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Gelling Agent and Growth Regulator Effects on Shoot Vitrification of 'Gala' Apple in Vitro

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Abstract. **Shoot tips of 'Gala' apple (*Malus domestica* Borkh.) were grown on proliferation medium containing Murashige and Skoog salts, supplemented with 0.56 mM myo-inositol; 1.2 μM thiamine-HCl; 2.2 or 4.4 μM BA; 0.005, 0.05, or 0.5 μM IBA; and 1.3 μM GA_3. The media were solidified with Gelrite at concentrations from 0.5 to 1.5 g·liter^{-1} plus agar (Sigma) from 0.5 to 4.0 g·liter^{-1}. Vitrification was influenced both by gelling agent and BA concentration, whereas the IBA concentration had little effect. Increasing either agar or Gelrite concentration resulted in a decreasing percentage of vitrified explants. The higher BA level generally increased vitrification, but increasing the agar concentration tended to reduce the difference in effect of the 2 BA levels. Certain combinations of gelling agents produced no vitrification with no reduction in shoot proliferation, and with a clarity of the medium approaching that of Gelrite alone. Chemical names used: *N*-(phenylmethyl)-1*H*-purin-6-amine [benzyladenine (BA)]; 1*H*-indole-3-butanoic acid (IBA); and gibberellic acid (GA_3).**

Gelrite gellan gum is a self-gelling hydrocolloid that forms rigid, brittle, transparent gels in the presence of soluble salts. Chemically, it is a polysaccharide comprised of uronic acid, rhamnose, and glucose (8).

Gelrite is used in place of agar because it costs less per liter of medium and its clarity makes it easy to observe plant growth and bacterial contamination. It is used commercially for in vitro propagation of some ornamental plants and of 'Paradox' walnut rootstock (4), but has resulted in shoot vitrification of several cultivars when used in apple proliferation medium (unpublished data). Vitrification is a physiological disorder frequently affecting plants vegetatively propagated in vitro, a subject recently summarized by Kevers at al. (9).

For *Malus* sp., vitrification (glassiness) was first mentioned by Jones (7), and other reports followed (1, 6, 11, 15). Leaves on vitrified shoots are translucent or water-soaked in appearance, malformed, strap-like, and often curled; stem elongation is sometimes inhibited (16).

The first comprehensive study of vitrification (3) reviewed different hypotheses with special reference to water potential and tested raising the agar content of the medium to overcome the problem. Ziv et al. (18) proposed 2 solutions to avoid vitrification on carnation: 1) culture apices in a stationary liquid medium for a few days and then subculture them onto agar solidified medium, and 2) use slanted agar cultures.

This study was undertaken to determine if combinations of Gelrite, agar, BA, and IBA could be found that would result in no vitrification of shoots, maintain adequate shoot proliferation, and produce a medium with the clarity of Gelrite.

Materials and Methods

Cultures of 'Gala' apple were established and proliferated using previously published methods (17). Shoot tips from existing in vitro cultures of 'Gala' apple were grown on proliferation medium containing Murashige and Skoog (10) salts, supplemented with 0.56 mM (100 mg·liter^{-1}) myo-inositol, 1.2 μM (0.4 mg·liter^{-1}) thiamine-HCl, 1.3 μM (0.45 mg·liter^{-1}) GA_3, 87.6 mM (30 g·liter^{-1}) sucrose, and various concentrations of BA and IBA. Iron was supplied in the form of ferric sodium salt of ethylenedinitrilotetraacetic acid to provide 0.1 mM Fe as in MS medium. The pH was adjusted to 5.2 prior to adding gelling agents and autoclaved for 15 min at 121°C and 1.1 kg·cm^{-2}. The media were solidified with Gelrite plus agar (Sigma) at concentrations specified below for each experiment.

