Seed production and gametophyte formation in Agave tequilana and Agave Americana

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Seed production and gametophyte formation in *Agave tequilana* and *Agave americana*

Rocio E. Escobar-Guzmán, Flor Zamudio Hernández, Katia Gil Vega, and June Simpson

Abstract: *Agave tequilana* Weber var. *azul* is the raw material used in the production of tequila. This species has a life cycle of approximately 6–8 years; however, owing to the practice of removing the inflorescence to conserve accumulated sugar reserves, the main form of reproduction is asexual. Little attention has, therefore, been paid to the process of flowering and the factors leading to low levels of germination and seedling viability have not been investigated in detail. The objective of this study was to document gametophyte development, seed production, and germination in *A. tequilana* under different pollination treatments and in an interspecies cross with *Agave americana* L. Seed production and germination efficiency was low for both *A. tequilana* and *A. americana* under the different pollination treatments, although interspecies crosses did produce some viable seeds. Development of the male gametophyte in both species is of the successive type, producing pollen grains with dicolpate morphology. Female gametophyte development is of the *Polygonum* monosporic type. The results obtained suggest that genetic incompatibility, inbreeding effects, factors affecting pollen development and germination, or errors in female gametophyte development may contribute to the low fertility observed for *A. tequilana* and *A. americana*.

Key words: Agave americana, *Agave tequilana*, female gametophyte, germination efficiency, male gametophyte, pollination treatments.

Résumé : L'*Agave tequilana* Weber var. *azul* constitue le matériel de base pour la fabrication de la tequila. Son cycle de vie s'étend sur environ 6–8 ans, cependant comme on élimine les fleurs pour conserver les réserves en glucides accumulées, la reproduction asexuelle domine. On a conséquemment accordé peu d'attention à la floraison et on n'a pas étudié en détail les facteurs qui conduisent aux faibles taux de germination et de viabilité des plantules. Les auteurs décrivent le développement du gamétophyte, la production des graines et la germination de l'*Agave tequilina* selon différents traitements de pollinisation et croisements interspécifiques avec l'*Agave americana* L. Les différents traitements de pollinisation conduisent à de faibles taux de production de graines et de germination chez les deux espèces d'agaves, bien que les croisements inter-spécifiques aient produit quelques graines viables. Le développement du gamétophyte mâle chez les deux espèces appartient au type successif, produisant des grains de pollen à morphologie bicolpés. Le développement du gamétophyte femelle est du type *Polygonum* monosporal. Les résultats suggèrent que l'incompatibilité génétique, les effets d'autocroisements, les facteurs affectant le développement du pollen et sa germination, ou des erreurs dans le développement du gamétophyte femelle peuvent contribuer à la faible fertilité observée chez les *A. tequilana* et *A. americana*.

Mots-clés : Agave americana, *Agave tequilana*, efficacité du gamétophyte femelle, efficacité de la germination, gamétophyte mâle, traitements de pollinisation.

[Traduit par la Rédaction]

Introduction

The Agavaceae family with 9 genera and 330 species is endemic to America (García-Mendoza 2004). Mexico is the center of origin for 76% of all *Agave* species, several of which have been exploited for the production of fibers or alcoholic beverages such as tequila and mezcal (Piven et al. 2001).

The preparation of alcoholic beverages is by far the most important economic activity associated with agave cultivation. Although mezcal can be prepared form a wide variety of species of agave, under the "Denomination of Origin" (Diario oficial 1993), at least 51% of the sugars used to produce tequila must come from *Agave tequilana* Weber var. *azul*. Despite the economic importance of *A. tequilana*, very little research into the genetics, breeding, and physiology has been carried out for this species. This is probably due to the long life cycle of the plant, the fact that inflorescences are difficult to access, and the practice in commercial *Agave* plantations of removing the inflorescence as soon as it emerges to conserve sugar reserves.

Agave tequilana plants are monocarpic perennials that produce flowers only once at the end of their life cycle of approximately 5–8 years, after which they die. However, *A. tequilana* var. *azul* is normally propagated asexually through offsets or suckers formed on rhizomes, and plants

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are only rarely found flowering in the field. Although Valenzuela-Zapata (1997) has reported low germination efficiencies, very few data are available. The occurrence of hybridization between *A. tequilana* and other *Agave* species has never been documented, although other interspecific crosses have been reported (Doughty 1936).

Sexual reproduction in *A. tequilana* normally begins in February or March when the vegetative apical meristem retracts or "sinks" marking the transition from vegetative apical meristem to floral meristem. Once initiated, the inflorescence or "quiote," which is covered with bracts, undergoes a period of rapid growth (up to 5 cm per day) until reaching a height of around 5–6 m. At a height of around 4 m, lateral branches or umbels begin to form on the inflorescence. Gentry (1982) reported 20–25 umbels·plant^{-1}, each capable of producing hundreds of flowers. Flowering proceeds from the lowest to highest umbels during the months of June and July and all stages of flower development can be observed simultaneously on a single plant. Flowers are protandric and composed of green tepals with purple tips and 6 anthers joined to filaments at the base of the tepals. The ovary is inferior and divided into three compartments with a long style terminating in moist stigmas. Since anthesis occurs before the stigmas mature, released pollen could fertilize receptive flowers lower on the inflorescence. This process is similar in *A. americana* and a more detailed description of flower structure and development in both species can be found in Gentry (1982).

Data on seed production and efficiency of germination are available for very few *Agave* species. Arizaga and Ezcurra (2002) reported the production of around 2800 seeds per plant in *Agave macrocantha* Zucc. with an efficiency of germination of 76%, whereas Nobel (1977) reported the production of around 65 000 seeds per plant in *Agave deserti* Engelm. In *Agave palmeri* Engelm., (Howell and Roth 1981) reported the production of 38 000 seeds per plant when natural pollinators such as insects, birds, and bats had access to the plants, but only 1600 seeds per plant when these pollinators were excluded, indicating the important role that pollinators play in the reproduction of *Agave* species.

However, nothing is known about seed production and efficiency of germination in *A. tequilana*, where inefficient pollination may also account for low seed set and germination efficiency. Other factors could be incompatibility between closely related genotypes or inadequate gametophyte development. Although detailed information on gametophyte development in *A. tequilana* is not available, (Dahlgren et al. 1985) reported that in general the embryo sac is of the *Polygonum* type, and endosperm formation is helobial in the *Agave* genus, whereas Piven et al. (2001) found that in henequen (*Agave fourcroydes* Lem.) and *Agave angustifolia* Haw., the female gametophyte is formed from two micropylar megaspore cells (bisporic type).

