are in the gel phase, and upon a sudden exposure to water a fast phase transition

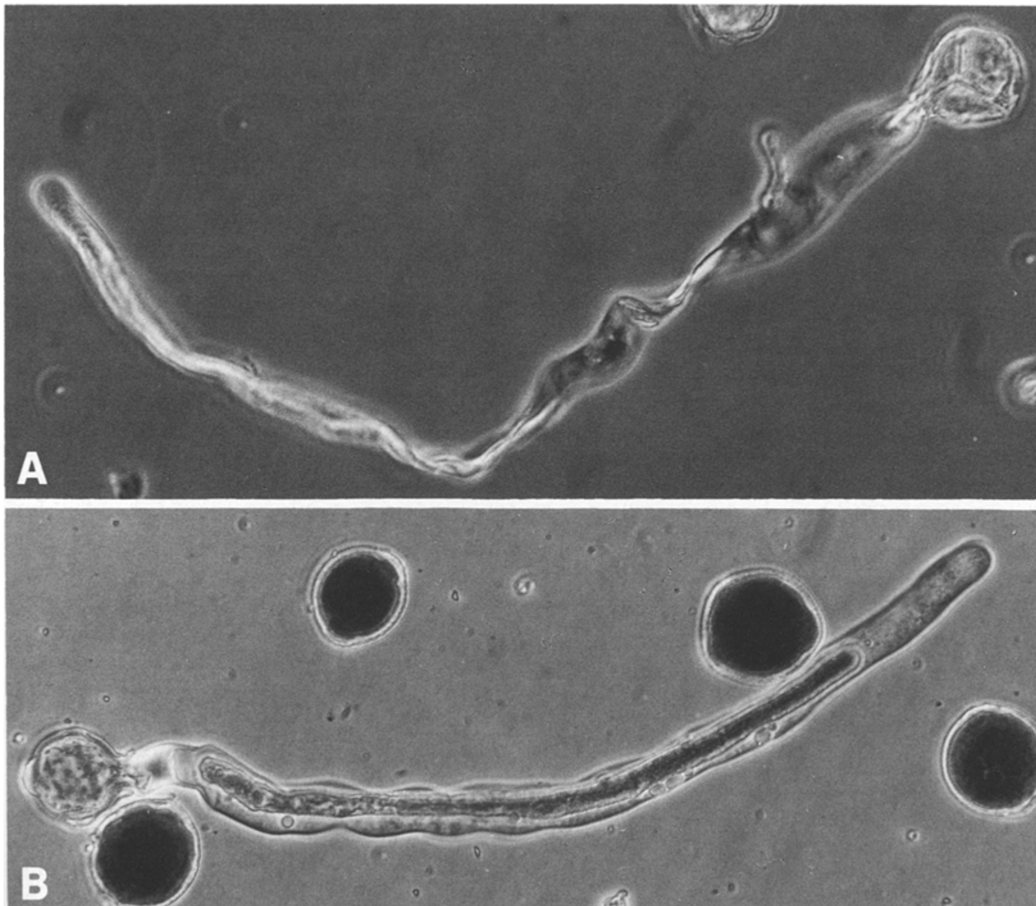

Fig. 6A, B. Light microscopy micrographs of *P. vera* L. pollen germinated in media containing PEG (**A**) or sucrose (**B**) (1000 mosmol/kg). ×250

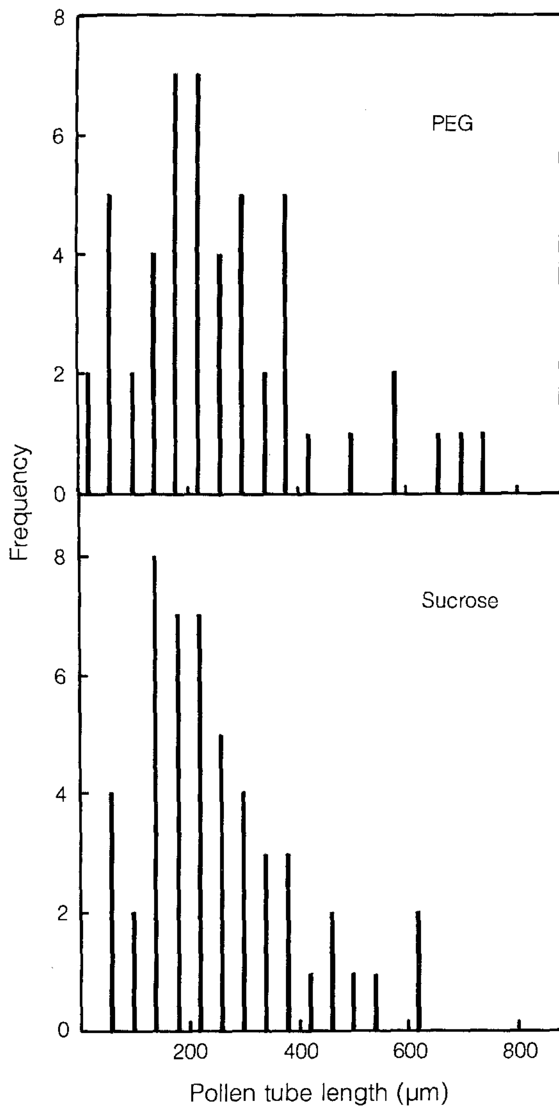

Fig. 7. Histograms of pistachio pollen tube length after 10 h of germination in media containing sucrose or PEG