Shoot tips, ≈15 mm long, were excised and collected in a jar of sterile distilled water, stirred gently to randomize, then placed horizontally on the medium. Culture vessels were glass-capped 120-ml glass jars wrapped with Saran plastic film. Each jar contained 40 ml of medium, with 4 shoot tips per jar. The cultures were grown for 3 weeks at 25° ± 2°C with 16-hr photoperiods provided by warm white fluorescent lights at a photon flux density of 40–60 μmol·s^{-1}·m^{-2}.

Five factorial experiments were conducted, one for each level of Gelrite. In each experiment, 2 levels of BA (2.2 and 4.4 μM), 3 levels of IBA (0.005, 0.05, and 0.5 μM), and several levels of agar were used. The amounts of gelling agent used (per liter) for each of the experiments were as follows: Expt. 1—0.5 g Gelrite plus 0.5, 2, 3, or 4 g agar; Expt. 2—0.75 g Gelrite plus 0.75 or 2 g agar; Expt. 3—1 g Gelrite plus 1, 2, 3, or 4 g agar; Expt. 4—1.25 g Gelrite plus 1, 2, 3, or 4 g agar; and Expt. 5—1.5 g Gelrite plus 1, 2, 3, or 4 g agar. Each treatment had 4 replicate jars.

Each explant with the axillary shoots it produced was examined and classified as vitreous or nonvitreous. Explants showing any vitreous characteristics were considered as vitreous. Data on the percentage of total original explants that were vitrified were analyzed following the arcsin transformation (14). All shoots, including the original explant, were counted and classified for length using 0.5-cm increments; those longer than 0.5 cm were considered usable. Data for percentage of vitrified explants, total number of shoots, and number of usable shoots were analyzed using the General Linear Means procedure of SAS (12).

Results

Vitrification. After 4 to 5 days of culture, leaves on affected explants became turgescent, dark green, thick, curled, and much longer and wider than normal. After 3 weeks, stems were thick

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and very brittle with a very small amount of callus growing out of the base, especially with a very soft gel (0.5 g·liter^{-1} Gelrite).

Generally, clumps composed of a small number of shoots were formed, mostly with one dominant shoot that could elongate to 5 to 8 cm. At other times, there was only the initial shoot, which might elongate to 5 to 8 cm, but with undeveloped axillary buds. The buds were very turgescent, dark green, and covered by 2 small leaves having the same characteristics described above for vitrified leaves. Moreover, there were sometimes 7 or 8 shoots per clump, 1.5 to 2.0 cm tall, with turgescent 1- or 2-node stems and translucent leaves, but no malformation in either stems or leaves.

The differences in percentage of vitrified explants among agar concentrations were statistically significant ($P = 0.1\%$) in each experiment. The percentage of vitrified explants decreased as Gelrite concentration increased and as agar concentration increased to 3 g·liter^{-1} (Fig. 1). Virtually no vitrification occurred at 4 g·liter^{-1} agar at any of the Gelrite concentrations tested. The higher levels of agar also tended to reduce the difference in effect of the 3 higher levels of Gelrite. Generally, the number of treatments with nonvitrified explants increased as Gelrite concentration increased and occurred only when agar concentrations were 2 g·liter^{-1} or more.

The BA concentration also significantly affected the percentage of vitrified explants in all experiments ($P = 0.1\%$ in Expt. 1 and 5; $P = 1\%$ in the others). Except for Expt. 3 (Table 1), 4.4 μM BA produced a higher percentage of vitrified explants than 2.2 μM BA, but the effect of BA concentration generally was far less than that of the gelling agents (Table 2). For any given combination of Gelrite and agar, the difference in vitrification between 2.2 and 4.4 μM BA exceeded 30% only for 2 g·liter^{-1} agar in Expts. 1 and 2 and for 1 g·liter^{-1} agar in Expts. 3 and 5, and for most combinations was ≤10%. All explants were vitrified when a very soft gel (0.5 g·liter^{-1} each of agar and Gelrite) was used, regardless of the BA concentration. So few explants were vitrified with 3 or 4 g·liter^{-1} of agar at 1 to 1.5 g·liter^{-1} of Gelrite that any effect of BA concentration was hardly discernible.