Basic information relating to micro- and mega-gametophyte development, details of seed and fruit development, percentages of germination of pollen grains, and efficiency of seed production and viability in *A. tequilana* is needed to develop research in genetics and breeding in this important crop plant, with a view to improvement in terms of disease resistance, broadening of the narrow germplasm base, and of traits such as sugar accumulation, production of offsets, and precocity.

The objectives of this investigation were (*i*) determine the levels of fruit and seed production and the viability of the seeds obtained by natural pollination, manual self pollination, manual cross pollination, and manual interspecific pollination treatments; (*ii*) determine the viability of the pollen produced by *A. tequilana* and *A. americana* plants; and (*iii*) study the development of male and female gametophytes in *A. tequilana* and *A. americana*.

Materials and methods

Plant material

Flowering *A. tequilana* plants are rare, since the inflorescences are removed as soon as they emerge to maintain sugar reserves. However, for this study, access was obtained to four *A. tequilana* plants (At2, At3, At4, At5) and one *A. americana* (Aa) plant that had reached maturity and produced fully developed inflorescences. All plants were located at Cinvestav, Campus Irapuato, where they had been planted as offsets obtained from a commercial plantation. Samples were pollinated between June and July 2003, and data on the number of umbels, capsules, and seeds per plant were recorded.

Determination of numbers of flowers, fruits, and seeds per plant

Estimations of total numbers of flowers, fruits, and seeds per plant were carried out on umbels and flowers, which were left to pollinate naturally. The number of flowers produced on five umbels of each *A. tequilana* or three umbels of the *A. americana* plant (all from the middle section of the inflorescence) were counted, and following natural pollination, the number of fruits formed were also counted. Based on the number of umbels of each plant, an estimation of the total number of flowers and fruits produced per plant was calculated. Fruits were left on the inflorescence until they were completely brown and dry, and were then harvested. An estimation of the number of black and potentially viable seeds produced per plant was done by counting the number of black seeds in different individual fruits taken randomly from each plant, and taking into account the estimated number of fruits produced under conditions of natural pollination.

Pollination treatments

Four different pollination treatments were applied to both *A. tequilana* and *A. americana* plants. Umbels from the middle sections of the inflorescence were used in all cases.

Manual self-pollination

Anthers were removed from unopened floral buttons, which were then covered with pollination bags (normally used for maize) and allowed to develop until the stigmas were receptive (moist stigmas). Anthers with mature pollen grains from around 10 flowers of the same plant were collected immediately before pollination and, using a paintbrush, pollen was transferred to the mature stigmas. Pollinated flowers were again bagged and left to develop

fruit. These procedures were carried out for *A. tequilana* plants At3, At4, and At5, and *A. americana* (Aa) plant.

Manual cross-pollination

Intraspecific crosses were carried out between At3 (female) and At5 (male), and At5 (female) and At4 (male), At5 (female) and At3 (male), and interspecific crosses were carried out with the At3, At4, and At5 *A. tequilana* plants as females, and the *A. americana* plant as the male, and with At3 and At4 plants as males, and the Aa plant as female. The procedure was essentially as described for the self-pollination treatment with the exception that pollen from different plants was used.

Open-pollination

In this group, flowers from At2, At3, At4, and Aa were labeled and allowed to pollinate naturally.

Emasculation without pollination

As a control, for all plants, anthers were removed from unopened flowers, which were then bagged as described above to prevent pollination.

Seed germination

Seeds were wounded by cutting open the seed coat with a scalpel to aid germination and then placed on wet cotton wool in darkness at 28 °C for 3 weeks, as described by Peña-Valdivia et al. 2006. The number of seeds germinated for each pollination treatment varied. For the open pollinated treatments, where many fruits were available, all black seeds from each fruit were germinated separately allowing comparison of germination and viability of seeds from different fruits. For the self pollinated treatments, all available black seeds from each fruit were germinated separately allowing comparison of germination and viability of seeds from different fruits. For inter- and intra-specific crosses, all available black seeds were germinated together and germination and viability of seeds from different fruits were not compared.

AFLP analysis

AFLP analysis was carried out to confirm that sexual reproduction had occurred between different individual *A. tequilana* plants, and between *A. tequilana* and *A. americana* plants, and to rule out accidental self-pollination. The protocol used was essentially that described by Vos et al. (1995), using the LI-COR Biosciences, IRDye™ Fluorescent AFLP® Kit (Lincoln, Nebr.) for Large Plant Genome Analysis, (Gil-Vega et al. 2006). Briefly, DNA was obtained from seedlings produced by both intra- and interspecific crosses and their parent plants using the procedure of Doyle and Doyle (1989). For the analysis of different seedlings, four selective AFLP primer combinations were used: *Eco*RI + ACA/MseI + AGT; *Eco*RI + ACA/MseI + ACC; *Eco*RI + AGC/MseI + AGT; and *Eco*RI + AGC/MseI + ACC. The *Eco*RI primers were fluorescently labeled and obtained directly from LI-COR.

Histological studies

Flower buds were collected at stages of development between 20 and 65 mm, and divided into two parts. The upper part, containing the anthers, was used to study male gametophyte development, and the lower part, containing the ovaries, was used for the analysis of female gametophyte development.

Anthers and ovules were extracted individually under a stereomicroscope and fixed for 24 h in FAA consisting of a 10:50:5:35 *v/v* solution of 40% formaldehyde – 95% ethanol – glacial acetic acid – distilled water; they were then placed in 70% ethanol, after which they were cleared successively for periods of one hour in 3:1, 1:1, 1:3 solutions of methyl salicylate and ethanol. The ovules were observed and photographed using a Leica DMR interference contrast microscope. Anthers were obtained from the 20 to 30 mm stages of development, stained with DAPI (4′, 6-Diamidine-2-phenylindole) and observed using an Olympus BX60 fluorescence microscope. A total of 100 anthers and 100 ovules obtained from different flowers and from each of the four individual plants (in the case of *A. tequilana*) were analyzed for each species. All *A. americana* samples came from the same plant.