occurs to a liquid crystalline phase that results in leakage. When the pollen were exposed to partial prehydration in a humid atmosphere, up to 50% of them germinated in the sugar solution (Fig. 5). According to Crowe's model (Crowe et al. 1989), these pollen grains did not undergo phase transition and therefore did not leak. One can not eliminate another possibility that the soluble fraction might have come from the wall layers partly derived from the haploid gametophyte and partly from its diploid parent. Evidence for this has come from the localization by immunofluorescence of the origins of the antigens present in the leachates from intact pollen (Heslop-Harrison 1975). Protein release during germination (Fig. 4) has been reported extensively (Heslop-Harrison 1975; Kamboj et al. 1984; Jackson 1989; and others). It has been suggested that these proteins may play a role in the interaction between pollen and stigma (Heslop-Harrison 1975). The SEM picture (Fig. 2) of water-washed pollen showing the removal of materials from the wall layers may support the possibility that at least part of the leachate originated in the pollen-wall layers and not in the cytoplasm. Furthermore, the presence of substantial amounts of solutes in the cell-wall layers was supported by the observation (data not shown) that large amounts of solutes were also quickly released from prehydrated pollen. At this hydration state it could be assumed (Crowe et al. 1989) that the membrane integrity was reestablished, which would have prevented the leakage of solutes from the cytoplasm.

In dry pollen only a minor amount of potentially osmotic-active substances is in a soluble form. When the water content of the pollen is 5%, the maximum solubility of glucose is three times lower than the amount present in the pollen. One-third of the sugars analysed consisted of glucose, which contributed approximately 38% to the osmotic concentration. The water content of the pollen was close to 50% after 44 h of hydration. An osmotic potential of -9.7 MPa could then be generated by the osmotica contained in the pollen. It seems unlikely that a major part of the solutes was found in the cytoplasm, because osmotic potentials much lower than -10 MPa could result during hydration of the dry pollen. Osmotic potentials of halophyte cells are -5 to -8 MPa, and osmotic potentials of most plant species lie between -0.4 and -2 MPa (Salisbury and Ross 1978).

The low water content of pistachio pollen and low osmotic potential of the medium required for germination may be related to their phytogeographical origin in the dry Irano-Turanian region. In species where high water content in the pollen is required for germination (e.g. *Zea mays*: 60%), the medium osmolality is also lower, probably in simulation of a wetter stigma. It has been speculated that in a wind-pollinated species like pistachio, high osmotic pressure is needed on the stigma to allow for a controlled hydration and release of solutes from the pollen and secretion from the stigma into the grain. This osmotic compatibility is critically important in the early interactions between the pollen grain and stigma (Heslop-Harrison 1979).

The importance of osmotic balance between the concentration of osmoticum in the medium and that contributed by the solutes contained in the pollen has been recognized since the earliest attempts to germinate pollen in artificial medium. The successful germination of *Pistacia vera* L. pollen required a high osmotic concentration in the medium. Low water potentials in the pollen provided the pollen with a great capacity to hold water vapour. This was certainly required for pollen hydration under low relative humidity conditions, conditions which this plant species is frequently exposed to. Maintenance of a high turgor pressure, a prerequisite for pollen tube elongation in stigma and style, will be promoted by a high internal solute concentration.

Sucrose could be substituted for by PEG for induction of germination (Fig. 5) (Roberts et al. 1983). Indeed, pollen germinated in PEG appeared shrunken (Fig. 6a), and a lower percent germination was obtained than in sucrose-containing medium (Fig. 5). We propose that the net osmotic effect is important in initiating pollen germination. The large variability in pollen tube size probably reflects the fact that pistachio is wind pollinated and

that the pollen grains leave the anther at different stages of maturity (Wagner et al. 1989), which leads to different rates of water uptake upon rehydration and different rates of germination.

Acknowledgments. The authors thank Prof. H.F. Linskens for valuable comments in the preparation of the manuscript. This research was supported by a USA-ISRAEL AID-CDR program grant No. DPF-5544-G-SS-7013-00 and by Hoffmann-La Roche & Co Basel, Switzerland.

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