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ORIGINAL ARTICLE

L.C. Boavida · J.P. Silva · J.A. Feijó

Sexual reproduction in the cork oak (*Quercus suber* L). II. Crossing intra- and interspecific barriers

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Abstract Intraspecific barriers promote outcrossing while interspecific mechanisms may contribute to the isolation of species; both control the exchange of genes between plants. In this paper we establish that post-pollination mechanisms promote outcross and act at different temporal and spatial levels to control seed set and quality in *Quercus* species. Controlled pollinations were performed to investigate intraspecific crossing barriers in *Q. suber* and pollen-pistil interactions following interspecific pollinations with some simpatric *Quercus* species. Cytological data of intraspecific crosses in *Q. suber* and *Q. ilex* have shown different kinetics on pollen tube growth after self and outcross pollination, but pollen tubes were able to reach the base of the styles in both species and seed set was successful. Although some prezygotic interaction is occurring at the style, the most important interaction takes place at ovary. The cross between *Q. ilex* and *Q. suber* is possible only in one direction, revealing a case of unilateral incongruity. We show that the lack of seed set observed in the cross *Q. suber* × *Q. ilex* is due to the inability of pollen tubes to penetrate the transmitting tissue after germination. With *Q. suber* mainly as female parent, pollinations with other simpatric *Quercus* species have shown different levels of constraint on pollen tube progression at stigma and style levels. Eventual hybridisation between *Quercus* species will depend on the compatibility of pollen-pistil interactions, on the competitive ability of pollen genotypes, and, most important, on the overlapping of geographical, phenological and ecological factors. Differences in seed set and seed allocation was evident in all crosses, and was particularly outstanding in interspecific and in self intraspecific crosses, determining the ultimate level of seed production and quality in *Quercus* species.

Keywords *Quercus* · Intraspecific · Interspecific crosses · Unilateral incongruity · Hybridisation · Early-acting inbreeding depression

Introduction

In flowering plants, the ability of female tissues to discriminate among the different genotypes that are sometimes randomly deposited on stigmata determines pollen rejection or recognition; this ability is crucial in the process of pollen choice and reproductive isolation. The ability to recognize gametes enables the species to fulfil two primary functions of sexual reproduction, maintaining the stability of the species and allowing a reasonable degree of genetic variability within the species. Post-pollination events occur either on the surface of the stigma by inhibiting pollen germination or pollen tube entry into the stigma, or in the transmitting tissue of stigma and style. Less frequently these events may occur in the ovary or ovule. These interactions establish the proportion of pollen tubes that eventually will reach and fertilise ovules (de Nettancourt 1977; Sage et al. 1999).

Beginning with pollen germination, several mechanisms limit successful fertilisation within and between species (Ascher 1986; Liedl and Anderson 1993). Intraspecific mechanisms encourage outcrossing within species, while interspecific mechanisms affect speciation and limit the exchange of genes between species.

Although significant progress has been made in understanding the factors involved in intraspecific incompatibility (de Nettancourt 1997; Newbigin et al. 1993), research on the details of inhibition following interspecific pollination lags behind. Self-incompatibility is the result of active recognition of pollen: self-pollen is recognised as a result of the interaction of *S*-allele products in the pollen and in the pistil, which in turn result in the

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L.C. Boavida · J.P. Silva · J.A. Feijó (✉)
Instituto Gulbenkian de Ciência, Apartado 14, 2780-156 Oeiras, Portugal
e-mail: jfeijo@igc.gulbenkian.pt
Tel.: +351-21-440-79-00, Fax: +351-21-440-79-70

L.C. Boavida · J.P. Silva · J.A. Feijó
Centro de Biotecnologia Vegetal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, Ed.C2, 1749-016 Lisboa, Portugal

activation of metabolic processes in the pollen and/or pistil, leading to pollen inhibition. In addition to affecting the patterns of intraspecific mating, these processes can also affect the probability of hybrid formation between closely related taxa (Lewis and Crowe 1958; Dhaliwal 1992). However, in the majority of interspecific crosses, the arrest of post-pollination events seems to be passive, not a result of active recognition of pollen, but as an incomplete pollen/pistil relationship (Hogenboom 1984). The high heterogeneity between and within populations of *Quercus suber* (also reported in other *Quercus* species) is frequently mentioned as the result of hybridisation. Moreover, edaphic variations introduce further complications at the species level (Elena-Rossello et al. 1992), frequently challenging the "biological species concept" (Burger 1975; Van Valen 1976). Recently the species status of two widespread European hybridising oaks was evaluated by microsatellite analysis, showing that these species maintain their individuality as separate taxonomic units beyond extensive hybridisation (Muir et al. 2000). Very rare cases of local hybridisation between cork oak (*Q. suber*) and some sympatric deciduous oak species have been described (Gil-Sanchez et al. 1996). However, cork oak was reported to hybridise mainly with other species of the evergreen-oak group and more particularly with holm oak (*Q. ilex*) (Camus 1938).

