Flavonoids, Alkali Earth, and Rare Earth Elements Affect Pecan Pollen Germination

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Additional index words. alternate bearing, nutrition, pollination, fruit set, viability, breeding, flowering

Abstract. The factors regulating pecan [*Carya illinoensis* (Wangenh.) K. Koch] pollen grain germination are poorly understood for both in vitro pollen viability tests and on receptive stigmatic surfaces of pistillate flowers. Potential regulating factors include flavonols, calcium (Ca), Ca-like alkali earth elements (AEEs), and rare earth elements (REEs). When various concentrations of certain naturally occurring simple flavonols (e.g., quercetin, kaempferol, myricetin, naringenin, and hesperetin) were tested in vitro by adding to standard pecan pollen germination medium, hesperetin, myricetin, and kaempferol functioned as a strong agonist at low concentration (0.12–2.0 μM for hesperetin and kaempferol, and 0.25 μM for myricetin), increasing pollen germination 2- to 3.9-fold over flavonol-free media. Hesperetin and myricetin were antagonistic at 16 μM . Kaempferol was not antagonistic at any concentration up to and including 16 μM . Naringenin was an antagonist at concentrations from 0.12 to 16 μM ; whereas, quercetin was an antagonist at 8–16 μM , but tended to function as an agonist at low concentration (0.12–0.50 μM). The equal molar replacement of Ca^{2+} in standard pecan pollen germination media by single REEs, resulted in certain REEs [e.g., yttrium (Y), gadolinium (Gd), and thulium (Tm)] partially replacing the obligate need for Ca^{2+} ; thus, functioning as agonists in absence of Ca. All non-Ca AEEs [beryllium (Be), magnesium (Mg), strontium (Sr), except for barium (Ba)], also partially substituted for Ca^{2+} at equivalent molar concentrations, but none were as efficacious as Ca^{2+} . Results are suggestive that a) pollen germination in in vitro test can be improved by incorporation of certain flavonols, and b) pollen germination on stigmatic surfaces of flowers in orchards might be influenced or regulated by flavonol composition and Ca-like metals in the liquid matrix of the wet (receptive) stigmatic surface.

Irregularity of fruit set is a common problem of commercial pecan (*C. illinoensis*) orchards and can be partially due to poor flower fertilization. Poor fertilization of pistillate flowers can be due to several factors, some of which are excessive self-pollination (Marquard, 1988), lack of pollen availability at time of stigma receptivity (Wood, 1997, 2000; Wood and Marquard, 1992), or pollen-stigma incompatibilities (Wood et al., 1997). Successful fertilization involves transfer of two male gametes (haploid sperm cells) from each pollen grain, with one to a haploid female gametophyte (egg) cell in the embryo sac to form the diploid zygote and the second fusing with the two polar nuclei at the center of the embryo sac to form the triploid endosperm, with the endosperm functioning

as a food source to nourish the developing embryo or nutmeat (Wilhelmi and Preuss, 1999). Failure of either fertilization leads to yield loss manifested by premature fruit drop or by absence of kernel (i.e., due to failure to produce the diploid zygote, producing a “pop”).

Pecan is a wind-pollinated species and therefore possesses “dry” pollen when grains are mature. The stigma and pollen of higher plants are of two broad types, i.e., “wet” or “dry,” with respect to the amount of secreted matrix on their surfaces (Lord and Russell, 2002). Depending on species, the stigma is “wet” and pollen is “dry” or conversely the stigma is “dry” and pollen is “wet.” Wind-pollinated species, like pecan, possess “wet” stigmatic surfaces when female flowers are receptive. The composition of this “wet” layer on stigmatic surfaces is poorly understood but contains a mix of organic and inorganic components (Lord and Russell, 2002) and almost nothing is known about pecan’s stigma secretions. This “wet” surface at time of receptivity provides the solvent (e.g., water and lipids), osmoticum (e.g., sugars), ionic ingredients [calcium (Ca^{2+}), protons (H^+), potassium (K^+), borate (BO_3^-), and chloride (Cl^-)] known to be requisite for

a degree of in vitro pollen grain germination and subsequent germ tube growth. Findings from other species are suggestive that other important growth promoting or inhibiting chemicals, such as flavonols, are also present under natural conditions (Crowe et al., 1989; Hepler et al., 2006).

