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# In vitro Douglas fir pollen germination: influence of hydration, sucrose and polyethylene glycol

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**Abstract** – Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) pollen stored at low moisture content experiences imbibition shock when put directly onto culture media. This can be overcome by rehydrating the pollen in 100 % relative humidity prior to culturing. The long-term effect of rehydration on pollen tube growth was investigated. The impact that osmoticants such as sucrose and polyethylene glycol (PEG) have on pollen elongation and tube formation was also studied, using culture media of increasing osmotic potentials obtained with different sucrose/PEG ratios. Rehydration not only improved survival and pollen development on all media but also enhanced pollen tube induction and growth on most of them. Douglas fir pollen produced tubes over a wide range of osmotic potentials (–0.73 to –1.88 MPa). Depending on the sucrose/PEG ratio, between 5 and 55 % of grains produced tubes. There was also a media effect on tube morphology. (© Inra/Elsevier, Paris.)

*Pseudotsuga menziesii* pollen / in vitro germination / osmotic potential / polyethylene glycol / sucrose

**Résumé** – Germination in vitro du pollen de douglas : influence de l'hydratation, du sucre et du polyéthylène glycol. Le pollen de douglas (*Pseudotsuga menziesii* (Mirb.) Franco), conservé sous forme déshydratée, subit un choc hydrique lorsqu'il est mis en culture. Une réhydratation préalable en atmosphère saturée d'eau supprime ce choc. Nous avons étudié l'effet à long terme d'une réhydratation douce sur le développement et la croissance des tubes polliniques. L'effet du potentiel osmotique du milieu de culture et celui d'agents osmotiques tels que le saccharose et le polyéthylène glycol (PEG) ont été analysés en utilisant une série de milieux de potentiels osmotiques croissants et contenant diverses combinaisons saccharose/PEG. La réhydratation a eu un effet positif non seulement sur la survie et le développement du pollen mais aussi sur le nombre de tubes polliniques. Le pollen du douglas a germé sur des milieux de potentiels osmotiques allant de –0,73 à –1,88 MPa. La production de tubes (5 à 55 %) et leur morphologie ont été largement influencées par la proportion saccharose/PEG utilisée. (© Inra/Elsevier, Paris.)

pollen de douglas / germination in vitro / potentiel osmotique / polyéthylène glycol / saccharose

## 1. INTRODUCTION

Many conifer pollen readily germinate on semi-solid media containing only Brewbaker and Kwack [5] minerals and 10 % sucrose, but the requirements of Douglas fir pollen are more complex. Pine or spruce pollen produces a tube in vitro in 24 h, but Douglas fir pollen needs more time as it first elongates over a period of about 5 days

before tube growth can be induced [8]. In nature, spruce pollen also germinates more rapidly, taking less than a week compared to Douglas fir which elongates over a period of at least 5 to 6 weeks before producing a pollen tube [2, 3]. The first study in which high percentages of Douglas fir pollen tubes were obtained in vitro used a two-step method (elongation and quercetin-induced tube induction) on media containing high concentrations of

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sugar and/or polyethylene glycol (PEG) [8]. Significant numbers of tubes have also been obtained using a one-step method [9].

Douglas fir pollen, particularly after storage, is more difficult to handle *in vitro* than most other conifer pollen. When Douglas fir pollen grains are first put onto culture media, they experience imbibition shock, which may result in low viability and poor development. To protect the plasma membrane of dry pollen against water stress damage occurring upon rapid rehydration [7], the pollen grains can be rehydrated in 100 % relative humidity (RH) prior to being put onto media. Charpentier and Bonnet-Masimbert [6] have shown that such a treatment substantially improved pollen performance during the first step of germination, that is elongation.

