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Abstract
The rocoto chili (*Capsicum pubescens*) is a species native to the highlands of South America, which is cultivated for its fruits. The species is regarded as self-compatible; however, self-incompatible strains and even a variable degree of self-incompatibility have been found. To characterise pollen grains and determine whether there is also variation in pollen performance and male fertility in the species, pollen morphology, cellular state, starch content, viability, longevity and germinability were analysed in plants obtained from the germplasm cultivated in Argentina. All the individuals studied were male fertile, showing a high percentage of vital pollen, capable of germinating *in vitro*. Some pollen features were uniform (e.g. exine sculpture, number of nuclei at maturity), but there were significant variations in pollen performance among the plants studied.

Keywords: *cultivated chili, ripe pollen, male fertility*

Sweet and hot chili peppers belong to the South American genus *Capsicum* L. (Solanaceae), which comprises *c.* 35 species (Carrizo García et al. 2013). Although the species of the *Capsicum annuum* complex (*C. annuum* L., *C. chinense* Jacq. and *C. frutescens* L.) are the most economically important worldwide, there are two other cultivated species, *C. pubescens* Ruiz et Pav. and *C. baccatum* L., which are consumed mostly in Latin America (Bosland 1996).

Capsicum pubescens, known as 'rocoto', is cultivated in the highlands of South America, from Mexico to northwest Argentina (Bosland 1996; CCG, personal observation, December 2011). The species is only known in cultivated forms, without a recognised related weedy race (Pickersgill 1971). It differs clearly from the other chilies in the presence of conspicuous leaf pubescence, black seeds and purple flowers (Eshbaugh 1993; Bosland 1996). The fruits are berries with a wide range of variation in size, shape and colour, although well-defined cultivars cannot be delimited (Rick 1950; DeWitt & Bosland 2009).

The processes involved in reproduction and fruit set of *Capsicum pubescens* are of major interest because the species is cultivated for its fruits (Eshbaugh 1979, 1993; Pickersgill 1991; Bosland & Votava 2000). Effective pollination is a prerequisite for fruit and seed set in most plants, and therefore, information on pollen biology such as pollen viability and germinability is useful to increase productivity (Cruzan 1989; Bolat & Pirlak 1999; Shivanna 2003; Abdelgadir et al. 2012). However, studies on flowering, pollination and fruit set in *Capsicum* have been mostly focused on the *C. annuum* complex (e.g. Quagliotti 1979; De Ruijter et al. 1991; Kubišová & Háslbachová 1991; Aleemullah et al. 2000; Raw 2000; Ercan & Onus 2003; Roldán Serrano & Guerra-Sanz 2006), whereas information is scarce for other species of the genus, either cultivated or wild (e.g. Saborío & Da Costa 1992; Carrizo García 2011).

Capsicum species are described as self-compatible and self-pollinating (Bosland & Votava 2000), with the only exception of *Capsicum cardenasii* Heiser et P.G.Sm. (Yaqub & Smith 1971; Onus & Pickersgill

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2004). However, although *C. pubescens* is regarded as self-compatible (e.g. Onus & Pickersgill 2004), self-incompatible strains have been found in the species (Yaqub & Smith 1971; Saborío & Da Costa 1992; Bosland & Votava 2000), and even a variable degree of self-incompatibility has been recorded (Saborío & Da Costa 1992). Further analysis of sexual functions are lacking for the species (e.g. pistil receptivity, pollen viability, pollen tube growth), which may provide key information on fruit and seed set. Therefore, as part of a comprehensive study about *C. pubescens* reproductive strategies, the aims of this work were to characterise pollen grains and to determine whether there is variation in pollen performance and male fertility in the species. We analysed germplasm cultivated in northwest Argentina, from which reproductive information is still not available.

Material and methods

The study involved eight individuals of *Capsicum pubescens* obtained in the Salta province (northwest Argentina), which showed variations in ripe fruit shape and colour. The plants were bought from the market of Salta or local farmers located around the city, and then taken to the Córdoba province (central Argentina), where they were maintained under glasshouse conditions (32 ± 2 °C, *c.* 35% relative humidity). Every variable was analysed for each individual plant so as to assess its degree of variation within the artificial population under study.

The flowers remain open for two to three days. The anthers open gradually along the afternoon of the day of flower anthesis (D1), and then pollen becomes fully available for dispersion on the next day (D2). Therefore, all the observations and analyses were performed using D2 pollen.