The IBA concentration had a significant effect on vitrification only in Expt. 1 ($P = 1\%$) and 3 ($P = 0.1\%$), but in those cases, there was no consistent pattern. In Expt. 1, more vitrification occurred at the lowest concentration of IBA (0.005 μM) at 2 and 3 g·liter^{-1} of agar than at the higher ones. In Expt. 3, both the interactions between IBA and BA (Table 1) and IBA and agar (Table 3) were significant. In addition, IBA interacted significantly with BA in Expt. 5 (Table 1), but the pattern was not the same as in Expt. 3 and the percentage of vitrified explants was much lower.

When comparison cultures in Expts. 1–3 were grown on medium solidified with 1, 1.5, or 2 g·liter^{-1} Gelrite alone, vitrification decreased from 100% to 80% as the Gelrite concentration increased. In contrast, vitrification never developed in comparison cultures grown on medium solidified with 6 g·liter^{-1} agar.

Shoot proliferation. The agar concentration significantly influenced the total number of shoots and the number of usable shoots produced in each experiment—the only factor to do so. About 30% of the total shoots produced were usable with the range being 19% (0.5 g·liter^{-1} each of Gelrite and agar) to 36% (0.5 and 1.5 g·liter^{-1} Gelrite plus 3 g·liter^{-1} agar). Since the response of explants to the different treatments was nearly the same for both measurements, only data on usable shoots are presented.