In previous studies of Douglas fir pollen, supplemental sucrose and PEG, which are often used as osmotica in tissue culture, have been shown to play important roles in pollen behaviour [24]. The primary role of sucrose is that of a substrate for respiration, but this compound is also an important contributor to the osmotic potentials of media. When sucrose is used in culture media at the high concentrations necessary to protect pollen grains from osmotic shock, it also inhibits pollen tube growth in plants such as *Petunia*, *Nicotiana*, *Brassica* [12, 15, 18, 26] or *Pseudotsuga* [8]. In pollen of *Petunia*, Zhang and Croes [26] showed that there is an increase in respiration rate due to the exogenous sucrose but that the extra energy is not used in pollen tube growth. Furthermore, high concentrations of sucrose may damage the membrane and increase its permeability. Zhang and Croes [26] reported that when sucrose was the osmoticum, leakages induced a 0.15 MPa decrease in the osmotic potential of the medium in as little as 3 h. The decrease was lower, only 0.06 MPa, when sucrose was replaced by PEG [26]. It is therefore desirable to totally or partially replace sucrose by another osmoticant, which is both non-penetrating and non-plasmolyzing in character.

PEG is such an osmoticum. It is an inert, non-ionic polymer ( $\text{HOCH}_2-(\text{CH}_2-\text{O}-\text{CH}_2)_x-\text{CH}_2\text{OH}$ ) which cannot be metabolized in plants [21]. High molecular sizes (e.g. PEG 4000, 6000) do not penetrate the walls of cultured plant cells [10]. PEG controls water uptake and regulates the permeability of the membrane. It also increases germination and stabilizes tube tips and protein-protein interactions in tobacco [15]. Any commercially available PEG is a mixture of short and long polymers, the average of which gives the molecular weight of the preparation. PEG, at various molecular sizes and at various concentrations, has been reported to enhance pollen tube growth and improve the morphology of the tubes in a number of angiosperm species [12, 15, 16, 18, 26].

In this study, we have analysed the effects that controlled rehydration in high RH and culture media (e.g. PEG, sucrose and osmotic potential) may have on pollen elongation and pollen tube emergence and growth.

## 2. MATERIALS AND METHODS

### 2.1. Pollen

Douglas fir pollen cones (clone 3202 obtained from British Columbia Ministry of Forests, Glyn Road Research Station, Victoria) were collected in the spring of 1997 and sterilized as indicated in Dumont-BéBoux and von Aderkas [8]. The dry (< 8 % MC) sterile pollen was stored in airtight glass vials kept over silica gel at 4 °C. Pollen taken from dry storage and put directly in culture is referred to here as dry pollen. The grains of dry pollen can be classified into four classes [24] depending on whether they elongate (classes 1 and 2), do not elongate (class 3) or show damage such as plasmolysis (class 4).

### 2.2. Rehydration of pollen

Pollen was rehydrated in sterile conditions essentially according to Charpentier and Bonnet-Masimbert [6]. Dry pollen was put into aluminium boats in an airtight plastic container lined with wet sterile filter paper. The pollen was rehydrated for 16 h at 24 °C in an atmosphere of 100 % RH before being cultured on various media. Pollen rehydrated before culture is referred to here as rehydrated pollen.

### 2.3. Osmolality

The osmolality of sugar and PEG solutions and of culture media were obtained with a Wescor 5100B osmometer. Expressed in  $\text{mosm kg}^{-1}$ , it was then converted to osmotic potential using the Van't Hoff relation  $\psi_\pi = -\mathcal{R}TC_s$ , where  $C_s$  is the solute concentration,  $\mathcal{R}$  is the gas constant and  $T$ , the temperature in K or °C [13]. Knowing that 1  $\text{osmo kg}^{-1}$  corresponds to an osmotic potential of about -2.5 MPa, the osmolality curves were used to estimate the respective importance of the media components (sucrose and PEG) as osmoticum. Sugar and PEG concentrations were expressed in percent (w/v).

### 2.3. Culture media

All culture media contained Brewbaker and Kwack [5] minerals diluted 1:10 and were solidified with 0.4 % phy-

tagel. Preliminary work having shown that best responses were obtained on media having osmotic potentials between  $-1.12$  and  $-1.48$  MPa, sucrose (S) and PEG were mixed in different ratios (S/PEG) to obtain osmotic potentials ranging from  $-0.43$  to  $-1.88$  MPa. The two 3/20 media were composed of 3 % sugar mixed with either 20 % PEG 1450 (3/20L) or 20 % PEG 4000 (3/20H). Media 5/5 and 8/8 contained PEG 400. Medium 8/15 contained PEG 6000. The other media contained PEG 4000.