Candidate bioactive components of the exudate of receptive stigmatic surfaces include the flavonoids. The flavonoids (a type of polyphenolic compound) is a large group of secondary metabolites found in essentially all higher plant species, exhibit diverse functionality—ranging from ultraviolet protection, to messenger and signaling molecules (Forbes et al., 2014), to affecting plant-pathogen interactions (Taylor and Grotewold, 2005). Flavonoid subgroups are the flavones, flavanones, dihydroflavonols, flavonols, and anthocyanins. Flavonoids are linked to pecan flowering in that pigmented flavonoids produce the anthocyanin pigments contributing to the coloration of stigmatic surfaces [i.e., stigma color is controlled by a single gene with green dominate to red/purple (Beedanagari et al., 2005)]. Because flavonoid biosynthesis occurs in pecan stigmas, nonpigmented flavonoids also possibly contribute to the “wet” matrix of receptive stigmatic surfaces and affect pollen germination and subsequent fertilization. Simple nonpigmented flavonoids play a critical role in pollen germination and germ tube growth of several nonwoody dicot species (Forbes et al., 2014; Taylor and Grotewold, 2005; Vogt et al., 1995; Ylstra et al., 1992); thus, raising the possibility they might increase (agonist) or decrease (antagonist) germination of pecan pollen and subsequent fertilization.

The role of metals in pecan pollen germination is poorly understood. Although Ca functions as an essential AEE metal for pollen germination of most species (Hepler et al., 2006), it is unknown how associated processes are affected by other AEEs (Be, Mg, Sr, Ba). Additionally, relative to most tree species, pecan hyperaccumulates REE metals possessing physiochemical traits similar to Ca (Wood and Grauke, 2011); hence, raising the possibility that one or more of these metals influence pollen germination and subsequent pollen tube growth. The REEs represent the largest group of elements in the periodic table, are common in the soil and plant environment, and have potential for yet undetermined essential or beneficial roles in metabolism and physiology of certain plant species (Brown et al., 1990). REEs are quantitatively abundant in pecan and possess physiochemical similarities to several essential nutrient elements [e.g., Mg, manganese (Mn), iron (Fe), and zinc (Zn)], but are especially similar to Ca (Bulman, 2003; Evans, 1983; Franklin, 2001; Lim and Franklin, 2004; Wybourn, 2004).

This study assesses the influence of several naturally occurring simple flavonols, AEE, and REE metals on pecan pollen grain germination in vitro and provides evidence for possible involvement of these factors under natural orchard conditions.

Received for publication 3 Oct. 2016. Accepted for publication 16 Nov. 2016.

I gratefully acknowledges field and laboratory assistance by James Stuckey and Kirby Moncrief. Use of trade names does not imply endorsement of the products named or criticism of similar ones not named.

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Materials and Methods

Pollen source. The influence of simple flavonols and REEs on pollen germination was investigated in vitro with pollen collected from 'Desirable' trees grown in a commercial-like research orchard at Byron, GA (lat. +32°39'54'' N, long. +83°44'31'' W). Pollen source trees were managed according to Georgia Extension Service guidelines for pests, fertilizers, etc. (Hudson et al., 2007). Pollen from five 'Desirable' trees was collected midmorning from catkins in late April 2015, as the catkins were beginning to shed pollen so as to ensure test pollen was fully mature. Collected catkins were immediately transferred to a Styrofoam cooler and then spread in a dark room, as a thin layer no more than 2–3 catkins deep, on a suspended nylon screen at 23 °C and in the dark. Dehiscent pollen was collected after 24 h from wax paper below the screens, bulked, cleaned by sifting through a no. 140 sieve, thoroughly mixed to ensure a homogenous mixture from the five source trees, and stored airtight in 15-mL polypropylene vials at 15 °C until needed.