The number of usable shoots produced at the 2 lowest Gelrite

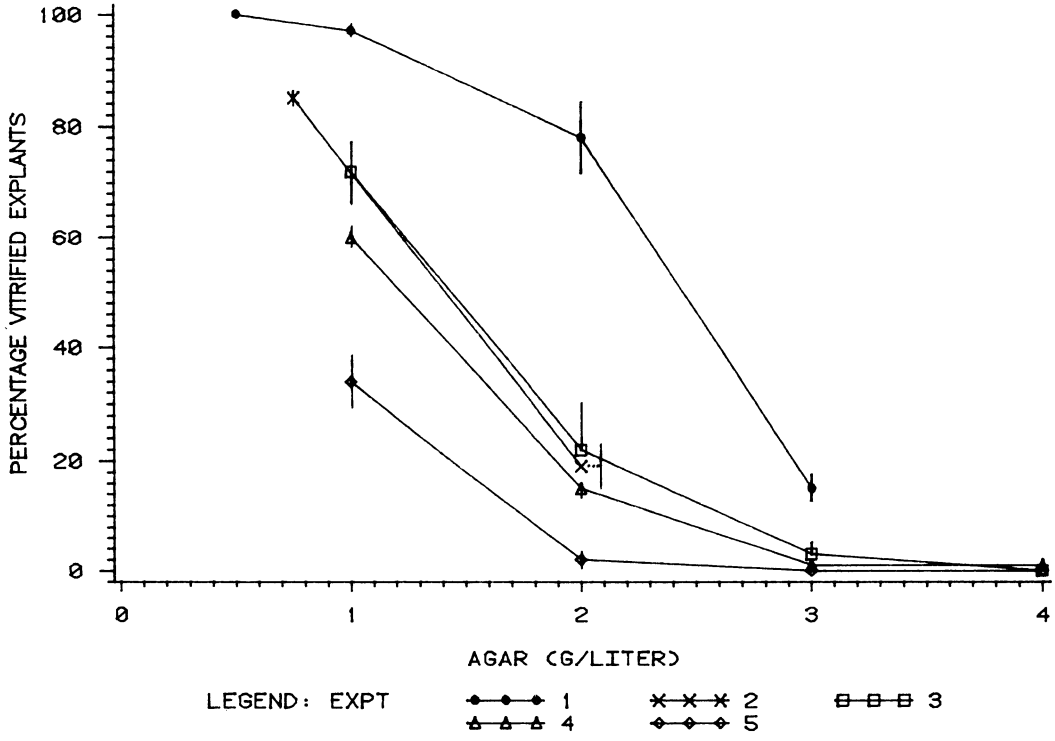

Fig. 1. The effect of Sigma agar concentration on percentage of vitrified explants at different concentrations of Gelrite. Linear component significant at $P = 0.1\%$ in Expts. 1, 3, 4, and 5, and quadratic significant at $P = 1\%$ in Expt. 1 and $P = 5\%$ in Expts. 4 and 5. Significant interaction between IBA and agar concentrations in Expt. 3 is probable reason for lack of significant quadratic component in this experiment. Vertical bars at each data point indicate ± 1 SE.

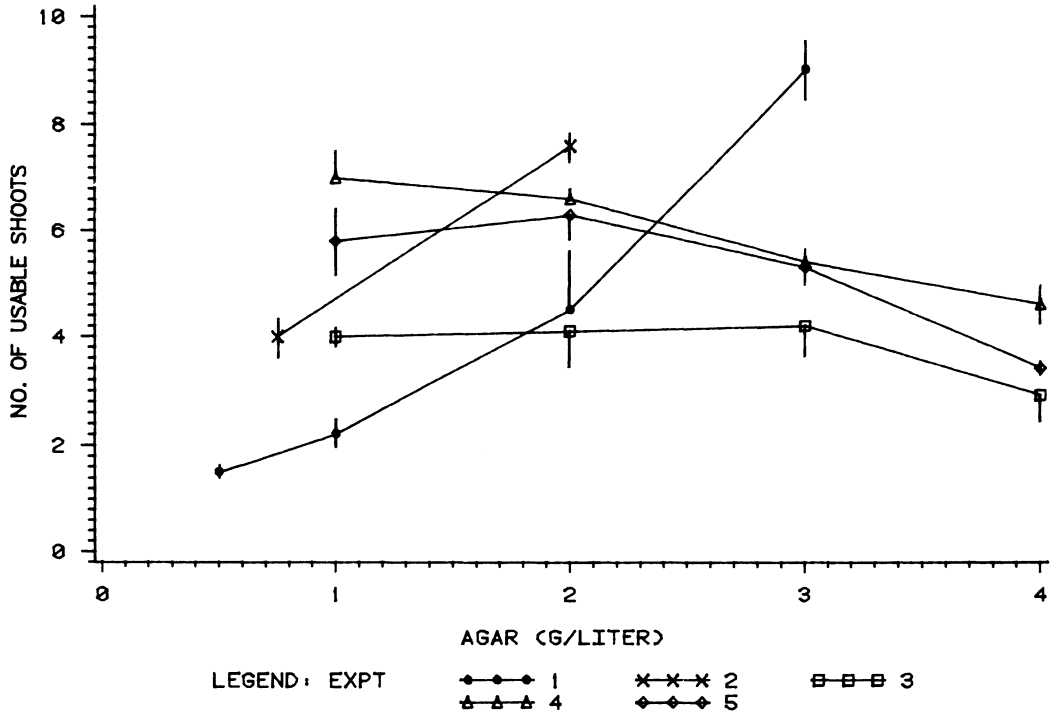

Fig. 2. The effect of Sigma agar concentration on number of usable shoots at different concentrations of Gelrite. Linear component significant at $P = 0.1\%$ in Expts. 1, 4, and 5 and at $P = 5\%$ in Expt. 3 and quadratic component significant at $P = 0.1\%$ in Expt. 5, $P = 1\%$ in Expt. 1, $P = 5\%$ in Expt. 3, and not significant in Expt. 4. Vertical bars at each data point indicate ± 1 SE.