The pH of the media was adjusted to a final pH value of  $5.3 \pm 0.2$  after autoclaving. PEG 400 and PEG 1450 were from Sigma Canada, PEG 6000 and PEG 4000 were from Fluka Canada.

## 2.4. Pollen culture

Rehydrated and dry pollen (four replicates) were cultured according to Dumont-BéBoux and von Aderkas [8] on elongation media containing various S/PEG ratios as described earlier. After 5 days on elongation media, they were transferred to tube induction media (elongation media supplemented with  $1 \mu\text{M}$  quercetin). The elongation media were used as controls. Generally, pollen was monitored for 5 days on elongation media and 4 days on induction media. On medium 0/25, it was monitored for an additional 7 days on induction medium.

## 2.6. Measurements and statistical analysis

The average percentages (number of viable grains and number of pollen tubes) were calculated from four lots of 100 pollen grains each. Pollen grains not showing any sign of plasmolysis were scored as being alive. The average length was calculated from samples of at least 20 pollen grains each, selected at random. Graphs (figures 1, 2 and 4), standard deviations and curve fittings were achieved with Kaleidagraph 3.0.2. The Wilcoxon two-sample test [20] was used on viability. Response variables (proportion germinating, length of all pollen grains after 5 days on elongation media and length of germinated pollen grains) were analyzed by multiple regression using the  $\text{lm}()$  function of S-Plus. The mean value for each Petri dish was used in the analysis to maintain independence of the data points. The proportion of pollen grains germinating was angularly transformed prior to analysis. None of the residuals deviated significantly from the expected normal distribution. Transformation of sucrose and PEG concentrations failed to improve the fit; thus, they were analysed on the original scale of measurement. The mean value of each response variable for the combinations of sucrose and PEG concentrations test-

ed were used to estimate the response surface using the method of Akima [1] as implemented by the  $\text{interp}()$  function of S-Plus.

## 2.7. Moisture content

Pollen moisture content was determined according to Webber and Painter [25]. Based on the initial (wet) weight of the pollen, it was calculated as follows:

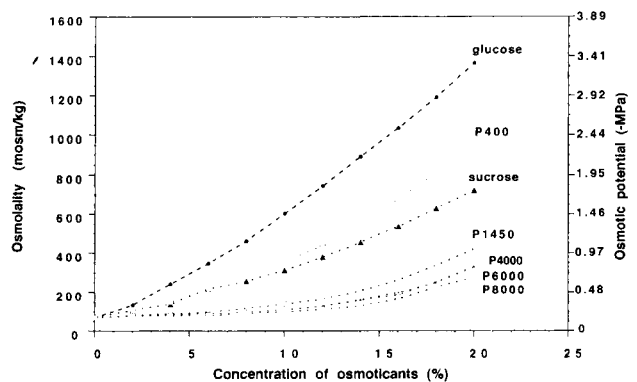
$$[(W_{t_w} - W_{t_c}) - (W_{t_d} - W_{t_c})] / (W_{t_w} - W_{t_c})$$

where  $W_{t_c}$  is the weight of the pollen container,  $W_{t_w}$  is the initial weight of the pollen plus the container and  $W_{t_d}$  is the weight of the dry pollen plus the container after 4 h at  $85^\circ\text{C}$ .