Pollen phenotyping

Pollen morphology was analysed using a LEO 1450VP scanning electron microscope at the Labmem, Universidad Nacional de San Luis, Argentina. Pollen preserved in 70% ethanol was spread onto carbon discs, air dried and coated with gold/palladium. Pollenkitt was detected by placing ripe pollen, taken directly from newly open anthers, in a drop of water onto a slide and observed under a light microscope (Dafni et al. 2005). Pollenkitt is evidenced as small yellow, translucent droplets all over the exine. The number of nuclei in ripe pollen was determined using DAPI staining (4′,6-diamidino-2-phenylindole 0.01 mg/ml in water; Goff & Coleman 1984).

Pollen starch content was estimated using Lugol's iodine (I_2KI). The percentage of pollen containing amyloplasts was quantified over 100 pollen grains, in triplicate, for each plant (aliquots taken from the total pollen of 5 to 10 flowers). According to that percentage, the pollen of each plant was identified as starchless (0%), mostly starchless (less than 50%), and mostly starchy (over 50%).

Pollen size was measured using hydrated pollen. Pollen samples collected from 5 to 10 flowers were preserved in 70% ethanol and small aliquots were placed onto slides in pure distilled water for measurement. The diameter of 100 pollen grains was measured using the ImageJ software package ('segmented line' tool).

Pollen performance

Pollen viability, longevity and germinability were estimated from a single pool of pollen grains for each plant. For that purpose, the total pollen produced by at least five flowers per plant was collected, mixed and kept isolated onto slides under laboratory conditions (25 ± 2 °C, *c.* 30% relative humidity). Aliquots of pollen were taken from every pool to perform each test at the specific time.

Pollen viability was determined using MTT (3,[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium-bromide 1 mg/ml in distilled water; Dafni et al. 2005). This vital stain is metabolised by active pollen to insoluble purple formazan dye crystals, staining vital pollen grains. Non-vital pollen grains, that is aborted without cytoplasm or non-aborted but with non-reactive cytoplasm, remain colourless; these two types of pollen grains could be clearly discriminated from each other. Pollen viability was determined at the stage D2. Pollen longevity was assessed by means of the pollen viability measured successively at three, seven, ten and 14 days after flower opening (D3, D7, D10 and D14, respectively). Pollen viability was calculated for 100 pollen grains, in triplicate, at each stage.

Pollen germinability was assessed at D2. Pollen was cultivated in a liquid culture medium adapted from Mercado et al. (1994), i.e. without sucrose and with 15% polyethylene glycol 3350. Sucrose was excluded from the culture medium to check germinability in autotrophic conditions, i.e. the capability to develop pollen tubes without nutrient uptake from the environment. Pollen grains were pre-hydrated at 93–94% relative humidity for one hour (in a sealed chamber with KNO_3 saturated aqueous solution) to avoid possible bursting by osmotic shock. Pollen tubes (i.e. length equal or longer than the hydrated pollen diameter) were counted after 90 minutes of incubation. Culture medium conditions and timing were defined through preliminary analyses (data not shown). Germinated pollen was counted for 200 pollen grains, in triplicate, for each plant.