The purpose of this study was to investigate post-pollination barriers following intra- and interspecific crosses, mainly in *Q. suber*, in order to estimate the importance of crossing barriers on the reproductive success of the species and on its natural diversity. This study evaluates pollen-pistil interactions using different genotype sources and estimates the contribution of pre- and post-zygotic events for seed-set and quality in intra- and interspecific crosses.

Materials and methods

Plant material

The geographic distributions of *Quercus* species in the central region of Portugal frequently overlap. Often, in transitional climatic regions (from high humidity to dry regions) *Q. suber* grows in natural conditions with other *Quercus* species: *Q. ilex*, *Q. coccifera* and *Q. faginea*. An additional species from the north of Portugal, *Q. robur*, was also included in our crossing scheme. One mixed stand of *Q. suber* and *Q. ilex* and a pure stand of *Q. suber* were selected as the main mature populations for carrying out controlled pollinations. Additional sites were carefully chosen to perform additional pollinations with the other selected species. Observations were made during the entire flowering season of the selected species. Flowering in these species overlaps with the spring vegetative growth. The female flowers emerge in a spike carrying 3–5 flowers, from the axils of new leaves.

Pollen collection, storage and viability

Pollen was collected during the flowering season of the selected species. Batches of pollen from trees selected to perform the pollination scheme were separated. Pollen was carefully isolated from anthers using a copper-screening sieve (approximately 100 µm). After collection, pollen was stored over dry silica gel for 24 h at room temperature. Viability rates were scored for approximately 100 pollen grains in fresh and stored pollen. Four non-overlapping fields were measured for each batch of pollen using the fluorochromatic reaction (Heslop-Harrison and Heslop-Harrison 1970). Cryo-preservation was achieved by freezing pollen through rapid immersion and storage in liquid nitrogen.

Pollination methods

Emasculation of male flowers was immediately followed by isolation of the new shoots in polyester pollination bags (PBS International). Emasculation was performed 1 week before female spikes emerged to prevent undesirable pollen contamination. Whenever possible, fresh pollen was used for pollinations. Flowers used in the experiments were pollinated when fully receptive stigmata emerged with an excess amount of pollen using a soft brush. Flowers were isolated until collection at specific times after pollination or after seed maturation. Using the species shown in Table 1, controlled pollinations were performed using *Q. suber* as the central species in the crossing scheme, which included outcross intraspecific (compatible), self, and interspecific pollinations with the selected species (*Q. ilex*, *Q. coccifera*, *Q. faginea*, *Q. robur*). Intraspecific pollinations were performed using a single pollen source, the same as the female parent for self pollinations, or a selected source of outcross pollen from a single tree different from the female parent. Interspecific crosses were performed using a mixture of pollen of different donors in accord with the desired pollination treatment.

Pollen tube growth

For every type of crossing (treatment), flowers (15–30) were collected and fixed at specific times after pollination. The material was fixed for 3 days at 4,0 ºC in a solution containing 4% paraformaldehyde (w/v), 1% $CaCl_2$ (w/v) in distilled water, saturated with $CaCO_3$ (Baker 1946) and autoclaved in 10% (w/v) sodium sulfite for approximately 1 h to soften the tissues. Squashed flowers were used to assess pollen tube germination and growth following incubation with 0.1% de-colorised aniline blue (Martin 1959) for 24 h. Slide preparations were observed under fluorescence microscopy (Olympus BH2 fluorescence microscope), with blue (500 nm) or UV (400 nm) excitation and photographed with Kodak Elite Gold 400 film. Digital images were collected using a Micromax-1300Y/HS cooled CCD camera (Roper Scientific, Tucson, Ariz.) controlled by Metamorph 4.55 software (Universal Imaging). In order to quantify pollen tube progression, at least ten pollen tubes per style were measured, or the length of the longest pollen tube was determined when direct measurements were impossible to perform. Comparisons of pollen tube growth were compiled, considering that effects within plants was not signifi-

Table 1 Crossing design with total number of flowers used for intraspecific and interspecific *Quercus* pollinations. Number of flowers analyzed for pollen tube progression/seed set

Species	CP	*Q. suber*	*Q. ilex*	*Q. coccifera*	*Q. faginea*	*Q. robur*
Q. suber	21/128	17/132	15/188	14/102	16/99	21/–
Q. ilex	20/126	22/130	18/170	–	–	–
Q. coccifera	18/–	15–				

Table 2 Flowering time and phenology of some *Quercus* species; *SE*, standard error

Species	Flowering time	Number of styles	Style length ± SE (μm)	Type of pollen	Pollen grain diameter ± SE (μm)
Q. suber	Early April–mid-May	4–5	2413.02±53.6	Bicellular	38.82±0.28
Q. ilex	Mid-February–end March	5	2543.15±26.26	Bicellular	28.54±0.23
Q. coccifera	Early May–end May	4	2184.05±25.09	Bicellular	28.05±0.18
Q. faginea	Mid-February–end March	3	1959.69±11.61	Bicellular	33.11±0.21