Pollen viability

Pollen viability was evaluated by in vitro germination of the pollen grains by the hanging-drop method on media containing 2% glucose, 200 $mg \cdot L^{-1}$ calcium chloride, 0.06% boric acid, 2 $mg \cdot L^{-1}$ glycine and the vitamins: 0.05 $mg \cdot L^{-1}$ folic acid, 0.5 $mg \cdot L^{-1}$ thiamine, 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$ pyridoxine, and 0.05 $mg \cdot L^{-1}$ biotin (Piven et al. 2001).

Results

Fruit and seed production in open-pollinated *A. tequilana* and *A. americana* plants

The number of umbels per plant was determined and, based on data from five umbels (*A. tequilana*), or three umbels (*A. americana*) of each plant where flowers were left to pollinate naturally, the number of flowers and fruits·plant^{-1} was estimated. This may lead to some overestimation of total flower and fruit production, since some umbels, especially those found on the lower and upper sections of the inflorescence, are smaller than those in the middle section. The estimated number of flowersper umbel varied between the three At plants, from a mean of 283 for At2 to a mean of 444 for At3. From these data the mean number of flowers per plant was estimated at 12 341 for At and 8268 for Aa. The data presented in Table 1 show that, only between 14.34%–18.69% of *A. tequilana* flowers left to pollinate naturally are converted into fruits (assuming each flower has the potential to produce a fruit) and 8.80% in *A. americana*.

Fruits from *A. tequilana* plants contained mainly degenerated wrinkled and white seed, with a small proportion (~10%) of black seeds of normal appearance (Table 1), as shown in (Fig. 1*a*). Fruits from *A. americana* contained a high proportion (around 89% in open pollinated fruits) of white seeds of normal size and shape, but which were found to be empty, and a low proportion of black seeds of normal appearance (Fig. 1*b*). No fruit was observed to contain all white or wrinkled seeds but contained at least one black

Table 1. Fruit and seed production in open pollinated *Agave tequilana* and *Agave americana*.

Plants	Flowers·umbel^{-1}	Flowers·plant^{-1}	Fruits·umbel^{-1}	Fruits·plant^{-1}	Fruit production (%)	Black seeds·fruit^{-1}	Black seeds·plant^{-1}
At2 (N = 5)	283.0±100.9	9056 (5,827–12.284)	40.6±13.0	1299 (883–1,715)	14.34	24.33±9.04 (n = 18)	31 604 (19 485–42 867)
At3 (N = 5)	444.0±43.4	16 428 (14 822–18 033)	83.0±23.1	3071 (2216–3925)	18.69	26.76±9.35 (n = 17)	82 179 (49 136–110 556)
At4 (N = 5)	330.8±32.6	11 908 (10 735–13 082)	58.8±19.1	2116 (1429–2804)	17.76	27.22±13.10 (n = 18)	57 597 (29 624–84 640)
Mean At	**352.6±82.6**	**12 341 (9450–15 232)**	**60.8±21.2**	**2128 (1386–2870)**	**17.24**	**26.10±1.55**	**55 540 (52 241–58 838)**
Aa (N = 3)	295.3±51.6	8268 (6823–9712)	26±5.5	728 (574–882)	8.80	36.70±9.28 (n = 78)	26 936 (16 744–37 128)

Note: Data are means±SD. Range of variation is provided in parentheses. N, number of umbels sampled; n, number of fruits sampled; At, *Agave tequilana*; Aa, *Agave americana*.

seed. Black *A. tequilana* seeds are opaque, whereas those of *A. americana* are shiny in appearance (Fig. 1*c*).

Development of fruits and seeds following different manual pollination treatments

When flowers were emasculated and covered to prevent pollination, no capsules formed on either the *A. tequilana* plants or the *A. americana* plant under these conditions, indicating that in the absence of pollinators the possibility of uncontrolled pollination during the experiment was low, and that the plants did not undergo apomixis.

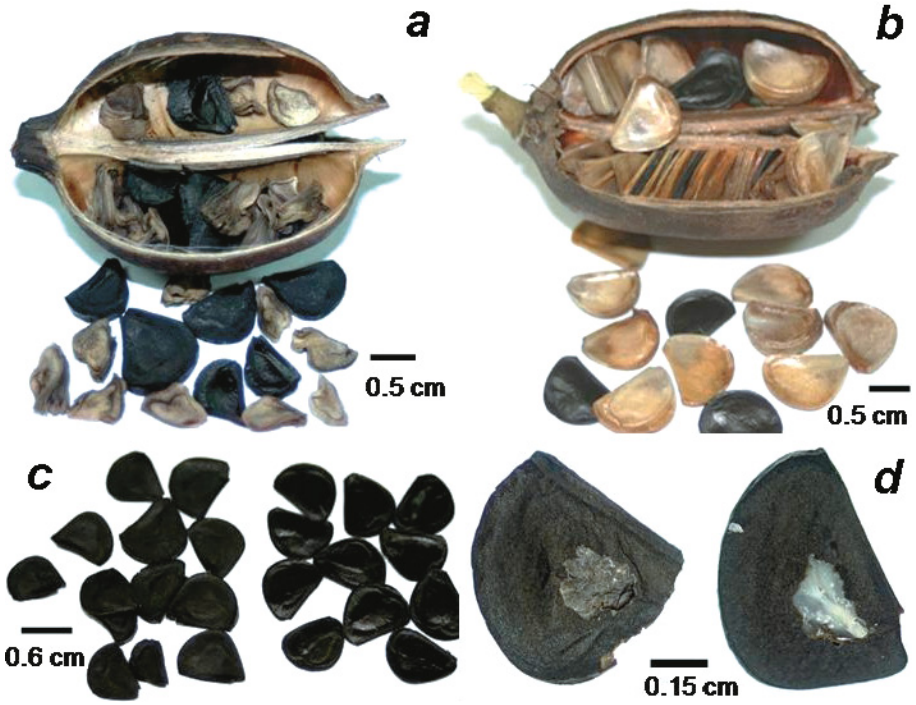
The percentages of fruits formed in relation to the number of flowers pollinated and the number of black seeds per fruit for each manual pollination treatment were low in comparison with the open pollinated samples. The At5 female × At4 male cross produced the highest percentage of fruits (15.78%), while the At3 female × Aa male cross produced the highest number of black seed (23.7 ± 11.14). All of the interspecific crosses with the At plants as female produced greater numbers of black seeds per fruit in comparison to the At × At crosses (Table 2). The percentage of fruits and the number of black seed produced when the Aa plant was the female parent was much lower than in the reciprocal crosses with Aa as the male parent. Although self-pollination was also carried out for the At3 and At4 plants, no capsules were obtained (data not shown). Only the At5 plant and the Aa plant produced low percentages of fruits and black seed when self pollinated (Table 2). No significant differences were observed in seed production between the open pollinated treatments, whereas significant differences in seed production were observed between open pollination and self pollination of the At5 and Aa plants, and when Aa or At plants were taken as the female parent in the interspecies crosses. These observations were confirmed by ANOVA (data not shown).