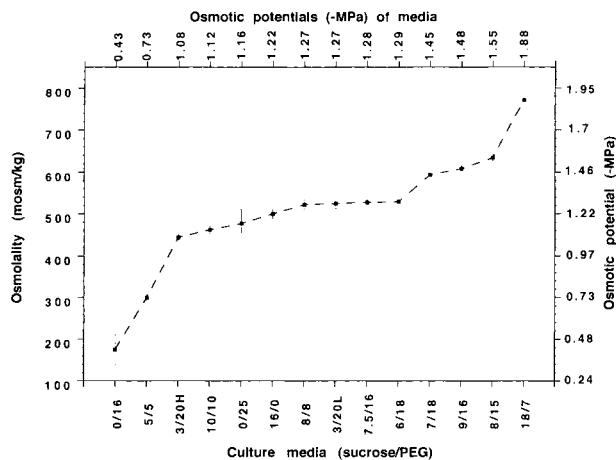
## 3. RESULTS

The osmolality of glucose was double that of sucrose while PEG 400 lay in between (figure 1). The osmolality of glucose, sucrose and PEG 400 increased almost linearly with concentration ( $R^2_{\text{glucose}} = 0.9904$ ,  $R^2_{\text{sucrose}} = 0.9799$ ,  $R^2_{\text{P400}} = 0.9607$ ). Exponential and polynomial curves better fitted the higher molecular weight PEGs (P1450 and P4000, 6000, 8000, respectively).

By changing the sucrose/PEG ratios of the culture media, and using different molecular sizes of PEG, a variety of media with osmotic potentials ranging between  $-0.43$  and  $-1.88$  MPa were generated (figure 2). The participation of either sucrose or PEG towards the final osmotic potential of the media varied according to their concentrations and related osmolalities. Based on the



**Figure 1.** Osmolalities and corresponding osmotic potentials of aqueous solutions of glucose, sucrose and several molecular sizes of polyethylene glycol (P400, P1450, P4000, P6000 and P8000).



**Figure 2.** Osmolalities and corresponding osmotic potentials of culture media containing different sucrose/polyethylene glycol (PEG) combinations. The upper x-axis shows the osmotic potential of each medium. Errors bars =  $\pm 1$  standard deviation.

osmolality curves (figure 1), four media had similar osmolalities of sucrose and PEG (media with S/PEG (%): 5/5, 8/8, 6/18 and 7/18), six had sucrose as the main osmoticum (S/PEG (%): 10/10, 16/0, 7.5/16, 9/16, 8/15 and 18/7) and four had PEG as the main or the sole osmoticum (S/PEG (%): 3/20L, 3/20H, 0/16 and 0/25).

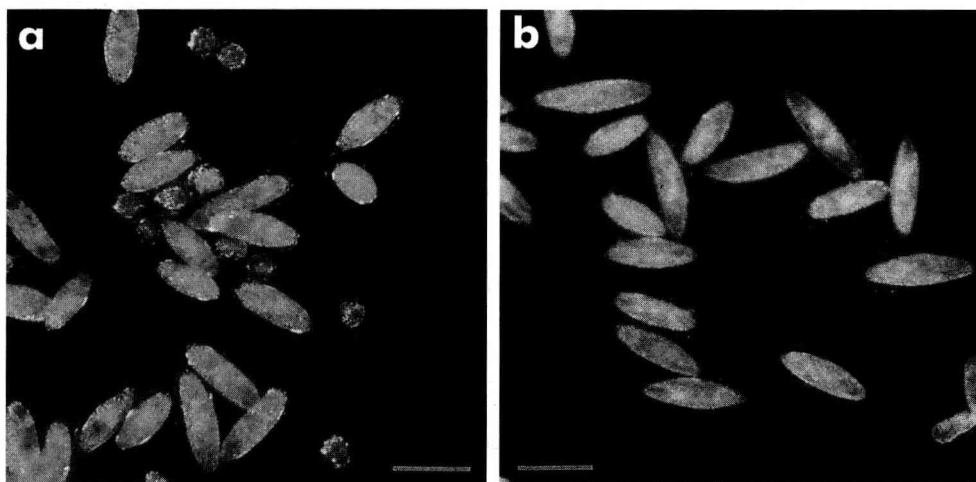
When first put onto media, dry pollen grains, having a moisture content  $< 8\%$ , were cup-shaped and measured about  $72 \pm 11 \mu\text{m}$  in diameter. After 16 h in 100% RH, the rehydrated pollen had a moisture content

of  $68\% \pm 5.5$ . The rehydrated grains were round and measured about  $100 \mu\text{m}$  in diameter.