Table 3 Average pollen tube progression following intraspecific, self and interspecific crosses in *Q. suber* and *Q. ilex*; *n*, number of pollen tubes measured for at least 15 flowers; *SE*, standard error; *ND*, not determined

Cross		Average pollen tube length (μm) ± SE (*n*)					
Female parent	Male parent	Time (days after pollination)					
		1	2	3	4	7	15
Quercus suber							
Intraspecific		311.74±3.46 (79)	499.26±3.54 (120)	ND	ND	819.84±2.37 (122)	2256.56±7.53 (64)
Self		328.59±4.82 (115) *ns*	502.5±11.53 (34) *ns*	ND	ND	714.04±4.76 (122)***	901.15±5.96 (81) ***
Interspecific	Q. ilex	105.62±1.89 (221) ***	98.83±1.85 (145)***	ND	102.54±1.65 (69)	103.16±1.99 (82)***	155.99±4.76 (81)***
	Q. faginea	ND	ND	313.25±2.58 (196)***	ND	653.39±4.81 (93)***	ND
	Q. coccifera	ND	ND	313.24±6.03 (114)***	ND	670.01±4.31 (80)***	*ND*
Quercus ilex							
Intraspecific		379.94±14.50 (103)	639.85±8.05 (81)	ND	760.95±20.66 (27)	1694.76±50.74 (46)	*ND*
Self		378.50±15.59 (61) *ns*	425.42±14.53 (60)***	*ND*	726.51±12.29 (95)*	1484.44±33.80 (47)***	*ND*
Interspecific	*Q. suber*	240.23±6.29 (47)***	454.94±11.27 (120)***	*ND*	674.97±15.40 (95) **	1431.03±21.29 (107)***	*ND*

**P*<0.05; ***P*<0.01; and ****P*<0.001; *ns* not significant

cant (data not shown). The number of samples considered was effective for detecting different pollen performances within genotypes.

Flower and pollen structure

Flower phenology of *Quercus* species involved in the crossing scheme was followed during the entire flowering period. Some flower features are presented in Table 2. Pollen grain diameter was determined using JAVA video analysis software (Jandel Scientific v. IBM 1.40). Pistil lengths were measured using light microscopy.

Seed set

At least 100 flowers were pollinated for each selected treatment (type of pollination). Flowers were isolated in polyester bags as described above until full maturation. After 5–6 months, fruits were collected, and all the material inside the bags was analysed for aborted flowers at different developmental stages and seed maturation. The success of a pollination method was scored on the basis of pollen tube progression and on the frequency and quality of seeds and flower development (early aborted flowers, flowers with developed ovules, mature seed set and seed weight).

Statistical analysis

Quantitative data from pollen tube progression with different types of pollination were statistically analyzed with Systat 7.0 for Windows to allow detection of significant differences between the different treatments and time intervals. The data was analysed using a two-way ANOVA to test for differences among treatments in the distance travelled by the pollen tubes. Regression analysis was used to analyse pollen tube progression through time. Simple correlation analyses were used to investigate the relationship between pollination treatments for the same variable (percent seed set and quality, percent flower abortion rate).

Results

The results of intraspecific crosses, self-pollinations and interspecific crosses on pollen tube progression and seed set are presented in Tables 3 and 4.

Differential pollen tube growth kinetics following self and outcross pollination in *Q. suber* and *Q. ilex*

To estimate the degree of compatibility of pollen–pistil interactions following outcross and self-pollinations,

Table 4 Seed-set following intra-, and interspecific pollinations and percent abortion at different development states

Cross		Percent early flower abortion	Percent late flower abortion	Percent seed set (by seed mass, g)			
Female parent	Male parent	(undifferentiated ovules)	(with developed ovules)	Low (<1.5)	Intermediate (1.5–2.5)	High (>2.5)	Total
Quercus suber							
Intraspecific		85.2	11.7	–	1.56	1.56	3.1
Self		96	3	1.0	–	–	1.0
Interspecific	*Q .ilex*	66.4	33.6	–	–	–	0
	Q. faginea	96	2	–	2	–	2
	Q. coccifera	92	5	2	–	1	3
Quercus ilex							
Intraspecific		58.5	6.5	–	17.5	17.5	35
Self		43	43	3	3	7.9	13.9
Interspecific	*Q. suber*	49	26	18	3.5	3.5	25