Pollen germination media. Pollen germination tests were conducted by thinly dispensing stored pollen onto plastic weighing boats, with pollen then acclimating to room temperature and hydrating for 4 h while suspended in the humid atmosphere within a desiccator containing a saturated $\text{Cu}(\text{SO}_4)_2$ solution before placing into germination media. The basic liquid germination media were similar to that used by other pecan researchers (Conner, 2011; Yates and Sparks, 1989, 1990; Yates et al., 1986, 1991), consisting of 20% sucrose, 0.05% $\text{Ca}(\text{NO}_3)_2$ (note that Ca is absent from treatments testing AEE and REE metals), 0.025% KCl, and 0.01% H_3BO_3 . Media (3 mL) was dispensed into small petri dishes (5 cm diameter) with different flavonols or AEEs or REEs added depending on treatment. A fixed amount of pollen (about 0.01 mg) was added to the liquid germination media and lids replaced to prevent evaporation and subsequent concentrating of the media solution. The pollen–substrate mix was then incubated at 23 °C, and in the dark for 24 h before assessing germination. Pollination percentage was assessed using a light microscope ($\times 100$ magnification). Pollen grains were considered germinated in all experiments if the length of the emerging pollen tube was at least as long as the diameter of the pollen grain. Percentages were based on counting of germinated vs. nongerminated grains within a zone at the center of the petri dish.

Flavonols. The naturally occurring flavonol aglycones evaluated consisted of several that have shown activity in certain other plant species. These are myricetin (CAS no. 529-44-2), kaempferol (CAS no. 520-18-3), naringenin (CAS no. 480-41-1), hesperetin (CAS no. 520-33-2), and quercetin (CAS no. 117-39-5) (Stanford Chemical Company, www.stanfordchem.com; Irvine, CA). It is presently unknown whether these specific flavonols are naturally present on the receptive stigmatic

surfaces of pecan pistillate flowers. These flavonols were added to the basic media described above to give flavonol concentration treatments of 0, 0.125, 0.25, 0.50, 1, 2, 4, 8, and 16 μM .

Experimental designs. The flavonol experiments consisted of "9 flavonol concentrations" and "5 flavonol forms" treatments totaling 45 treatments for each experiment. Experiments were replicated four times to assess flavonol influence on germination percentage. The effect of flavonols on germination percentage was evaluated using regression analysis and standard error of means.

The AEE and the REE studies were conducted using nitrate salts of each of these elements at a concentration equivalent to the molar metal equivalent of the 0.05% $\text{Ca}(\text{NO}_3)_2$ control. These experiments were repeated four times to assess metal impact, relative to that of Ca, on pollen germination as represented by percentage of grains germinating. Data were arcsin transformed and analyzed by standard least squares analysis of variance and treatment means separated by LSMeans differences Student's *t* test.

Results

All five tested flavonols influenced pecan pollen grain germination percentage. The presence of hesperetin at relatively low concentration (i.e., 0.12–2 μM) increased germination 2- to 3.9-fold compared with the absence of hesperetin (Fig. 1). Myricetin increased pollen germination by about 2-fold and kaempferol increased 2.3- to 3.1-fold at low concentration (i.e., 0.25 μM for myricetin and 0.12–2.0 μM for kaempferol) (Figs. 2 and 3). This is suggestive that hesperetin, myricetin, and kaempferol are potentially agonists at low concentration; thus, potentially increasing germination percentage of pecan pollen in vitro. Conversely, quercetin also appeared to function as an agonist at low concentrations, but an antagonist at high concentration (i.e., 8–16 μM ; Fig. 4). Alternatively, naringenin functioned as an antagonist at all tested concentrations (0.12–16 μM ; Fig. 5).

The germination of pecan pollen depends on the presence of Ca^{2+} ions in the germination medium, with only about 1% of grains germinating in absence of the Ca^{2+} but much higher percentages in the presence of Ca^{2+} (Fig. 6). This differential differs depending on cultivar, year, storage conditions, pollen maturity, and many other factors. Ca^{2+} is physiochemically similar to Be^{2+} , Mg^{2+} , and Sr^{2+} and the results in Fig. 6 indicate they partially substitute for Ca^{2+} ions in the pollen grain germination (25% to 45% vs. 78%) process, although none of these AEEs were nearly as promotive as Ca^{2+} . Be^{2+} was more effective at replacing Ca^{2+} requirements than was Mg^{2+} . The heaviest AEE, Ba^{2+} , did not substitute for Ca^{2+} to any degree. When individual trivalent (M^{3+}) metals (i.e., Al^{3+} , Ga^{3+} and Cs^{3+}) and REEs (Sc^{3+} , Y^{3+} , La^{3+} , Ce^{3+} , Pr^{3+} , Nd^{3+} , Sm^{3+} , Eu^{3+} , Gd^{3+} , Tb^{3+} , Dy^{3+} , Ho^{3+} , Er^{3+} , Tm^{3+} , Yb^{3+} , Lu^{3+}) replaced Ca in the germination media, at the same molar

