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ABSTRACT

Pollen germination medium was further refined. Using our current methods we have been able to obtain greater than 80% germination for freshly collected pollen of nearly every cultivar tested. The medium contains 25% sucrose, 100 ppm boric acid, 1.0mM CaCl₂ and is solidified with 0.65% agar. Pollen viability, as indicated by its ability to germinate on this medium, declined rapidly when pollen was held at ambient conditions in the laboratory. Indications were that this loss of viability was a consequence of water loss by the pollen. Mean germination fell from 95% at time of collection (water content 5-12%) to 80% at 24 hr (water content ca. 4.5%) and less than 50% at 48 hr (water content ca 4%). By 72 hr germination had dropped to zero and mean water content was approximately 3.5%. Regression analyses indicate that pollen viability as a function of water content conforms to a catastrophic model with 50% fatality occurring at 3.94% water.

Pollen from several cultivars was stored at -20°C (-4°F, freezer storage) and -196°C (-321°F, liquid nitrogen storage). Relative humidity of the freezer storage environment was regulated by maintaining the pollen in closed vessels over saturated solutions selected to provide a range of relative humidities from 8% to 84%. Survival after one year in storage varied among the varieties but for all varieties RH of approximately 30% provided optimum storage conditions. Survival where RH was not maintained near 30% was uniformly low. Similar variation was noted among varieties held at -195°C and, varieties that responded well under one storage regime did not necessarily respond well in the other. 'Chandler' pollen, for example, showed the lowest percent germination (2%) after one year at -20°C, 30% RH but was among the best (39%) following liquid nitrogen storage.

Pistillate flower initiation and differentiation was followed in several cultivars. Results indicated that initiation of the following season's pistillate flowers begins within six to eight weeks after pistillate anthesis. By eight to eleven weeks pistillate flower differentiation stops, not resuming until shortly before bloom the following spring. Cessation of pistillate flower differentiation corresponds to the time that the growing fruits attain full size and begin a series of developmental events that include shell lignification, rapid embryo growth and, ultimately, accumulation of sugars and lipids in the embryo. Termination of pistillate flower differentiation as the growing fruits attained full size was noted in each of the clones examined regardless of the mode of dichogamy characteristic for that clone. In each of the protogynous clones, however, perianth initiation occurred before cessation of organ initiation in the spring. By contrast, in protandrous clones tepals did not form until growth resumed the following year. Thus, protandrous cultivars require more organogenetic activity during the weeks prior to anthesis, a factor...
which is likely a central developmental basis for heterodichogamy in walnut.

PROCEDURES

Pollen germination and pollen tube growth in vitro. Pollen was collected at the time the first staminate flowers of the catkin had begun to shed pollen. Catkins were brought into the laboratory and spread out on sheets of white butcher paper such that pollen was shed onto the paper as the anthers dehisced. Pollen was collected within 4 hours and cleaned by being passed through a 70um-mesh sieve. After screening pollen was sprinkled evenly over the surface of agar-solidified medium or immediately prepared for storage experiments.

Previous work had indicated that only boron (as boric acid), Calcium (as CaCl₂) and sucrose (which serves as an osmoticum as well as an energy source) are required for successful germination and growth of walnut pollen on agar-solidified medium. Extensive experimentation was conducted using permutations of concentrations of these components as well as the agar. Concentration ranges investigated were: sucrose, 5-35% in 5% increments; boric acid, 0-100 ppm in 50 ppm increments; CaCl₂, 0.001-10.0mM in 0.3pCa increments; and DifCo agar, 0.35-0.95% in 0.15% increments.

Pollen germination percentages were determined by counting 1,000 pollen grains after 24 hr incubation. Pollen tube lengths were determined either by use of a computer-interfaced digitizer or as a multiple of pollen grain diameters.

Pollen viability testing. All estimates of pollen viability after storage were based on germination percentages in the medium determined as described above. It should be noted that one must be cautious in using the in vitro germination as an indicator if viability as inability to germinate in vitro does not necessarily indicate inviability in vivo.