pollen tube progression was evaluated over time. *Quercus suber* and *Q. ilex* were the primary species considered in this analysis. To identify intraspecific barriers and their potential contribution to interspecific mating, a complete diallele cross was performed. Pollen tube measurements were carefully made, considering previous data which have shown that pollen tube reduction across the pistil would determine that no more than 2–3 pollen tubes enter the ovary (Boavida et al. 1999). All measurements were made during pollen tube progression within the style and prior to entry of the latent phase, which precedes ovary penetration and fertilisation. In both *Q. suber* and *Q. ilex*, pollen tube progression was relatively constant over time (P<0.001). However, when the different treatments (self and outcross) were taken into account, differences in the kinetics of pollen tube progression were observed. In *Q. suber*, pollen germinated readily following both cross- and self-pollination, indicating that no inhibition of self-pollen occurs on the stigma level and on the transmitting tissue penetration level. Differential growth rates between self and outcross pollen were mainly observed at day 7 and later (P<0.001), corresponding to progression through 2/3 of the style (Table 2), with no apparent modification on pollen tube morphology. In *Q. ilex*, differences in pollen tube growth rates between self and outcross pollinations were particularly evident in the earliest stages of stigmatic pollen tube penetration, and during further progression within the style (P<0.001; P<0.05) (Table 3). The differential kinetics of pollen tube growth outline an evident delay of adhesion and germination processes and continued growth through the transmitting tissue. Basically, pollen tube appearance following both treatments was similar, with bright fluorescing plugs spaced at regular intervals (100–150 µm) along the entire length. The number of self-tubes observed reaching the base of the styles was a similar to the number of outcross tubes. Important pollen-pistil interactions occur at style level that restrict pollen tube growth, but later mechanisms may occur in the ovary that determine seed set differences in *Q. suber* and *Q. ilex* (Table 4).

Unidirectional successful cross between *Q. suber and Q. ilex*

Concerning interspecific crosses, special attention was given to pollen-pistil interactions in *Q. suber* and in *Q. ilex* following reciprocal pollinations. When *Q. ilex* was used as female parent, pollen grains of *Q. suber* were able to germinate, penetrate the stigmatic surface and progress along the transmitting tissue. Nevertheless, a decrease in growth rate was observed as soon as pollen tubes penetrated the transmitting tissue (P< 0.001); curled pollen tubes and small ramifications were observed before stigma penetration (Fig. 1). No further ab-

Figs. 1–6 Fluorescence micrographs of pistils following interspecific pollinations, stained with aniline blue for detection of callose.

▶

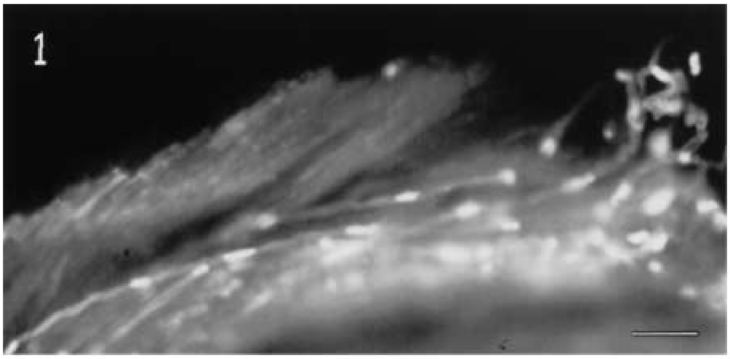
Fig. 1 Interspecific successful pollination, *Q. ilex* × *Q. suber*, visible reaction at the stigma penetration level, where pollen tubes show a slight lack of directionality; however, further progression within the transmitting tissue is comparable to that in outcross pollinations. *Bar* 200 µm.

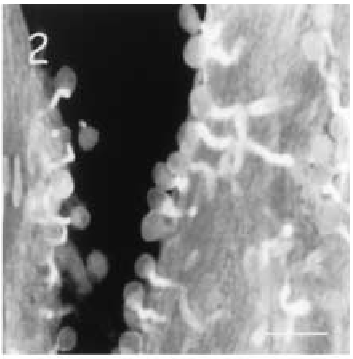
Fig. 2 *Q. suber* × *Q. ilex* cross. Arrested *Q. ilex* pollen tubes after stigma penetration in *Q. suber* pistils. There was successful adhesion, hydration and germination of pollen grains, and no abnormal morphology of pollen tubes or visible changes at the stigma level, but pollen tubes were arrested when transmitting tissue was reached. *Bar* 200 µm.

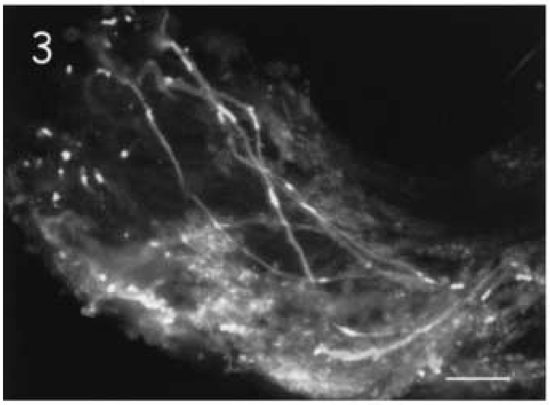
Fig. 3 *Q. suber* × *Q. faginea* cross. The number of pollen tubes growing within the style is substantially reduced for outcross intraspecific pollination. There is visible callose deposition at the stigma surface. Few pollen tubes reach the base of the style. *Bar* 400 µm.