Fig. 1. Influence of different concentrations of the flavonol, hesperetin, on germination of 'Desirable' pecan pollen in vitro.

Fig. 2. Influence of different concentrations of the flavonol, myricetin, on germination of 'Desirable' pecan pollen in vitro.

Fig. 3. Influence of different concentrations of the flavonol, kaempferol, on germination of 'Desirable' pecan pollen in vitro.

Fig. 4. Influence of different concentrations of the flavonol, quercetin, on germination of 'Desirable' pecan pollen in vitro.

Fig. 5. Influence of different concentrations of the flavonol, naringenin, on germination of 'Desirable' pecan pollen in vitro.

concentration as Ca^{2+} in the control, certain REEs partially substituted for Ca^{2+} , but with reduced germination (e.g., <22% for the most promotive metal vs. 78% for Ca^{2+}). The non-REE trivalent metals (M^{3+}) exhibited little or no ability to substitute for Ca^{2+} . Although most REEs did not exhibit a promotive effect, a few did improve germination compared with the negative Ca control. The greatest agonists among the REEs were Y^{3+} , Gd^{3+} , and Tm^{3+} , enabling germination of about 15% to 20% over the negative Ca^{2+} control at 1% vs. 78% for the positive Ca^{2+} control. Additionally, although not statistically significant, there is evidence that Er^{3+} and Yb^{3+} might exhibit a small stimulative response. None of the REEs were as effective as was Ca^{2+} in stimulating pecan pollen grain germination.

Discussion

Pecan, as do woody angiosperms in general, possesses a well-developed mechanism

Fig. 6. Efficacy of alkali earth elements (AEE) and rare earth element (REE) metals for replacing Ca functionality in germination of pecan pollen in vitro. Means with different letters statistically differ using Student's t test with $\alpha = 0.05$.

for enabling pollen grain germination and subsequent control of fertilization. Aspects of this process appear to involve both flavonols and Ca , and possibly Ca -like elements. The observed promotive and inhibitory bioactivity of naturally occurring flavonols at micro- or nanomolar concentration indicates great sensitivity of pecan pollen germination physiology to flavonols. These data indicate that efforts to maximize germination of pecan pollen in vitro might benefit from inclusion of nanomolar concentrations of an agonistic flavonols, such as hesperetin, myricetin, quercetin, or kaempferol in the germination media. The addition of certain Ca -like REEs might also enhance in vitro germination of pecan pollen grains.

These results are suggestive that flavonols possess a signaling function in pecan's pollination/fertilization process, similar to what has been observed in certain other diploid species (BéBoux et al., 2000; Derksen et al., 1999; Forbes et al., 2014; Karapanos et al., 2006; Sedgley, 1975; Taylor and Hepler, 1997). The pollination/fertilization process begins when a relatively desiccated "dry" pollen grain landing on the "wet" surface (i.e., receptive) of a pistillate flower's stigma. The pollen grain then hydrates on exposed to substances present in the liquid matrix on the receptive stigma. Findings from other species indicate that short-chain lipids within the stigmatic exudate act to trigger hydration and subsequent opening of aquaporin-like channels on the pollen surface allow movement of water through pollen membranes, which then sets the pollen grain

up for bioregulatory interactions with flavonols (Wilhelmi and Preuss, 1999). As with other higher plant species, the present study is suggestive that pecan likely possesses a recognition mechanism operating at the molecular level where interspecific pollination is rigidly controlled so that pollen from species dissimilar to that of the pistil are rejected (Franklin-Tong, 1999). Pollen from many different sources, to include other *Carya* and non-*Carya* species, undoubtedly lands on receptive stigmas of pecan; however, it is unknown whether these pollens germinate and produce germ tubes. It is possible that the qualitative and quantitative mix of flavonols in the stigmatic matrix on the stigmatic surface, and perhaps within style's microphyle channel, ensures that only pecan and certain other *Carya* sp. (due to the ability to make pecan-hickory hybrid seed) are able to make complete pollen grain hybridization and production of a germ tube capable of growing down the stigma's micropyle passage to the ovary, where two parental haploid nuclei are delivered to complete the double fertilization process required to produce the diploid zygote (to become the nutmeat) and the triploid endosperm (to become the endosperm that nourishes the developing embryonic tissues and organs).