Table 1. Effect of BA and IBA concentrations on percentage of vitrified explants produced at 2 different concentrations of Gelrite after 3 weeks of culture.[z]

BA (μM)	IBA (μm) 0.005	0.05	0.5	Mean	BA	IBA	
			Expt. 3				
2.2	28	18	28	24	**	***	
4.4	45	3	2	12			
x̄	36	9	11			***	
			Expt. 5				
2.2	1	0	4	1	***		*
4.4	12	11	3	8			
x̄	5	4	4			NS	

[z]Gelrite concentrations (g·liter^{-1}): Expt. 3 = 1; Expt. 5 = 1.5.
***,**,*,NSSignificant at the 0.1%, 1%, and 5% levels and not significant, respectively.

Table 2. Effect of BA and agar concentrations on percentage of vitrified explants produced at 3 different concentrations of Gelrite after 3 weeks of culture.[z]

BA (μM)	Agar (g·liter^{-1}) 0.5	0.75	1	2	3	4	Mean	BA	BA × agar
				Expt. 1					
2.2	99	---	94	48	8	---	68	***	*
4.4	100	---	99	97	23	---	89		
				Expt. 2					
2.2	---	82	---	4	---	---	---	**	*
4.4	---	87	---	41	---	---	---		
				Expt. 5					
2.2	---	---	12	1	0	0	1	***	**
4.4	---	---	61	4	0	0	8		

[z]Gelrite concentrations (g·liter^{-1}): Expt. 1 = 0.5; Expt. 2 = 0.75; and Expt. 5 = 1.5.
***,**,*Significant at the 0.1%, 1%, and 5% levels, respectively.

Table 3. Effect of IBA and agar concentrations on percentage of vitrified explants produced at 1 g·liter^{-1} Gelrite (Expt. 3) after 3 weeks of culture.

IBA (μM)	Agar (g·liter^{-1}) 1	2	3	4	Mean	IBA	IBA × agar
0.005	93	69	8	0	36	***	***
0.05	69	4	0	0	9		
0.5	47	8	7	0	11		

***Significant at the 0.1% level.

concentrations increased as the agar concentration increased (Fig. 2). With 1 g·liter^{-1} Gelrite (Expt. 3), increasing agar concentration from 1 to 3 g·liter^{-1} had no effect, but further increase reduced the number of usable shoots. At the 2 highest concentrations of Gelrite, the number of usable shoots decreased as agar concentration increased, except for the lowest agar concentration in Expt. 5.

The concentration of BA had a significant effect on the number of usable shoots in Expts. 1, 3, and 5, where 2.2 μM BA produced more usable shoots than 4.4 μM (Table 4). Significant interactions between the concentrations of BA and agar occurred in all experiments except Expt. 2. In Expts. 1 and 3, the explants responded differently to BA concentrations at agar levels of 2 g·liter^{-1} or higher, whereas in Expts. 4 and 5, the difference occurred only at 1 g·liter^{-1} agar (Table 4).

The concentration of IBA produced a significant overall effect only in Expts. 3 and 5, but no consistent pattern was evident

Table 4. Effect of BA and agar concentrations on number of usable shoots produced at 4 different concentrations of Gelrite after 3 weeks of culture.[z]

BA (μM)	Agar (g·liter^{-1}) 0.5	1	2	3	4	Mean	BA	BA × agar
				Expt. 1				
2.2	1.6	2.4	6.5	9.5	---	5.0	**	**
4.4	1.4	2.0	2.5	8.5	---	3.6		
				Expt. 3				
2.2	---	3.8	5.1	5.0	3.4	4.3	**	*
4.4	---	4.2	3.1	3.5	2.4	3.3		
				Expt. 4				
2.2	---	7.8	6.4	5.1	4.8	6.0	NS	*
4.4	---	6.1	6.8	5.8	4.5	5.8		
				Expt. 5				
2.2	---	6.9	6.1	5.5	3.3	5.4	*	**
4.4	---	4.7	6.5	5.1	3.6	5.0		

[z]Gelrite concentrations (g·liter^{-1}): Expt. 1 = 0.5; Expt. 3 = 1.0; Expt. 4 = 1.25; and Expt. 5 = 1.5.
**,*,NSSignificant at the 1% and 5% levels and not significant, respectively.