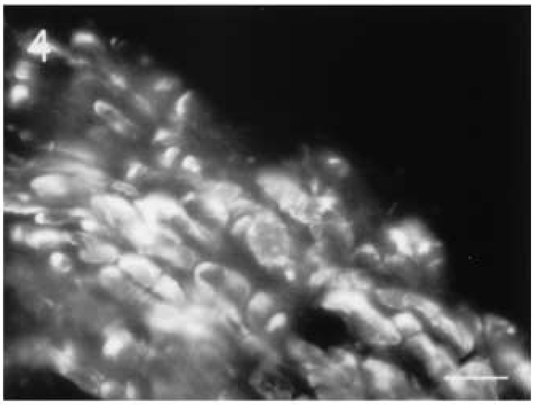
Fig. 4 Aspect of callose deposition in stigmatic cells following *Q. robur* interspecific pollination. Most pollen grains are unsuccessful at the adhesion/penetration level on the stigma surface, and few pollen tubes overcome this early barrier and progress through the transmitting tissue. *Bar* 100 µm.

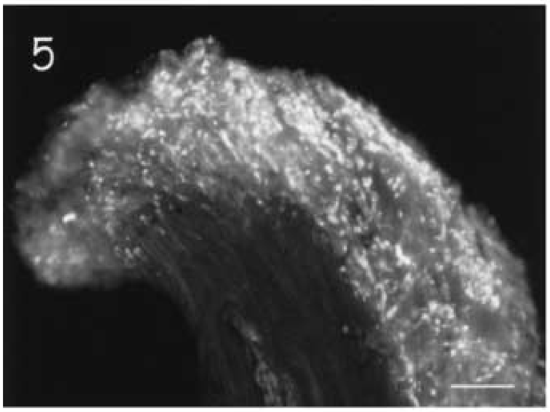
Fig. 5 General aspect of a pistil after *Q. suber* × *Q. robur* pollination. All of the stigmatic surface shows abnormal deposition of callose, pollen grains are detached from the surface and just a few short fragments of pollen tubes are visible by their intense callose deposition. *Bar* 400 µm.

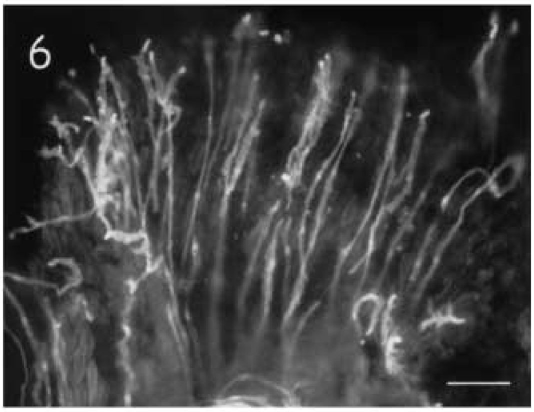












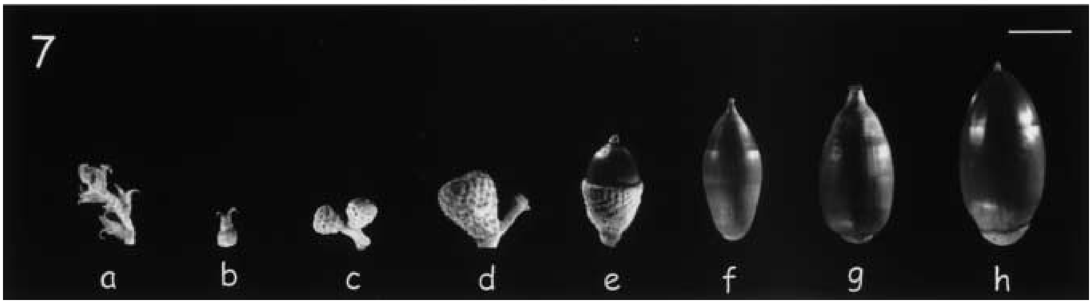


Fig. 6 *Q. suber* × *Q. coccifera* cross. Pollen tube progression within the style is successful, although a reaction at the stigma level is visible during pollen tube penetration. The number of pollen tubes is reduced along the style. *Bar* 200 µm.

Fig. 7 Developmental stages of flower/fruit abortion and seed allocation in *Q. suber*. The image shows the relative size of flowers and fruits. *a, b*. Premature drop of flowers a few weeks after pollination. *c, d*. Developmental states corresponding to completion of ovule development and fertilisation. *e–h*. Differential allocation of resources to seeds (intraspecific and interspecific) from low to normal weight. *Bar* 1 cm normal alterations were observed during pollen tube growth within the style. When *Q. ilex* pollen was used to pollinate *Q. suber* pistils, pollen tubes germinated and penetrated the stigma, but as soon they reached the transmitting tissue, pollen tube growth was prevented. No further significant pollen tube progression was observed in this cross (Table 3). Pollen tubes show a normal appearance during stigma penetration and no abnormal callose deposition was observed at stigma level (Fig. 2). There was no seed set in this cross, although the reciprocal cross, using *Q. ilex* as female parent, was able to produce 25% seed set. This represents a 10% decrease in seed set compared with intraspecific outcross pollination (35%), but a considerable difference from the 13.9% obtained from self-pollination (Table 4).