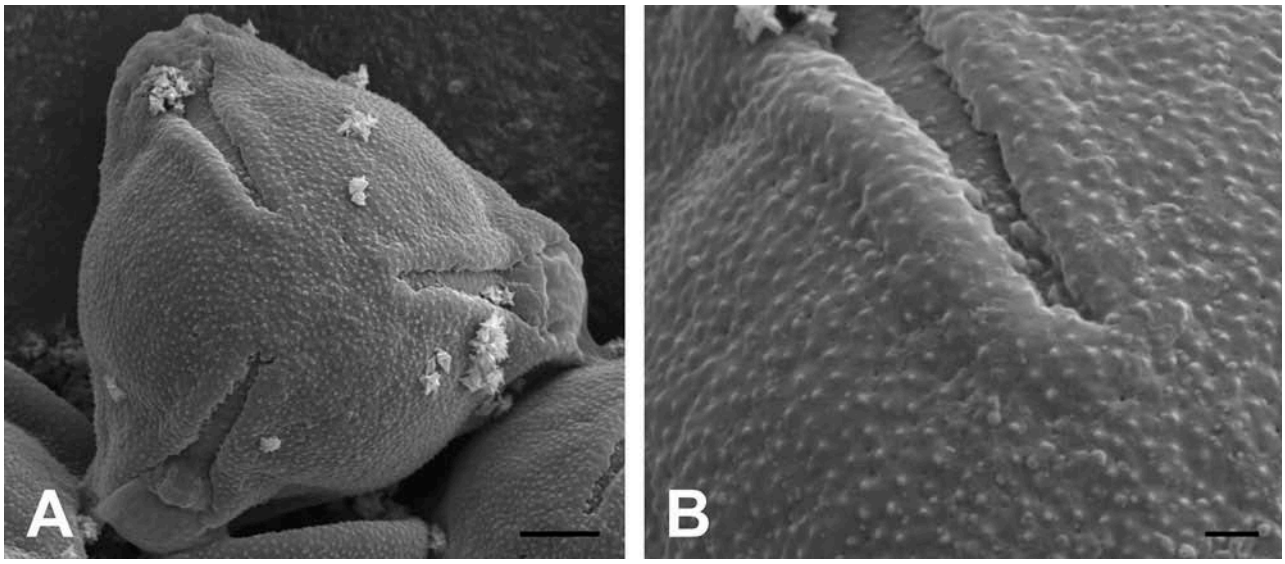

Figure 1. Pollen morphology of *Capsicum pubescens*. **A.** Polar view of pollen in hydrated condition. **B.** Detail of the micro-echinate, perforate exine. Scale bars – 4 μm (A), 1 μm (B).

Statistical analyses

Mean values were calculated for pollen diameter, pollen viability (including the stages to determine pollen longevity), non-aborted pollen, and pollen germinability, individually for each plant. An analysis of variance (ANOVA) (Tukey's test, $P = 0.05$) was performed to test all possible pair-wise differences of means to determine if at least one was significantly different. All of the variables were analysed following this method, except for pollen longevity. To determine pollen longevity, changes in viability over time were analysed using the Student's two-sample *t*-test ($P = 0.05$), contrasting pairs of consecutive stages within each plant.

The correlation between the following pair of variables was evaluated with the Pearson correlation test ($P = 0.05$): non-aborted versus vital pollen grains, pollen viability versus pollen germinability, pollen viability versus pollen type according to the starch content, and pollen germinability versus pollen type according to the starch content.

Results

Pollen phenotyping

Pollen grains are spheroid in hydrated conditions, tricolporate (Figure 1A), with micro-echinate, sparsely perforated exine sculpturing (Figure 1B). Pollen grains are small-sized (Table I), with an average diameter in hydrated conditions of 22.71 ± 0.81 μm, estimated from all individuals. The pollen diameter shows small differences among plants; pollen grains are significantly bigger in plant 6 and smaller in plants 2, 5 and 8 (Table I). Pollen grains are loosely arranged over the anther walls, owing to the presence of a little amount of pollenkitt, but they are dispersed in monads. Pollen is partially dehydrated during exposure, then acquiring a prolate shape due to the infolding of the furrows (Figure 2A). Pollen is binucleate when it is ready for dispersion (Figure 2B).

The type of pollen according to starch content was variable among individuals; it was starchless in plants 1 and 4, mostly starchless in plants 2 and 8, and mostly starchy in plants 3, 5, 6 and 7 (Figure 2C–E, Table I). In turn, the amount of amyloplasts per pollen grain was variable within each individual (Figure 2D, E), but this variable was not quantified.

Pollen viability and longevity

All the plants were male fertile, showing a high quantity of non-aborted pollen, although it was significantly lower in plants 6 and 8 (Table I). At stage D2, pollen viability was over 80% in most individuals, being significantly higher in plants 1, 3 and 7, and significantly lower in plant 6 (Table I). There was a significant positive correlation ($r = 0.8$) between the percentages of vital and non-aborted pollen grains (i.e. vital plus non-vital but with cytoplasm). On the contrary, there was no significant correlation between viability level and pollen type, according to the starch content ($r = 0.11$).