Pollen water content. Pollen water content was determined by weighing a sample of pollen, placing it in a vacuum oven at 60°C (= 140°F) and reweighing at intervals until there was no further weight loss. The difference between initial weight and final weight represents the water content of the pollen. Pollen viability was investigated as a function of water content and time at ambient conditions after anthesis.

Pollen storage. Previous results had indicated that pollen stored for one year in sealed vials at -20°C (-4°F, freezer) did not retain the ability to germinate in vitro. These results were confirmed on the modified medium. We attempted to extend freezer storage life to at least a year by regulating the relative humidity (RH) of the storage environment. This was accomplished by keeping the pollen over saturated salt solutions selected to provide a range of RH environments. Salts used and approximate RH at -20°C for each were: LiCl.H₂O (18%), MeCOK.l5H₂O (28%), MgCl₂.0 (33%), NaI.2H₂O (48%), NaBr₂.H₂O (70%) and NH₄Cl (84%). NaOH.H₂O was used to provide a low RH (ca. 8%). Small amounts of pollen were placed in 2ml shell vials capped with cotton. Six such vials,
each containing pollen from a different source, were placed in a 15ml shell vial containing one of the salt solutions. Solution volumes were sufficient to bring the level approximately halfway up the outside of the 2ml vials. The larger vial was sealed with a rubber stopper and paraffin film (Parafilm). Samples were removed from the freezer and allowed to return to room temperature before being opened and assayed for viability after 3, 8 and 12 months.

Tests of liquid nitrogen (−196°C, −321°F) storage of pollen were also done using a long-term liquid nitrogen facility and controlled rate freezer made available to us by the Botany Department at UCD. Pollen was cooled to liquid nitrogen temperatures (−195°C) at a rate of 1°C per minute and held at that temperature until being assayed. Vials were removed from the freezer, returned to room temperature, opened and assayed.

Pistillate Flower Differentiation. Mixed buds containing floral primordia were collected throughout the year from selected protandrous and protogynous clones. Trees were sampled from either the exposed upper portion of the canopy or the outer southern row of the orchard. Samples were taken from six protogynous clones: 'Chico', 'Sharkey', 'Amigo', 63-378, 'XXX Mayette', and 'Meylan'. These cover the range of leafing and bloom times with the first two being early, the second two midseason, and the remaining pair late-season cultivars. Three protandrous cultivars were included for comparison: 'Ashley', 'Chandler', and 'Howard'.

Bud scales and unexpanded leaves were removed from the buds and material was prepared for light and scanning electron microscopy. Later stages of floral differentiation were easily identified under the highest magnification of a dissecting microscope following dissection of the bud and these were typically not processed further except for occasional samples used for documentation.

Pollen tube growth, fertilization and fruit set. Pistillate flowers of 'Chcio' were pollinated with 'Serr' pollen and collected at two-day intervals for the next two weeks. Flowers were fixed and embedded in paraffin for microscopic analysis of pollen tube path and details of ovule development and fertilization.

RESULTS AND CONCLUSIONS

Pollen germination and pollen tube growth invitro. The medium determined to be the most suitable for pollen germination and maintenance of pollen tube growth consists of 1.0mM CaCl₂, 100 ppm boric acid, 25% sucrose solidified with 0.65% DifCo™ agar. It is our opinion that the primary factor in the failure of previous workers to devise a suitable medium for walnut pollen was due to overemphasis on the chemical components of the medium while neglecting the hydraulic environment. The critical event in pollen germination is controlled rehydration and reestablishment of membrane integrity in the pollen grain. Chemical requirements are simple and predictable (Ca²⁺, boron and an energy source). The critical factors in attaining controlled hydration invoice osmotic and matrix effects and were manipulated here by altering concentrations of sucrose and agar respectively. We have shown that walnut pollen has very narrow toleration limits for these factors. In
our opinion this is related to the extremely low water content of walnut pollen at anthesis (see below).