Seed viability

To determine whether the black seeds obtained under each pollination treatment were viable, seeds were germinated as described in Materials and methods. Initial attempts to germinate seeds obtained from an open pollinated *A. tequilana* plant gave a germination efficiency of 12% (data not shown). Based on previous reports (Peña-Valdivia et al. 2006) describing the effects of vernalization and scarification on germination efficiency, the effect of wounding the seeds before germination was tested and shown to increase the efficiency of germination of seeds obtained from other open pollinated plants to around 30% (data not shown). During the wounding process it was observed that many of the black seeds were in fact empty and did not contain either embryo and (or) endosperm. Examples of empty and normal embryo containing seeds are shown in Fig. 1*d* (left and right, respectively). Seeds of this type were obtained from both species.

Figure 2 shows the percentages of viable, unviable (embryo present but no germination observed) and empty seeds observed for each pollination treatment. Although the open pollinated umbels of the At plants produced more seeds (Table 1) than the other pollination treatments involving At plants, only between 30%–40% of these seeds were capable of germinating (Fig. 2). On the other hand the open polli-

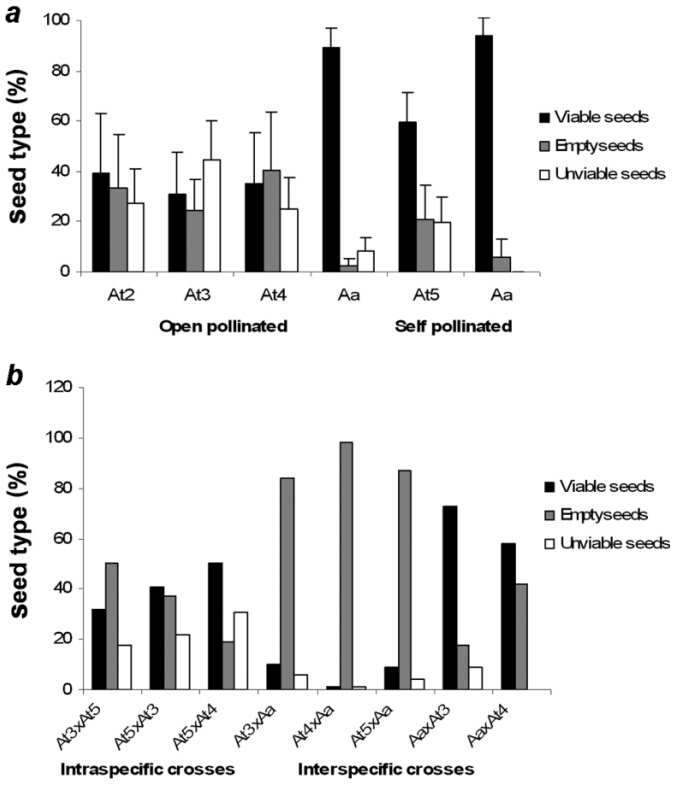
Fig. 1. Examples of *Agave* seeds. (*a*) Fruits and seeds of *Agave tequilana*. (*b*) Fruits and seeds of *Agave americana*. (*c*) Opaque *A. tequilana* seeds and shiny *Agave americana* seeds. (*d*) Comparison of seeds with no embryo or endosperm (left) and normal embryo containing seeds (right).



nated Aa umbel produced a higher number of seeds per fruit and a higher proportion of viable seeds (>80% of germination) in comparison to the open pollinated At plants as shown in Fig. 2*a*.

The results from the self pollination experiments are unclear, since although umbels on the At3 and At4 plants were self pollinated, no seeds were obtained. The self pollinated At5 and Aa umbels produced low levels of seeds (Table 2), but the seeds obtained showed high levels of viability (>60% for At5 and >90% for Aa, Fig. 2*a*). For the intraspecific crosses, the reciprocal crosses between At3 and At5 produced similar numbers of viable, empty, and unviable seeds, whereas the At5 female × At4 male cross produced a slightly higher number of viable seeds (Fig. 2*b*). For the interspecific crosses, a clear difference in germination efficiency was observed depending on which plant was used as the female. In all crosses involving the At plants as female, >80% of empty seeds were obtained and very low levels of viable seeds were observed (Fig. 2*b*). However, when the Aa plant was the female parent, the levels of empty and unviable seeds were lower and the germination efficiency rose to >60% (Fig. 2*b*). Although the At × Aa crosses produced greater numbers of black seeds in comparison with the At × At crosses, very few were viable (Fig. 2*b*). In general we found an inverse relationship between the number of seeds produced and the germination efficiency, i.e., few seeds, high germination efficiency and many seeds, low germination efficiency.

Fig. 2. Percentage seed germination and occurrence of empty and unviable seeds under different pollination treatments for *Agave tequilana* and *Agave americana*. Data in 2*a* are means ± SD of seeds from different individual fruits. Data in 2*b* were obtained from mixtures of seeds from different capsules.



Genetic and morphological analysis of inter- and intraspecific crosses

The emasculation experiment suggests that the levels of spurious pollination were extremely low. However to con-

Table 2. Fruit and seed production in *Agave tequilana* and *Agave americana* under different pollination treatments.