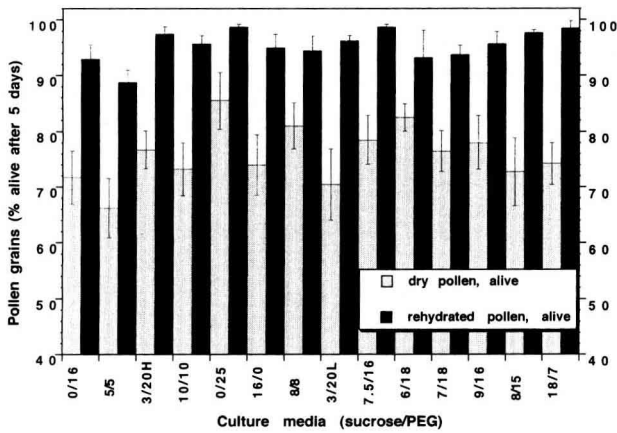
Dry (figure 3a) and rehydrated (figure 3b) pollen were both able to produce elongated grains, but there were differences. Rehydrating the pollen in 100% RH consistently led to better survival and to a more overall homogeneous population of elongated grains, all belonging to class 1 grains (figure 3b). Conversely, the dry pollen population was heterogeneous and contained all four classes of grains. After 5 days on elongation media, more than 98% of the rehydrated pollen grains were elongating. In contrast, the percentage variation for dry pollen was greater and depended on the medium (not shown).

Both dry and rehydrated pollen survived on media with osmotic potentials ranging from  $-0.43$  (0/16) to  $-1.88$  (18/7) MPa. Between 75 and 95% of the dry pollen and between 89 and 97% of the rehydrated pollen were alive after 24 h on the different media (not shown). After 5 days, those percentages decreased sharply for dry pollen but there was no change in the survival of rehydrated pollen (figure 4). The Wilcoxon two-sample test [20] showed that, after 5 days, the difference in viability between dry and rehydrated pollen was highly significant ( $\alpha = 0.01$ ).

After 24 h on elongation media, the average length of elongating pollen grains ranged, depending on the treatment, between  $120$  and  $190 \mu\text{m}$ . No differences were found among media, nor between dry and rehydrated pollen (not shown). After 5 days on elongation media, the length of pollen grains was not well explained by the multiple regression (figure 5). Only 35% of the variation was accounted for. There was an effect of sucrose concentration (slope =  $-6.18$ ;  $t = -2.2$ ;  $P = 0.04$ ) and PEG concen-



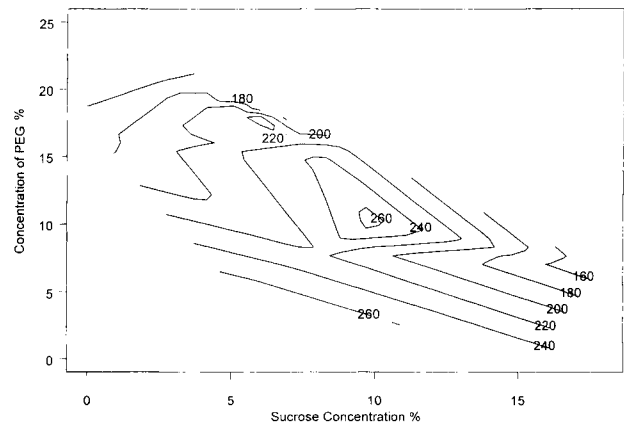
**Figure 3.** Dark field micrographs of dry (a) and rehydrated (b) pollen after 9 days on elongation medium 3/20L. Scale bars =  $160 \mu\text{m}$ .



**Figure 4.** Viability of dry and rehydrated pollen. Percentages were calculated after 5 days on elongation media. Error bars =  $\pm 1$  standard deviation. PEG: polyethylene glycol.

tration (slope =  $-6.32$ ;  $t = -3.2$ ;  $P = 0.004$ ) but the interaction term, hydration of the pollen grains, or the additional effect of osmotic potential could not be detected (all  $P > 0.2$ ). On media containing high percentages of PEG (0/16, 0/25, 3/20H, 3/20L, 7/18) or sucrose (18/7), pollen grains elongated little, while they doubled or almost tripled their original size on all other media.