The exudates on pecan's floral stigmas likely present an early barrier to germination and tube growth and function as part of a complex mechanism to prevent self-fertilization or fertilization by other species. For example, maize and petunia plants are self-sterile when the female plant is unable to

produce flavonoids, but fertility is restored by addition of flavonol aglycones, a flavonoid subgroup (Derksen et al., 1999; Mo et al., 1992; Taylor and Jorgensen, 1992; Xu et al., 1997; Ylstra et al., 1992). In the case of *Petunia hybridia*, only flavonols with unsubstituted hydroxyl groups at positions 3 and 7 could induce pollen germination, whereas hydroxylation of the position 5 and the B ring inhibited germination (but methylation promoted germination) (Vogt et al., 1995). Flavonoids occur in 4000+ molecular forms (Vogt et al., 1995), and it is presently unknown which molecular species occur on pecan stigmatic surfaces or as constituents of the “wet” stigmatic matrix.

The “wet matrix” on receptive stigmas appear to be lipid based, as it resists removal by water (e.g., rain) but is easily removed by a mild soap solution that results in a flower that is no longer capable of being fertilized (personal observation). It is possible that in situations of prolonged rainfall spanning several days, that successful fertilization of flowers is impaired by either washing off, or dilution of, the postulated flavonol containing “wet matrix” on stigmatic surfaces.

The diploid pecan, and especially the tetraploid *Carya* species, hyperaccumulate REE metals (Wood and Grauke, 2011). This hyperaccumulation likely confers heretofore unidentified survival advantages for these *Carya* species in certain environments, and may function to replace or supplement certain physiological roles for Ca. In vitro pecan pollen germination studies clearly indicate that Ca^{2+} is essential for germination. This is suggestive that the liquid exudate on receptive stigmas contains Ca^{2+} and possibly other Ca^{2+} -like ions, such as the REEs or AEEs. It is noteworthy that certain REEs can replace Mg and Zn in certain proteins and enzymes, and Gd and La act as Ca analogues in Ca-channel studies (Lim and Franklin, 2004), and they also bind with Ca-dependent protein kinases involved in key cellular processes (Polya et al., 1987). The REEs are surprisingly similar in ion size, coordination environment, ligand preferences, and Lewis acid activity (giving good hydrolytic activity) to Ca, but are trivalent. It is noteworthy that the other trivalent metals were substituted for Ca in the germination medium; they were highly inhibitory to germination. Additionally, the ability of Be^{2+} , a relatively rare metal in the environment, to elicit substantially greater pollen germination than Mg^{2+} , a relatively abundant alkali earth metal, is suggestive that the Ca^{2+} -requiring pollen germination processes are finely tuned to exclude much interference by physiochemically similar Mg^{2+} .

The observed effect of certain REEs on promoting pollen grain germination hints of a potentially beneficial role in pecan pollen germination, such as in environments where Ca is limiting. Because no other AEE or REE metal was as effective as Ca for meeting the physiological needs for pollen germination, it appears that although the physiological role for Ca in germination is tightly regulated, it is not so tight as to exclude a backup option

afforded by certain REEs which are typically readily available in most soils. Additionally, the great variability in pollen germinated encountered in in vitro germination studies may be linked in part to differences in pollen grain sensitivity to flavonols or to AEE and/or REE metals in the germination medium.

In summary, the flavonols in particular, but also the AEE and REE composition of pecan pollen germination medium affects germination percentage. This is suggestive that presence of these factors in the liquid matrix on receptive stigmas potentially influences pollen compatibility and germination in orchard and wild pecan trees and therefore flower fertilization, fruit set, and kernel quality.

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