Interspecific crosses with other *Quercus* species reveal prezygotic interactions during the progamic phase

We analysed interspecific crosses with other related *Quercus* species, which in some climatic regions share geographical distribution with *Q. suber*. Some flower features and phenology are shown in Table 2. Several similarities between the species considered, including the pollination mechanism (anemophilous), individual and separated female and male flowers within the same plant (monoecious), a partial protandrous system (geitonogamy), a dry stigmatic surface, bicellular pollen and lack of ovule differentiation at the time of pollination are evident from observation of structural and phenological features (Table 2).

Controlled pollinations were performed using primarly *Q. suber* as female parent and using *Q. coccifera*, *Q. faginea* and *Q. robur* as pollen donors. In all crosses considered, differences were observed at distinct levels, with a visible constraint on pollen tubes. In crosses using pollen from *Q. faginea* and *Q. robur,* the number of pollen tubes entering the transmitting tissue decreased considerably at the stigma level, due to both lack of pollen grain adherence and abnormal callose deposition (Fig. 3, Fig. 4). The constraint was evidently severe in several samples where no pollen tubes were observed in the style (Fig. 5). *Quercus suber* pistils pollinated with *Q. coccifera* pollen showed abnormal growth mainly at the style level. Despite small ramifications at the stigma level, some severed pollen tubes growing in the transmitting tissue presented thinner walls, branching and closer callose plugs, reducing the number of pollen tubes growing within the style (Fig. 6). The reciprocal cross, using *Q. coccifera* as female parent only, presented similar ramifications at the stigma penetration level, but no constraint within the style was observed. Pollen tube growth rates of interspecific genotypes were usually slower than those obtained for outcross intraspecific pollination in *Q. suber* ($P<0.001$) (Table 3). Pollen tubes that survived the viability barrier exhibited delayed pollen germination and stigma penetration or slow pollen tube growth, indicating poor competitive ability but not the loss of fertilisation potential. Effectively, if only a few pollen tubes were able to reach the base of the style, those were able to set seed (Table 4). Important pollen-pistil interactions occur at different levels (stigma and style), with different restriction attributes, and represent early and strong interactions.

Patterns of flower/fruit abortion at different developing states and seed set allocation in *Q. suber* and *Q. ilex*

The results for seed set, and during flower/fruit development was strictly marked by an abortion pattern (Fig. 7). Therefore, flower/fruit abortion was classified according to aborted developmental state, and further classes were added at the seed (mature fruit) level, to allow an analysis of seed-set allocation (Table 4). *Quercus suber* and *Q. ilex* showed a strong drop of flowers a few weeks after pollination, around 90–99% and 50–60%, respectively. A second class of later flower abortion represents the stage corresponding to completion of ovule development and fertilisation. Both outcrossed *Q. suber* and *Q. ilex* produced a higher number of seeds, exhibiting a higher degree of investment in seed mass then self-pollinated flowers (Table 4). *Quercus ilex* self-pollinated flowers produced 13.9% seed set, which was evidently favoured in outcrossed flowers (35%), representing around a 50% decrease in seed set. While *Q. suber* seed set after outcross pollination was higher (3.1% vs. 1%), also representing a decrease in a similar range, the results are not so impressive, due to considerable abortion of flowers just after pollination. Correlation data between treatments show that the percentage of seeds with higher weight was clearly larger for seeds resulting from outcross pollination, even if different classes of seed allocation are present for all types of crosses (Table 4). Seed set from interspecific pollination produced a higher number of seeds than self-pollinations, although a decrease in seed quality was similar for both treatments (Table 4).

Discussion

Intra and interspecific pollen tube performance in *Q. suber* and *Q. ilex* may reveal different competitive advantages for achieving fertilisation

Both intraspecific (self) and interspecific genotypes, which overcome the strong stigmatic and early style restrictions, have shown substantially slower pollen tube growth rates than outcross genotypes; this probably represents attrition of pollen tube growth due to pollen-pistil interactions. The outcome of distinct pollen tube growth rates occurring between different genotypes at style level should, however, be carefully analysed. The delay in fertilisation in *Quercus* species and other angiosperms such as *Corylus*, *Hamamelis*, and *Myrica* (Wilson and Burley 1983; Boavida et al. 1999; Cecich 1997) may be extremely long. Under these circumstances, resumption of growth acts as a suppressor of competition, annihilating the advantage of some genotypes, allowing all the tubes to “catch up” at the base of the style (Hormaza and Herrero 1994). In addition, pollen tube competition for ovules resumes in the last phase of pollen tube growth for those tubes which were not selected against during the latent phase (Marshall and Folson 1991). Although mixtures of pollen were not used for lack of appropriate markers to distinguish different genotypes, the assumptions of these findings should be weighed when mixtures of pollen are likely to be present, as in naturally pollinated flowers. The distinct competitive abilities found at style level in *Q. suber* and *Q. ilex* suggest modulation of genetic interactions by female tissues, resulting in reduced number of pollen tubes growing within the style, and presumably favouring genotypes with higher competitive abilities (Mulcahy and Mulcahy 1975; Schlicting et al. 1987). Regardless of the strength of other internal barriers, we expect that pollen competition strongly increases assortative mating, providing a late-acting advantage to outcross pollen at the ovary level, as observed in several other species (Weller and Ornduff 1991; Williams et al. 1999). If no other post-pollination mechanism is ruling at the pistil level, we would expect the seed frequencies to be equal after single self, outcross and interspecific pollinations. However, when pollen tubes are able to reach the ovary after pollen tube competition, additional interactions should account for the differences in seed set.