Tubes were obtained on all induction media except on 0/16. Pollen tube growth occurred over a wide range of osmotic potentials ( $-0.73$  to  $-1.88$  MPa). Generally, the first tubes appeared after 24 h on induction media and percentages were highest at 96 h. The percentages of tubes then declined as, on quercetin-treated media, tubes started to disintegrate and grains to plasmolyze. Pollen raised on 0/25 was slow to germinate; it took 72 h for the first tubes to appear and percentages were highest (30 and 40 %, dry and rehydrated pollen, respectively) at 268 h. Pollen that had been rehydrated prior to testing was more likely to germinate ( $t = 3.3$ ;  $P = 0.0034$ ) than dry pollen. Depending on the media composition, a two- to seven-fold increase in the number of tubes was observed between dry and rehydrated pollen. Increasing sucrose and PEG concentrations caused a decline in the proportion of germinating pollen grains ( $t = -4.0$  and  $-4.2$ , respectively; both  $P < 0.001$ ). The non-linearities in these relationships were reflected in a significant interaction term between sucrose and PEG concentrations ( $t = 2.9$ ;  $P = 0.008$ ). There was also an overall effect of osmotic potential with higher germination rates at lower potentials. In combination, these effects led to the highest germination rates at intermediate levels of both sucrose and PEG concentration for both dry and rehydrated pollen



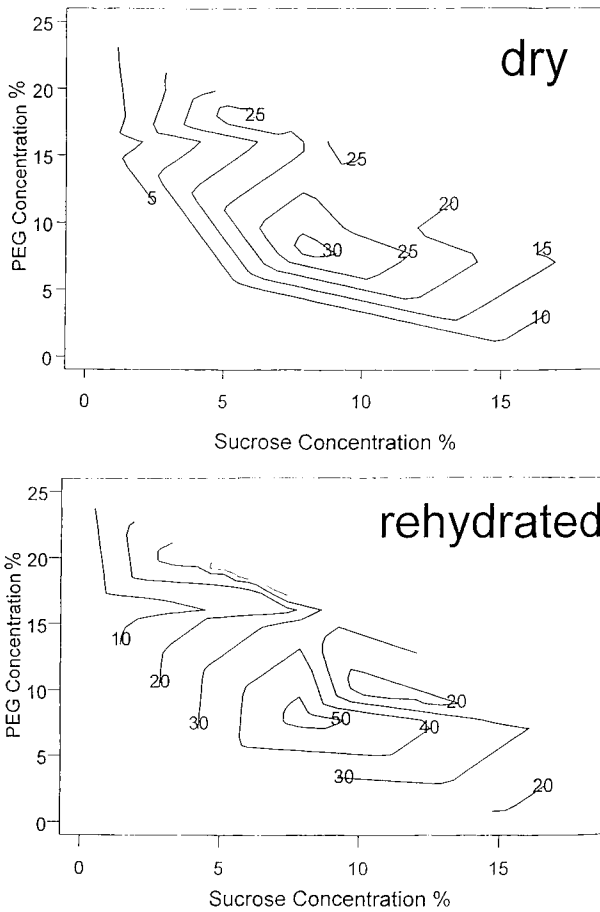
**Figure 5.** Average length of elongating pollen (dry and rehydrated taken together) as a function of polyethylene glycol (PEG) and sucrose concentrations. Measurements were taken after 5 days on elongation media.

grains (figure 6). The statistical relationship accounted for over 71 % of the variation in germination rate.

Tubes grew in the medium as well as on it or vertically in the air. Division of the generative cell occurred on all media with the sole exception of 18/7. Generally, gametes, as detected by light microscopy (not shown), appeared more quickly in rehydrated pollen.