Self-pollination	*N*	Fruits (%)	Black seeds·fruit^{-1}	*n*	Intraspecific crosses	*N*	Fruits (%)	Black seeds·fruit^{-1}	*n*	Interspecific crosses	*N*	Fruits (%)	Black seeds·fruit^{-1}	*n*
At5	315	6.66	8.55±2.92	20	At3(f) × At5(m)	206	9.70	13.60±3.30	10	At3(f) × Aa(m)	328	12.19	23.70±11.14	10
Aa	94	4.25	12.25±9.42	4	At5(f) × At3(m)	139	13.66	5.20±1.61	10	At4(f) × Aa(m)	81	9.87	12.62±5.60	8
					At5(f) × At4(m)	57	15.78	4.66±1.58	9	At5(f) × Aa(m)	263	9.12	16.50±6.70	10
										Aa(f) × At3(m)	168	2.38	3.00±1.40	4
										Aa(f) × At4(m)	91	2.19	5.50±6.00	2

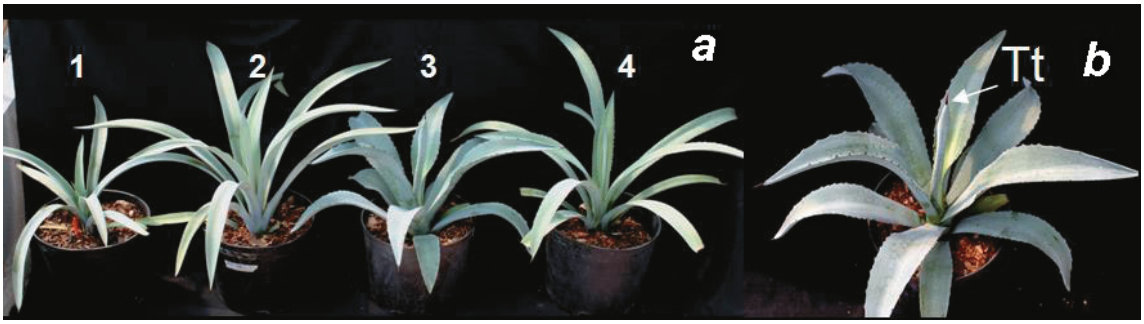
Note: Data are means ± SD. *N*, number of pollinated flowers; *n*, number of fruits sampled; At, *Agave tequilana;* Aa, *Agave americana;* (f), female; (m), male.

Fig. 3. AFLP analysis showing recombinant genotypes of plants obtained from seeds produced by an interspecific cross between *A. tequilana* (At5) and *A. americana* (Aa). P1, P2, P3, indicate plants obtained from different individual seeds. ~, At5 alleles; *, Aa alleles.

firm that the intraspecific and interspecific crosses had been successful, molecular marker analysis using AFLP was carried out on parent plants and samples of the F_1 progeny. An example of these results for the At5 female × Aa male cross is shown in Fig. 3. As can be observed, novel combinations of bands exclusive to one or the other parent are observed in the progeny, indicating that they carry genetic information from both parents owing to successful sexual reproduction.

In addition, germinated seedlings obtained from inter- and intra-specific crosses, self-pollinations, and open-pollinations, have been growing under greenhouse conditions for around 3 years. In general, plants obtained by self-fertilization are smaller and less vigorous compared with those obtained by open-pollination or from the intraspecies crosses. All plants obtained by natural pollination or from the intraspecies crosses have the morphological characteristics of normal *A. tequilana* plants. Although few of the seeds obtained from the interspecific crosses where *A. tequilana* was the female germinated, as described above, surviving plantlets show morphological characteristics typical of *A. americana*, such as wide leaves and thick terminal thorns confirming the success of the interspecific cross. In addition, seeds produced from interspecific crosses using At as female produced opaque seeds, whereas those with Aa as female produced shiny seeds. Examples of plants

Fig. 4. Three year old *Agave* plants grown from seeds of plants pollinated by different treatments. (*a*) Plants showing phenotypes typical of the following: 1, self-pollinated *A. tequilana* plants; 2, open-pollinated *A. tequilana* plants; 3, interspecific *A. tequilana* × *A. americana* crosses; 4, intraspecific *A. tequilana* × *A. tequilana* crosses. (*b*) Close up of plant 3 showing the typical wide leaves and thick terminal thorn of *A. americana* plants; Tt, terminal thorn.



obtained from germinated seedlings of each type of cross are shown in Fig. 4.

Histological analysis of gametophyte development

The unviable, empty, or embryo-less seeds could be produced because of errors in the development of the gametophytes, in the viability of the pollen, or following fertilization. Although gametophyte development has been studied in other *Agave* species (Piven et al. 2001), the process has not been previously documented in either *A. tequilana* or *A. americana*. To provide information on this process and attempt to identify possible errors that could lead to the formation of the aberrant black seeds, a histological study of male and female gametophyte development in both *A. tequilana* and *A. americana* was carried out. The viability of pollen produced in both species was also determined.

The process of female gametophyte development in *A. tequilana* is shown in Figs. 5*a*–5*g*, (no difference was observed by comparison with *A. americana*, and these figures are not shown). The process is similar to that observed in other *Agave* species where the megaspore mother cell differentiates from the nucelar tissue (Figs. 5*a* and 5*b*) and undergoes meiosis to form a tetrad of haploid megaspores (Fig. 5*c*). While the three micropylar megaspores degenerate, the megaspore closest to the chalazal zone survives to become the functional megaspore (Fig. 5*d*). Development of the female gametophyte in *A. tequilana* and *A. americana* can therefore be classified as *Polygonum* monosporic. The single nucleus of the functional megaspore undergoes several rounds of mitosis (Figs. 5*e*–5*f*) and eventually cell walls are formed to produce the typical 7 celled structure of the *Polygonum* type female gametophyte.

In *A. tequilana* it was observed that around 2%–5% of the ovules analyzed did not produce a mature gametophyte. Only elongated nucellar cells were observed (Fig. 5*g*). This phenomenon was not observed in *A. americana.*

Male gametophyte development is essentially the same in *A. tequilana* and *A. americana.* Completion of meiosis in the pollen mother cell leads to the formation of a tetrad of haploid cells (Figs. 6*a* and 6*b*). The tetrad of cells separates to gives rise to the individual microspores (Fig. 6*c*) and the single nucleus within each microspore undergoes a mitotic division to produce two cells. The generative cell is completely enclosed within the larger vegetative cell (Fig. 6*d*). Mature *A. tequilana* pollen grains show a reticulate, dicolpate architecture (Fig. 6*e*), as do pollen grains from *A. americana* (data not shown).

Viability of pollen

To determine the viability of the pollen produced by the At and Aa plants, in vitro germination assays for both species were carried out as described in Materials and methods.

Pollen grains placed on germination medium began to germinate within 30 min for both *A. tequilana* and *A. americana* (Fig. 7*a*). Approximately 36% of the pollen grains of *A. tequilana* and 79.5% for *A. americana* germinated under these conditions. After 1.30 h of growth on germination medium, the pollen tubes began to burst and degenerate in both *A. tequilana* and *A. americana* (Fig. 7*b*). When older pollen, which had been stored for up to 24 h, was germinated, the formation of aberrant pollen tubes (Figs. 7*c* and 7*d*) was observed and a much lower percentage of germination was obtained (11% and 54% in *A. tequilana* and *A. americana*, respectively).