Pollen viability at ambient conditions. Water contents of 18 cultivars of walnut pollen at the time of shedding ranged from 4.8 to 12.2%. The top of this range is low and the mean and minimum values are exceptionally low when compared to other species. Indeed, the lowest values place walnut pollen among the driest reported. This observation is confirmed in the literature. In spite of the wide range of values and low water content, germination at anthesis was uniformly high (ca. 90%) for freshly shed pollen. Results further indicated that water above approximately 4-5% is relatively loosely bound, as we found that after 24 hr at ambient conditions the range of pollen water content had decreased to 3.9 to 5.2%. Pollen at this time had germination percentages of approximately 80%. By 48 hr mean germination fell to less than 50% and mean water content was approximately 4.0%. By 72 hr germination had dropped to 0 and mean water content was approximately 3.5%. Regression analyses indicate that pollen viability as a function of water content conforms to a catastrophic model with 50% fatality occurring at 3.94% water and that water loss in the critical range is linearly correlated with time.

It is concluded that the relatively short longevity of walnut pollen is a direct consequence of (1) its exceptionally low water content at anthesis and (2) the relatively rapid rate of water loss from the pollen. Additionally, although not directly tested here, indications from the literature suggest that the period of viability under field conditions would be much less than that determined here in the lab.

Pollen Storage. Controlling the relative humidity of the storage environment greatly enhanced the storage life of pollen from all varieties. Optimum RH for storage was in the range of 30% which provided significant improvement over freezing without regulation of RH. While varieties with high retention of viability under uncontrolled storage did improve greatly, the effect was most dramatic in varieties that failed to germinate at all after a short time in storage. 'Chandler' and 'Tehama' were two such varieties going from 0 to 6.2% in the case of 'Chandler' and 23.6% in the case of 'Tehama'. We conclude that storage at -20°C is possible if the relative humidity of the storage environment is maintained at approximately 30% by using a saturated solution of MeCOOK\(\cdot\)1.5H\(_2\)O (potassium acetate) or MgCl\(_2\)\(\cdot\)6H\(_2\)O. Although not tested here, CaBr\(_2\)\(\cdot\)6H\(_2\)O may also be suitable as it too provides RH in the acceptable range.

A problem was encountered with the 18% RH storage environment. The LiCl\(\cdot\)2H\(_2\)O solution used to provide 18% RH apparently created a toxic situation as pollen stored over that solution was completely unable to germinate by 3 months and, circumstances make it unreasonable to attribute this loss of viability to RH alone. Thus, as it may be possible that storage in the 15-25% RH range is preferable to 30% it would be reasonable to investigate that possibility using salts other than LiCl\(\cdot\)2H\(_2\)O.

Even under these conditions of controlled RH viability in storage varied
among cultivars and, in the case of some cultivars (eg. 'Chandler'), will be insufficient for effective use in a field situation. However, where the objective of pollen storage is to have material for hand pollinations, as in the walnut breeding program, it seems likely that the method described should be useful. We further conclude that under storage without controlled RH the likelihood of having a adequate percentages of viable pollen for any purpose is low.

Our results for liquid nitrogen storage also indicated variability among the varieties tested. Interestingly, 'Chandler' pollen, which had the poorest response to storage at -20°C, was extremely responsive to storage at -195°C. As the behavior of free water in a cell is a critical factor in determining the capacity of that cell to survive freezing, the possibility exists that the variation in the ability of cultivars to survive at -195°C may be related to variations in the amount of loosely bound pollen water (estimated to range from 0 to 7%, see above) at the time of freezing rather than to strictly genetic differences. If this is the case it may be possible to hold pollen for 24 hr before freezing and thereby attain high survival rates. At 24 hr most loosely bound water has been lost but germination is sufficiently high. We intend to explore the implications of these results further this spring.