The highest percentage of tubes with a regular morphology were obtained with media 7/18 and 0/25. The shape of the tubes varied between media treatments but not between rehydrated and dry pollen. Depending on the S/PEG ratio, 10 to 50 % of the tubes were branched. Media 10/10, 8/8 and 5/5 had the most deleterious effect on tube shape. On these media, tubes were either fat, heavily branched, club-shaped or short and bulb-like. On 18/7 and 16/0, tubes were either arrested early in the development or abnormal. After 5 days on medium 7/18, tube length varied between 150  $\mu\text{m}$ , 1.5 times the diameter of the pollen grain, and 800  $\mu\text{m}$  (figure 7a). On media favouring long-term survival (e.g. elongation media 7/18, 0/25), tubes measuring up to 3 mm have been seen after 20 days of culture (not shown).

Pollen tubes appeared on grains of varied length, ranging between 250 and 400  $\mu\text{m}$ , with the middle 50 % measuring 301 to 339  $\mu\text{m}$ . On one occasion, on medium 3/20H, a pollen grain did not elongate but still produced a nice-looking tube (figure 7b). Germinating grains were on average shorter at higher concentrations of sucrose (slope =  $-3.55$ ;  $t = -2.5$ ;  $P = 0.02$ ) and at higher concentrations of PEG (slope =  $-4.99$ ;  $t = -5.2$ ;  $P < 0.0001$ ) (not shown). There was also a significant interaction term ( $t = -3.6$ ;  $P = 0.002$ ) but no discernible independent effect



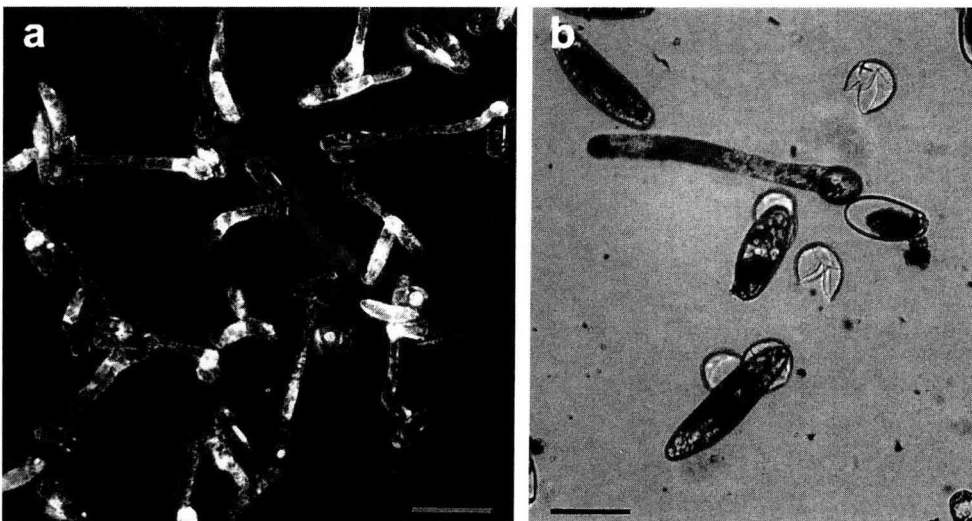
**Figure 6.** Germination frequency on quercetin-induced dry and rehydrated pollen grains after 96 h on induction media. Results are shown relative to polyethylene glycol (PEG) and sucrose concentrations.

of hydration or osmotic potential. Taken together, these variables accounted for 82 % of the variation in the length of germinating pollen grains.

#### 4. DISCUSSION

This study provides evidence that both pretreatment in 100 % RH and medium composition strongly influenced pollen response. These include pollen grain survival, elongation and vigour. Short-term effects due to rehydration and to the osmoticum did not appear to be additive: survival and development were directly linked to rehydration. The extent to which the grains elongated was solely affected by the composition of the media.