Unidirectional incongruity and interspecific pollen-pistil interactions show constraint at the stigma/style level

Our study presents for the first time evidence of pollen–pistil interactions and post-pollination barriers acting in interspecific crosses in *Quercus* species. Observations of flower structure and phenology of the selected *Quercus* species suggest that these species may share similar breeding systems, as already described for other *Quercus* species (Boavida et al. 1999; Cecich 1997). Pollen-pistil interactions in interspecific crosses act at different temporal and spatial levels than intraspecific interactions and correspond to strong and early interactions between male genotypes and the female tissues. If some kind of self-incompatibility (SI) mechanism is acting in interspecific crosses, our assumption is that the mechanisms prevailing are distinct from those acting in self-pollinations, due to differences in the timing, location and morphology of pollen-pistil responses (Liedl et al. 1996). Nevertheless, it is pertinent to ask whether there might truly be interspecific barriers that depend upon physiological controls similar to those operating in the biochemical basis of SI systems, although not necessarily sharing elements of genetic control (Grun and Aubertin 1966; Liedl et al. 1996). The incompatibility barrier appears to function on a species–specific basis. The different patterns of responses observed may represent different kinds of interspecific barriers: (1) ramifications at the stigma penetration level and through progression within the style (*Q. coccifera*); (2) lack of adherence of pollen grains, strong restraint on pollen tube penetration, callose deposition in receptive cells (*Q. faginea* and *Q. robur*); and (3) pollen tube arrest at the transmitting tissue level with no further pollen tube elongation (*Q. ilex*).

The cross between *Q. ilex* and *Q. suber* presents evidence of unidirectional compatibility. This is confirmed by lack of seed set on the cross using *Q. ilex* as male parent, showing that this species is usually (if not exclusively) the female tree in hybridisation events. Reports accounting for unilateral introgression between these two species were also provided by enzyme analysis and DNA markers (Elena-Rossello et al. 1992; Dumolin et al. 1995).

Several reports have described interspecific and intergeneric barriers operating in one direction, whereas the reciprocal cross is successful; this is usually referred as unilateral incongruity (UI) (de Nettancourt 1977; Hogenboom 1984; Dhaliwal 1992). Incongruity as defined by Hogenboom (1975) encompasses passive reproductive barriers (which evolve due to isolation of taxa) resulting from lack of genetic information in one parent about some relevant character of the other. Hence, they vary from system to system, and are considered a by-product of evolutionary divergence. The limited production of hybrid progeny can be due to lack of fertilisation, poor competitive ability of heterospecific pollen, or other processes such as selective abortion by maternal plants or seed inviability if hybrid seeds were less fit than conspecific seeds (Walsh and Charlesworth 1992; Carney et al. 1994). Under such circumstances, the evolution of selective abortion should be equivalent to the reinforcement of prezygotic isolating mechanisms.

Pre or postzygotic mechanisms determine seed set and seed allocation in *Q. suber* and *Q. ilex:* is this late-acting self-incompatibility or early-acting inbreeding depression?

The combined results of hereditary and physiological influences operating within individual oak trees present numerous unsolved problems. The pattern of flower abortion a few weeks after pollination in both species considered (*Q. suber* and *Q. ilex*) was very consistent within the population, even in naturally pollinated flowers; less than 30% of the flowers reached the fully developed state (completion of ovule development) and very few remained until fruit maturity. Several reports on oak species examined the correlation between weather conditions and fruit set; however, the results are far from conclusive (Sharp and Sprague 1967; Wolgast and Stout 1977; Feret et al. 1982). Premature flower/fruit abortion has been reported in several species as due to pollen limitation, interference of self-pollen (Campbell and Halama 1993; Seavey and Carter 1994; Ramsey 1995; Barret et al. 1996), or to selective allocation of resources (Stephenson 1981; Stephenson and Bertin 1983). We presume that the source of this huge abortion rate may result mainly from physiological conditions or from an early resource competition with the vegetative bloom, considering the range of aborted flowers in all treatments and the prevalence of this pattern in the population.