Table 5. Effect of IBA and agar concentrations on number of usable shoots produced at 2 different concentrations of Gelrite after 3 weeks of culture.[z]

IBA (μM)	Agar (g·liter^{-1}) 1	2	3	4	Mean	IBA	IBA × agar
			Expt. 3				
0.005	3.4	4.2	5.9	2.4	4.0	**	**
0.05	4.4	5.6	3.8	4.3	4.5		
0.5	4.2	2.6	3.2	2.0	3.0		
				Expt. 5			
0.005	4.8	7.2	5.0	3.4	5.1	*	*
0.05	5.7	5.4	4.8	3.6	4.9		
0.5	6.8	6.3	6.2	3.4	5.7		

[z]Gelrite concentrations (g·liter^{-1}): Expt. 3 = 1.0; Expt. 5 = 1.5.
**,*Significant at the 1% and 5% levels, respectively.

(Table 5). Significant interactions between the concentrations of IBA and agar occurred in Expts. 3 and 5, but again no pattern was apparent (Table 5).

No interaction between BA and IBA was found, but in Expt. 5 a 3-factor interaction among concentrations of BA, IBA, and agar existed. Again, no pattern emerged except that far less variability among treatments occurred at 4 g·liter^{-1} agar—the level that produced the fewest usable shoots.

Discussion

A reduction in the incidence of vitrification was obtained by increasing the amount of gelling agent in the culture medium, but, in so doing, shoot proliferation and growth were usually reduced, as also found by other workers (2, 3, 5). As Gelrite concentration increased at any agar concentration or as agar concentration increased at any Gelrite concentration, the percentage of vitrified explants decreased (Fig. 1), with the effect being greater at lower total concentration of gelling agents. In contrast, shoot proliferation increased with increasing agar concentration at the 2 lower levels of Gelrite and decreased with increasing agar concentration at the 2 higher levels (Fig. 1), indicating that an optimal level of gelling agent could be selected to maximize shoot proliferation and minimize vitrification. This conclusion is strengthened by the fact that no vitrification was produced by combinations of 1 to 1.5 g·liter^{-1} Gelrite plus 2 to 4 g·liter^{-1} agar. These results contrast with those of Singha (13), who found that increasing agar above 3 g·liter^{-1} decreased shoot proliferation of 'Almey' crabapple and 'Seckel' pear.

The high concentration of BA generally increased the percentage of vitrified explants and reduced the number of usable shoots by 10% to 30%. However, BA concentration interacted significantly with agar concentration (in all but Expt. 2) with regard to the number of usable shoots. As the agar or Gelrite concentration increased, the differences in the amount of vitrification produced by the 2 BA concentrations decreased. At 3 or 4 g·liter^{-1} of agar plus 1 to 1.5 g·liter^{-1} Gelrite, virtually no difference in percentage of vitrified explants occurred between 2.2 and 4.4 μM BA. These results support the hypothesis of Debergh (2) that cytokinin affects vitrification only at particular concentrations of the gelling agent. Thus, it seems that the effect of BA on vitrification can be overcome by increasing the gelling agent concentration.

IBA had no apparent effect on inducing vitrification, and an inconsistent (although significant in Expts. 3 and 5) effect on number of usable shoots. For practical purposes then, IBA can be incorporated in the medium in the concentration range tested without concern about detrimental effects on explant growth.