When directly placed onto culture media, pollen water uptake is extensive, rapid and usually highly damaging to plasma membranes. Crowe et al. [7] showed that the rapid swelling of the grains stresses the lipid by-layers as the phospholipids of the membranes go through a rapid gel/liquid crystalline phase transition. The membranes are damaged in the process and cytoplasm leaching occurs. However, partial rehydration over water vapour has been shown by the same authors to allow the phospholipids to go through phase transition before being exposed to bulk water. Webber and Bonnet-Masimbert [24] showed that, while respiration was not affected by a preconditioning of pollen in 100 % RH, significantly less leaching occurred in rehydrated pollen than in dry pollen. Furthermore, a slow rehydration of the pollen improved pollen grain elongation [24] and promoted ultrastructural changes [6]. In our study, rehydration substantially improved both pollen grain survival and development, independently of media composition.



**Figure 7.** (a) Dark field micrograph of pollen tubes after 5 days on induction medium 7/18. (b) Light micrograph of a non-elongated pollen grain with pollen tube after 3 days on induction medium 3/20H. Scale bars = 333  $\mu\text{m}$  (a) and 142  $\mu\text{m}$  (b).

The influence of controlled rehydration was not limited to the first step of Douglas fir germination, but extended to the second step, tube induction and growth. Rehydrated pollen was generally more receptive to the quercetin induction signal than dry pollen and tube emergence was enhanced on most media. The extent to which induction and tube growth were improved by rehydration depended on the media. Rehydration has a long-term effect which affected the whole germination process.

Pollen grains survived on all media and pollen tubes appeared over a wide range of osmotic potentials ( $-0.73$  to  $-1.88$  MPa). Below  $-0.43$  MPa, osmotic potential did not play any major role in either the development of Douglas fir pollen grains or the production of tubes. Similar results have been reported for *Petunia* pollen, which germinated on media having osmotic potentials between  $-0.25$  and  $-1.5$  MPa [26]. Tomato cells have also been reported to grow under PEG-initiated water stress and to withstand water potentials between  $-1.5$  and  $-2.2$  MPa [10].

The nature of the osmotica (sucrose and/or PEG) had a significant influence on pollen behaviour. Generally glucose, sucrose and PEG 400 are strong osmotica while the larger PEGs are not. Because PEGs with a molecular weight of 1 450 or larger do not follow van't Hoff's law [21], they have little influence on the osmotic potential of media, unless present at very high concentrations. Large molecular-size PEGs bind water [15] and are known to slow water uptake [23]. In this way, they protect the plasma membrane and may regulate its permeability [15]. It has been suggested by Jacomini et al. [11] that high molecular-weight PEG may accumulate in extracellular spaces of water-stressed tomato plants and may pump water out of the cell, inducing a cellular dehydration as opposed to a tissue dehydration. By binding water, either out of the medium or out of the cell, PEG decreases the amount of water available. Compared to sucrose, PEG's main feature is its inertness. Even when penetrating the extracellular spaces and circulating through the apoplast [11], PEG has no recognized effect on the metabolism of the cell. This is not the case of sugars which are rapidly metabolized. Exogenous sucrose, often included in pollen culture media, is incorporated into pollen tube walls [14, 17]. Furthermore, sucrose is known to hydrolyze upon autoclaving [19] and over time [22] into glucose and fructose, the latter being detrimental to pollen [8].

As shown in this study, the effects of sucrose and PEG and their combined influence appear to be very complex as they have drastic, sometimes antagonistic, effects on pollen germination and tube growth. Dumont-BéBoux and von Aderkas [8] reported that, in Douglas fir, high molecular-weight PEG slowed development and diminished the number of tubes while greatly improving tube

morphology. Depending on its concentration, sucrose has been reported to interfere with pollen tube growth in both angiosperms and conifer species [4, 8, 12, 15, 18, 26], resulting in poor tube morphology or in partial or total inhibition of its growth.

In both flowering plants and gymnosperms such as Douglas fir and Scott pine [4], there are similar media requirements for successful in vitro germination. Pollen tube growth in Douglas fir is enhanced and improved by a slow rehydration prior to culturing. Over a wide range of osmotic potentials, it is the S/PEG ratio of the medium that governs the number of tubes, their rate of appearance and their morphology.

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