Pistillate Flower Differentiation. Pistillate flower differentiation begins with the initiation of a bract and two bracteole primordia that grow together to form a ridge of involucral tissue around the flank of the floral meristem. Through this stage the floral apex is flat. Subsequently, the four oppositely positioned tepals of the perianth arise within the ring of the primordial involucre. The developmental sequence is completed by the eventual emergence of the gynoecium, which arises from the center of the floral apex.

In our material pistillate flowers were found to be initiated early in the spring with differentiated floral primordia evident by six to eight weeks after pistillate anthesis for each of the clones examined. During this period the young fruits present on the tree undergo rapid increase in size marked primarily by increases in the involucral tissues and endosperm until, depending on cultivar, about eight to eleven weeks after pistillate bloom when the fruits reach their full size. This growth is mainly characterized by increase in size driven by water uptake. As fruits attain maximum size there is a shift in their development that leads to an increase in the requirement for photosynthate by the growing fruits. Developmental events occurring at this time include shell lignification and rapid embryo growth with accumulation of sugars and, ultimately, lipids in the embryo. Examination of buds collected after this period indicated that differentiation of new organs in the developing pistillate flowers had stopped and further development of organs that had been initiated slowed greatly as the fruits reach full size. Pistillate flower organogenesis did not resume until the weeks prior to bloom the following season.

This sequence of events suggest that nutrient partitioning phenomena
may be important factors leading to the cessation of organ initiation in the primordial flowers such that the developing pistillate flowers become less favored sinks as the nutritional demands of fruits increase.

This general pattern of early floral initiation with no differentiation during the period of pericarp lignification and embryo development was evident in each clone examined regardless of the pattern of dichogamy typical of that clone. The major difference between the protogynous and protandrous clones was in the degree of pistillate flower differentiation completed before differentiation ceased in late spring. For each of the three protandrous cultivars organogenesis stopped following the differentiation of the involucral ring. In all of the protogynous clones initiation of perianth parts was completed by eight to ten weeks.

Thus, protogynous individuals enter the next growing season with Pistillate flowers having attained a developmental stage that allows gynoecial differentiation to proceed immediately. By contrast, the protandrous cultivars require more organogenetic activity during the weeks prior to anthesis as perianth parts must be initiated in addition to the gynoecium (Fig. 4). As the time required for this additional developmental activity is sufficient to account for the relative differences in pistillate bloom times between protandrous and protogynous walnuts, it appears that a fundamental basis for heterodichogamy in walnut is in the extent of pistillate flower differentiation that occurs during the postanthesis period.

Wetzstein and Sparks at the University of Georgia have noted a similar phenomenon in the expression of heterodichogamy in pecan, a close relative of the walnut. They found that the mode of dichogamy correlated with the timing of staminate flower organogenesis. However, the timing of pistillate flower differentiation in pecan did not vary with the mode of dichogamy; rather, differentiation was closely correlated to the stage of bud development and essentially similar for each of the cultivars examined. We did not include an examination of staminate flower development in our walnut study but, in light of Wetzstein and Spark's conclusions, such an analysis would seem to be appropriate.

Pollen tube growth and fertilization. Since the work of German plant morphologists at the turn of the the century, the pollen tube growth path in Juglans has been considered to be chalazogamous. That is, rather than pollen tubes entering the ovule through the micropyle as is typical for nearly all flowering plants, the pollen tubes are supposed to grow around to the basal (chalazal) end of the ovule, through the vascular tissue supplying the ovule and enter the ovule through the side opposite the micropyle. This view of pollen tube growth is so well entrenched in the literature on floral biology that walnut is typically listed in textbooks as a prime example of chalazogamous fertilization. We, as well as others interested in the transmission of blackline virus in walnut, have assumed that this is the case and have designed our analyses based on this assumption. Over the past two years we have come to believe that this is not the case after all. We believe that we have identified the artifact that led earlier workers to suppose that the species is chalazogamous and have
reasonably good evidence that the usual mode of fertilization, entry of the pollen tube through the micropyle, is the case in walnut. We expect to have the definitive results necessary to challenge such a widely held view after next spring's bloom period.