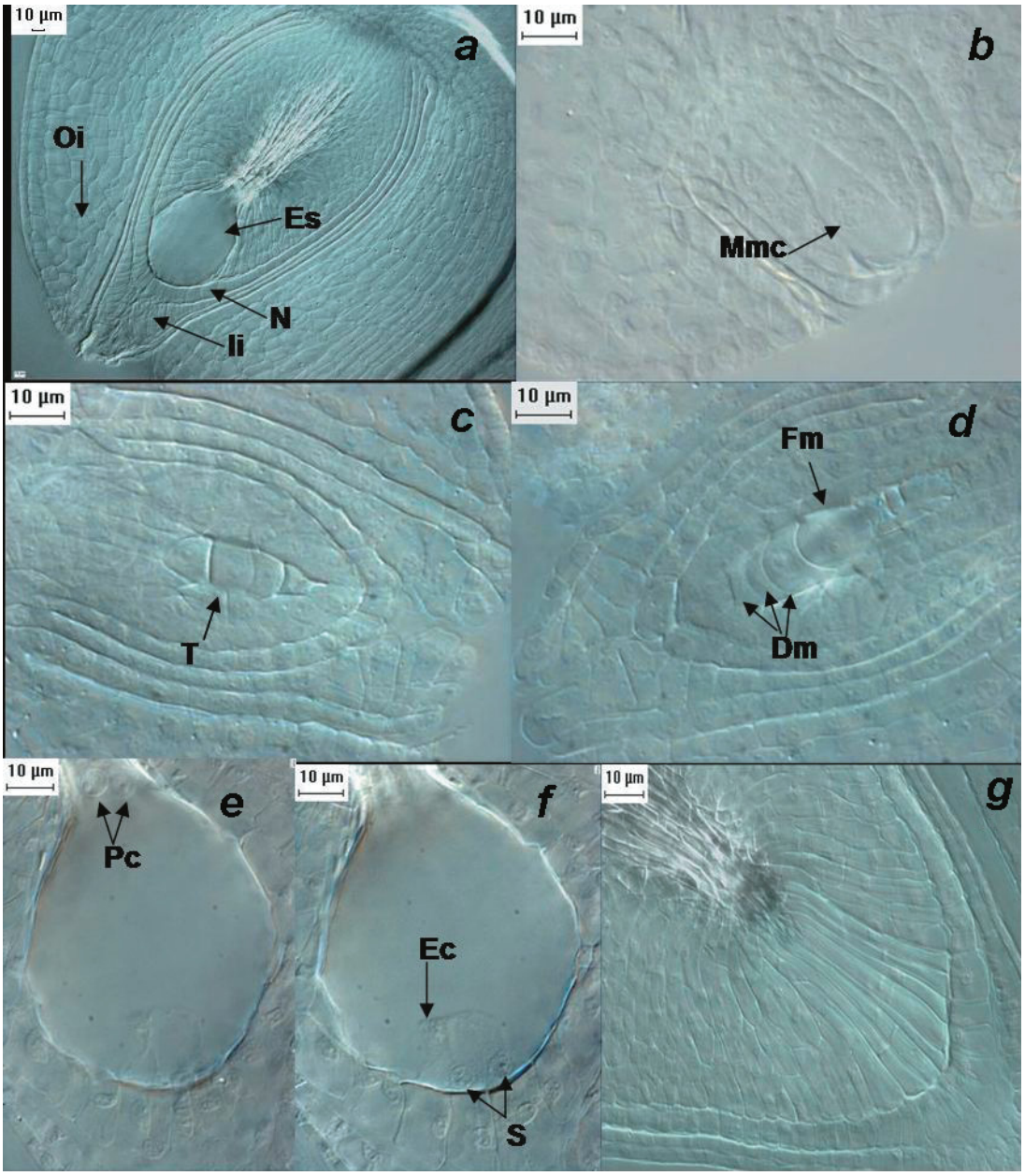
Discussion

As has been reported for other *Agave* species (Sutherland 1987; Arizaga et al. 2000*a*, 2000*b*), both the *A. tequilana* plants and the *A. americana* plant analyzed in this study produced many more flowers than fruits when left to pollinate naturally. Sutherland (1987) suggested, based on studies in *A. mckelveyana* Gentry, that this situation leads to an increase in male fitness, since excess flowers contribute to pollen production and dissemination but not to the production of mature fruits (an indication of female fitness).

Fruit set was low by comparison with studies in *A. angustifolia* (Molina-Freaner and Eguiarte 2003), where in the majority of cases values of between 20%–34% were reported. Low fruit set could be due to several factors, such as lack of adequate pollinator species and errors during gametophyte development, pollination, and fertilization.

Although hummingbirds and a variety of insects were observed to visit flowers during the day, and bats are common in the area at night, we cannot rule out the effects of lack of effective pollinators. However, the results estimating fruit- and seed-set in the open pollinated flowers are within the range reported for other *Agave* species, (Arizaga et al. 2000*a*, 2000*b*; Molina-Freaner and Eguiarte 2003; Rocha et

Fig. 5. Megasporogenesis and development of the embryo sac of *Agave tequilana*. (*a*) Developing ovule outer integument (Oi), inner integument (Ii), embryo sac (Es), nucellus (N). (*b*) Megaspore mother cell (Mmc). (*c*) Linear tetrad (T). (*d*) Development of the functional megaspore (Fm) and degenerate megaspores (Dm). (*e*) Embryo sac showing the polar cells (Pc). (*f*) Embryo sac showing the synergids (S) and egg cell (Ec). (*g*) Ovule in which the embryo sac failed to develop.



al. 2005). Previous reports have considered black seeds to be equivalent to fertile seeds, and the phenomenon of empty or embryo-less seeds has not been reported previously for other *Agave* species, although Trame et al. (1995) showed a reduction of around 40%–50% between the mean number of mature seeds per fruit and the mean number of emerged seedlings per flower pollinated in *Agave schottii*. It is possible that a similar phenomenon is also occurring in that species.