The crossing experiment provided strong evidence of maternal choice for particular genotypes through differential seed abortion or differential seed allocation in the maturing fruits. Restriction of self-pollen tubes in the style is not sufficient to prevent pollen tubes from reaching the ovary, indicating that if self-incompatibility operates in *Q. suber* and *Q. ilex*, this event should happen in a later stage (ovary level) and pre-zygotically. Otherwise, another mechanism is acting in early embryos leading to seed abortion (Westoby and Rice 1982). While it is conceptually straightforward to distinguish between these phenomena (SI and early inbreeding depression), in practise it can be difficult, especially in cases where rejection of self pollen occurs in the ovary (late-acting or ovarian self-incompatibility) (Kenrick et al. 1986; Gibbs and Bianchi 1993; Seavey and Bawa 1986; Sage et al. 1994). Inbreeding depression is a frequent event in flowering plants and often acts between fertilisation and seed maturation to decrease seed-set (Hamilton and Mitchel-Olds 1994; Husband and Schemske 1996).

The critical issue in understanding the basis of self-sterility is whether self-rejection occurs pre- or post-zygotically. Several reports have shown that a high percentage of mutations in plants are expressed in the embryo as lethal mutations (Jurgens et al. 1991; Meinke 1991). Self genotypes could be selected against, when in the embryo/zygote one or more combinations of lethal or deleterious homozygous alleles are present (Charlesworth and Charlesworth 1987). Inbreeding depression is a more likely explanation than self-incompatibility, presuming that developmental failure occurs at a variety of stages and different numbers of fruits are produced after selfing and outcrossing (Seavey and Bawa 1986).

Hybridisation between sympatric *Quercus* species depends on pollen-pistil interactions and on geographical and phenological overlap

Non-synchronous flowering and/or geographical isolation are critical to parental species. If the flowering periods overlap and the species share the same geographical distribution, possible hybridisation must depend on the compatibility of pollen-pistil interactions between those species. We have shown that *Q. suber* is a very selective female parent, but a more successful male parent. Here, we analysed in detail the potential hybridisation between two sympatric species, in which the limiting factors can be extrapolated to interactions with other oak species. With *Q. suber* and *Q. ilex* the flowering period occurs earlier in holm oak (*Q. ilex*) than in cork oak (*Q. suber*), usually with no overlap (Elena-Rossello et al. 1992), although considerable phenological plasticity in both species may occasionally provide flowering overlap (Corti 1959; Elena-Rosselo 1993). Moreover, as trees of both species are protandrous, the first *Q. suber* trees flowering may pollinate female flowers from the last *Q. ilex* trees flowering. Our results suggest that hybridisation may be a fairly unfrequent event due to: (1) rare phenological overlap; (2) unidirectional compatibility or strong pollen-stigma interactions; or (3) competitive disadvantage of interspecific pollen tubes. The combination of all these factors probably will substantially decrease the frequency of hybrid progeny under natural conditions.

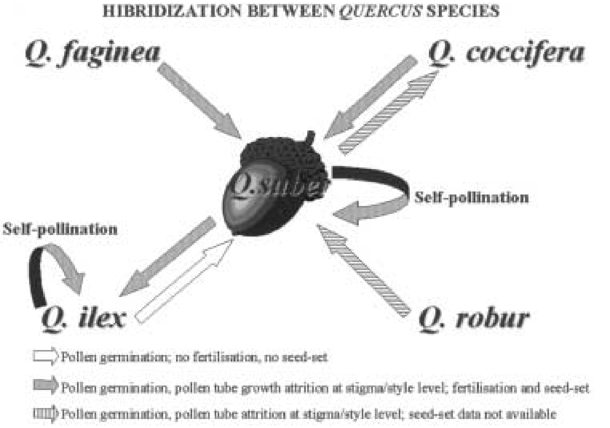


Fig. 8 Hybridisation scheme of interspecific crosses showing possible directionality of hybridisation events between *Quercus* species following controlled pollinations. Results show that strong stigma/style interactions may contribute to unsuccessful pollen tube growth, and poor seed-set and seed quality in interspecific crosses using *Q. suber* as female parent under natural conditions

Conclusions

By analysing pollen tube performance, we were able to spatially and temporally map pollen–pistil interactions acting in intra- and interspecific crosses (Fig. 8). Moreover, we evaluated whether relative pollen tube growth rates may act as a pre-zygotic mechanism and infer whether pollen-pistil interactions contribute as isolating barriers in interspecific crosses. Our results show that *Q. suber* and *Q. ilex* have very similar breeding systems; they are primarily outcrossing species, where genotypes with poor competitive ability are modulated by interactions with the female tissues. Our results present different ways by which the fertilisation process can be affected, proceeding evidence that barriers to crossing between different species are critical steps for reproductive isolation, and supporting the classical model in which speciation is a consequence of genetic divergence in reproductive genes within isolated populations. Early-acting inbreeding depression is an important process by which preferential abortion or resource allocation may limit the number of seed progeny if intraspecific (self) and interspecific seeds are less fit than outcross seeds.

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