Since 2.2 μM BA induced greater shoot proliferation than 4.4 μM in certain treatment combinations as well as inducing less vitrification of explants, it seems that the lower amount should be used to maximize apple shoot proliferation with these gelling agents. In these experiments, a satisfactory combination appears to be 1.5 g·liter^{-1} Gelrite plus 2 or 3 g·liter^{-1} Sigma agar, 2.2 μM BA, and 0.5 μM IBA. This combination contrasts with previous results obtained in our laboratory using 4.4 μM BA in medium gelled with 6 to 7 g·liter^{-1} Difco Bacto-agar (16, 17).

The results of this study point to a method for examining the phenomenon of vitrification. Since growing apple explants on Gelrite alone results in the reliable production of vitrified shoots, doing so provides a means of reproducibly inducing the condition in apple explants on a solid medium. At the same time, transferring the cultures to an appropriate mixture of Gelrite and Sigma agar should reverse or halt the development of vitrification. The technique also provides a means for time-course studies of the development of vitrification.

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Pollen Yield from Olive Tree cvs. Manzanillo and Swan Hill in Closed Urban Environments

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Abstract. **Airborne pollen concentrations (grains/m^3) within and near trees of 2 cultivars of *Olea europaea* L. were studied during the 30-day pollination period at 2 urban sites in Tucson, Ariz. 'Manzanillo', the dominant horticultural cultivar, was compared to the fruitless 'Swan Hill'. Air sampling using a Burkard trap was undertaken from 2 Apr. until 1 May 1985; during this period, 95% of the 1985 *Olea* pollen was airborne. Peak atmospheric *Olea* pollen concentrations at both sites occurred on 14 Apr. 1985. Pollen concentrations around the 'Manzanillo' site ranged from 7 grains/m^3 to 6196 grains/m^3 per day. At the 'Swan Hill' site, daily totals were an order of magnitude less, from 5 to 309 grains/m^3 per day. Hourly pollen concentrations for the 'Manzanillo' site on the peak day varied from 1000 to 18,133 grains/m^3 per hr. Hourly values at the 'Swan Hill' site on the peak day varied from 7 to 896 grains/m^3 per hr. Both sites exhibited rapidly increasing pollen concentrations at sunrise with a sharp increase for the 'Manzanillo' site between 1100 to 1300 HR. Both cultivars produced about 85,000 pollen grains per anther. An unknown anatomical or physiological factor in 'Swan Hill' inhibits stomial rupture, resulting in 85% inhibition of anther dehiscence and pollen-shedding.**

In 1984, the Board of Supervisors passed an ordinance banning the sale or planting of *Olea europaea* L. in Pima County, Ariz. This action was taken to protect the public health of those individuals susceptible to ''allergy'' induced by *Olea* pollen. Airborne pollen concentrations of *Olea* resulting in allergy symptoms occur in the southwestern United States and Mediterranean region (2, 8, 9, 16, 21). To date, studies focus on *Olea* as an agronomic crop and horticultural ornamental. The nutrient requirements and growth–fruit production patterns are well-documented for *Olea europaea* L. (5). However, little is known about the physiological–environmental control of anther dehiscence and pollen-shedding, the aerodynamics of pollen dispersal, the efficiency of pollen capture by female flowers, or the minimum number of pollen grains needed to fertilize and set fruit in *Olea*. Long-term phenology and aerobiological data are needed for the dominant cultivars of plants grown in different edaphic and environmental conditions. Griggs et al. (5) collected data on bloom phenology for 5 olive cultivars; they investigated pollen viability by in vitro germinability assays, and the effect of temperature on pollen storage. From 1951–1954, these authors used a gravity slide approach to study pollen dispersal in olive orchards near Davis, Calif.

In this study, we compared pollen production and dispersal in the common *Olea europaea* L. cv. Manzanillo with a newly derived fruitless olive tree, *Olea europaea* L. cv. Swan Hill (6, 17). Pollen production was measured for both cultivars by counting the number of pollen grains per anther. Pollen dispersal was measured by Burkard and Tauber samplers installed at 2 sites in urban Tucson, Ariz. They monitor airborne pollen concentrations hourly, daily, and seasonally.

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