Two of the self pollinated *A. tequilana* plants failed to produce any fruits or seeds, and the self-pollinated At5 and Aa plants produced very few fruits and seeds. These results are similar to those reported by Molina-Freaner and Eguiarte (2003), where no fruits or seeds were obtained from manual self pollination of *A. angustifolia* and *Agave subsimplex* plants, suggesting that self-pollination is not an efficient method for fertilization and seed production in both *A. tequilana* and *A. americana*. This low efficiency may be due to genetic incompatibility between the pollen and the stigma of the same plant, although Trame et al. 1995 determined that this was not a factor in reduced fertility observed in *A. schottii*. An alternative explanation is the effect of inbreeding, where recessive alleles could have deleterious effects leading to abortion following fertilization.

In general we find an inverse relationship between the number of seeds produced and the number capable of germi-

nation, suggesting that embryo development may be affected in the very early stages (empty seeds), but also that in many cases apparently normal embryos are unable to develop further (unviable seeds).

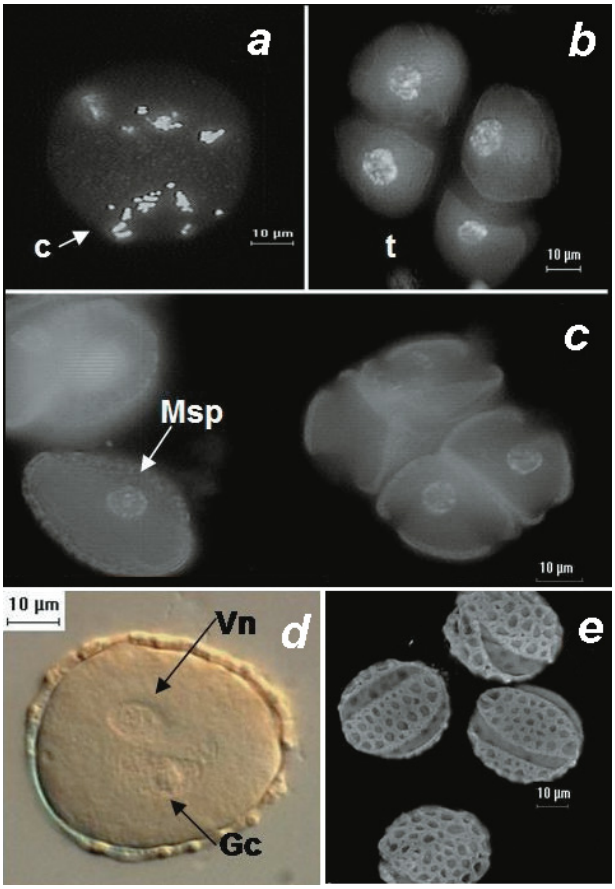
It is likely that effects of inbreeding and (or) genetic incompatibility also exist between the At3, At4, and At5 plants, since they were obtained from the same commercial plantation and owing to predominantly asexual reproduction, the level of genetic diversity within cultivated *A. tequilana* plants is extremely low (Gil-Vega et al. 2001, 2006). This may be a generalized problem for *A. tequilana*, since this species is propagated asexually on a massive scale in commercial plantations, and this may contribute to reduced fertility under natural conditions. The generation of crosses between different *A. tequilana* cultivars could improve the level of fertility and help to maintain a broader germplasm base. However, for such hybrids to be commercially acceptable the restriction to use only *A. tequilana* var. *azul* for tequila production would have to be addressed

Both *A. tequilana* and *A. americana* have a diploid chromosome complement of $2n = 2x = 60$ (Castorena-Sánchez et al. 1991; Granados 1993; Palomino et al. 2003), and although at least one manually produced hybrid between *A. amaniensis* and *A. angustifolia* has been recorded (Lock 1962; Boulanger 1985), little is known about cross-species hybridization in *Agave* under natural conditions. The results presented here clearly indicate that crosses between *A. tequilana* and *A. americana* can occur and produce viable offspring, confirmed by the phenotypes of the offspring and the AFLP analysis. This should be taken into account if attempts to increase the genetic variability of *A. tequilana* by allowing at least some plants to undergo sexual reproduction in the field. Although using the At plants as female in the At × Aa crosses produced more black seeds than the At × At crosses, very few of these seeds were viable, suggesting that although fertilization in the interspecies crosses is relatively efficient, embryo development is poor. Crosses involving *A. americana* as the female parent are less fertile than crosses involving any of the *A. tequilana* plants as female, producing fewer fruits and seeds per fruit.

Although some form of genetic incompatibility may affect fruit set and seed production in both the inter- and intraspecies crosses, the occurrence of a high proportion of apparently normal but empty seeds suggests that other factors such as the formation of viable gametophytes or inbreeding effects may also contribute to the low levels of fertility.

As has been documented in other taxa (Haga and Channell 1982; Saunders and Sipes 2006), the white, shriveled seeds found in *A. tequilana* fruits and the white seeds found in *A. americana* fruits probably correspond to ovules that were not fertilized, whereas black seeds correspond to fertilized ovules. The empty seeds could correspond to seeds that were fertilized successfully, but which aborted at very early stages of development. One possible explanation for this occurrence is the fertilization of ovules by defective pollen sperm cells. The in vitro pollen germination assay indicates the proportion of pollen grains capable of producing pollen tubes, but does not discriminate between the cells within the germinated tubes that are viable or nonviable. Ruvalcaba-Ruiz and Rodríguez-Garay (2002) reported that errors in meiosis during pollen formation are common for

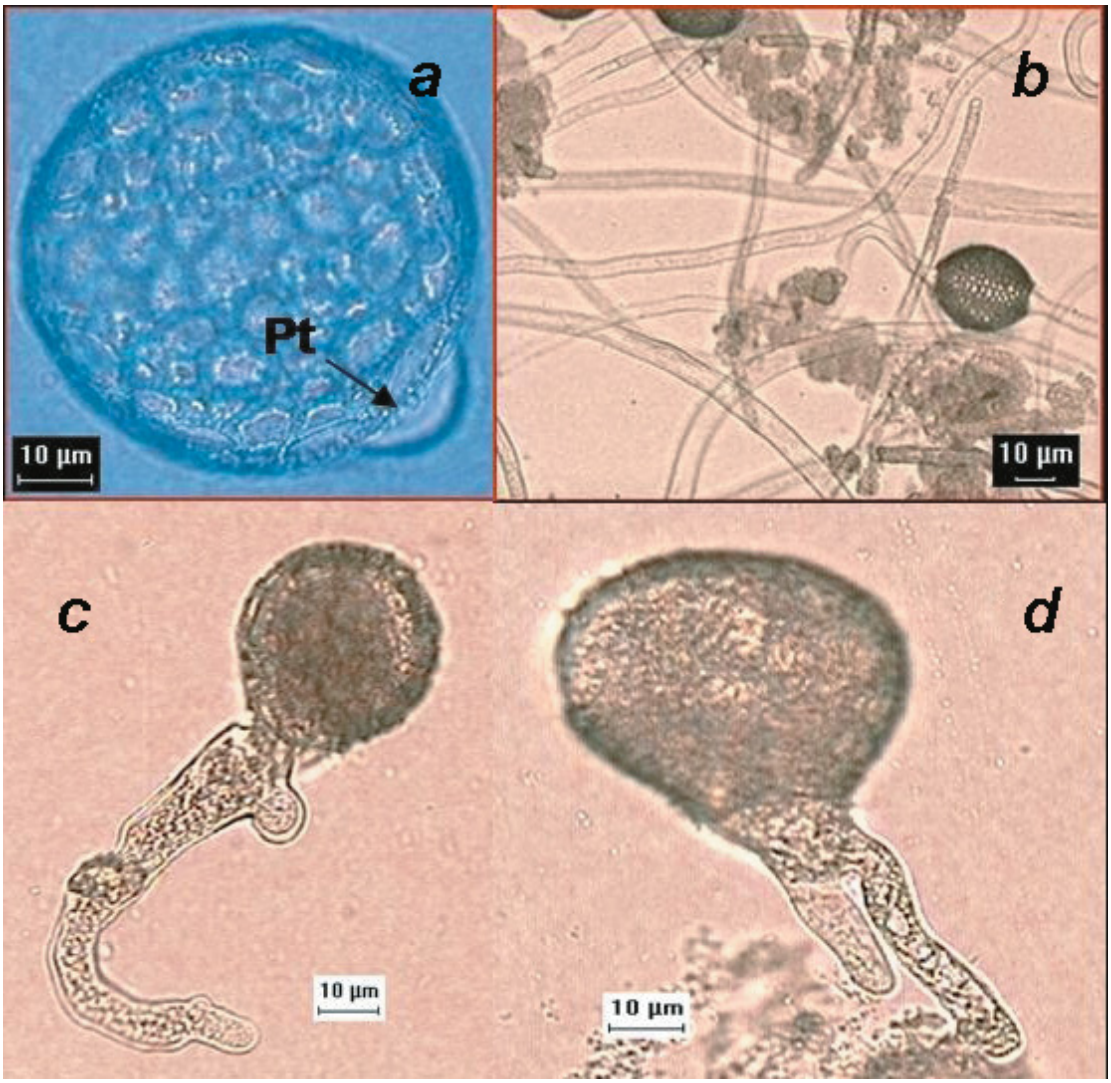
Fig. 6. Microsporogenesis and development of pollen grains in *Agave tequilana*. (*a*) Pollen grains stained with DAPI showing pollen mother cells during the first meiotic division and condensed chromosomes (*c*). (*b*) Example of tetrads produced by meiosis. (*c*) Tetrad and free, mature microspore (Msp). (*d*) Cleared mature pollen grain showing the generative cell (Gc) and the vegetative cell nucleus (Vn). (*e*) Mature *A. tequilana* pollen grains showing reticulate dicolpate architecture.



A. tequilana, and by staining with aniline blue showed that up to 42% of the grains produced had a defective morphology. Piven et al. (2001) also reported up to 66.4% of defective grains for the pentaploid *Agave foucroydes*. It is possible that a certain proportion of the remaining apparently viable grains could harbor less severe chromosomal aberrations, which could lead to problems during pollen tube germination and subsequent mitoses to form the sperm cells or following fertilization, leading to problems in early development. These observations could in part account for the lower pollen viability determined by the in vitro pollen germination assay described above for *A. tequilana* (36% as compared to the 58% observed by Ruvalcaba-Ruiz and Rodríguez-Garay 2002).

In this study a higher proportion of *A. americana* pollen grains germinated, by comparison with *A. tequilana*, indicating fewer defective grains. This may have led to preferential self-pollination in the open pollinated Aa umbel and competition between At and Aa pollen in the open pollinated umbels.

Fig. 7. In vitro germination of pollen grains from *Agave tequilana.* (*a*) Pollen grain after 30 min of contact with the germination medium, emerging pollen tube (Pt). (*b*) Pollen tubes after 90 min in germination medium. (*c*) and (*d*) Formation of aberrant pollen tubes.



Failure in female gametophyte development could also be a factor affecting seed development. Although Piven et al. (2001) report observations of possible chromosome fragmentation during female meiosis in *A. fourcroydes*, and in this work a low level of aborted female gametophytes were observed for *A. tequilana*, no detailed data are available on the occurrence of aberrant female meioses in *Agave*.

The descriptions of pollen grain anatomy and female gametophyte development reported in this work contrast with previous reports. Pollen for the *Rigidae* group of the *Agave* subgenus to which *A. tequilana* belongs, is normally described as monocolpate with only a single furrow; however, observations in this work show dicolpate morphology. In addition, Piven et al. (2001) describe female gametophyte development in *A. fourcroydes* as bisporic, whereas our results indicate that for both *A. tequilana* and *A. americana* female gametophyte development is of the *Polygonum monosporic* type.

Wounding of the seed coat greatly increased germination efficiency for both *A. tequilana* and *A. americana.* Peña-Valdivia et al. (2006) previously reported accelerated germination in scarified *A. salmiana* seeds; however, a biological basis for the need for scarification under natural conditions is unclear. Seeds from most *Agave* species are normally distributed by the wind as capsules open to release them and not by birds or other animals. Mechanical damage however could occur once they have reached the ground owing to the movement of animals or the activities of insects.

The results presented here suggest that genetic incompatibility and (or) effects of inbreeding between genetically closely related genotypes and errors during gametophyte development, especially pollen development, could all contribute to low seed set and germination efficiencies in *A. tequilana.* This also occurred for the *A. americana* plant although pollen from this plant showed much higher viability, however other *A. americana* plants should be tested to confirm this hypothesis. This is the first report of fruit and seed set and seed viability in *A. tequilana* and also of male and female gametophyte development. This emphasizes the need for much more basic research at the genetic and physiological level on a plant with an extremely important role in the Mexican economy.

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