Pollen viability declined at different rates among plants over successive days after flower opening (Figure 3), although it was always high throughout the flower life-span (i.e. D2–D3; Figure 3). Pollen viability dropped to values close to or lower than 5% by D10, except in plant 4, in which the steepest reduction occurred at D14 (Figure 3). After the

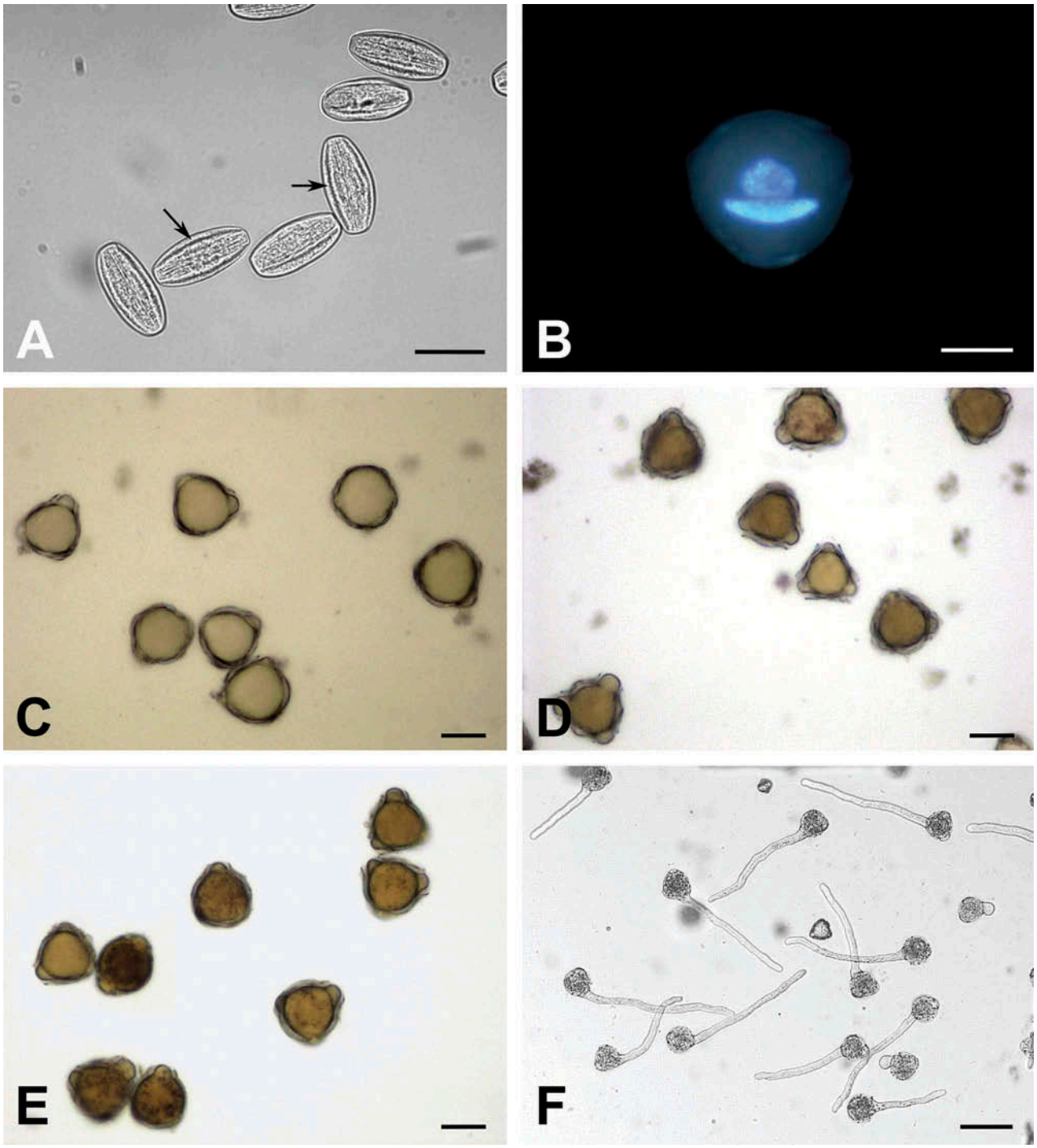

Figure 2. Features of *Capsicum pubescens* pollen. **A.** Dehydrated pollen in equatorial view (the arrows show infolding of furrows). **B.** Binucleate pollen at the time of dispersion (DAPI staining). **C–E.** Variability of pollen starch content (reaction with Lugol's iodine) among plants: starchless pollen (**C**; plant 4), mostly starchless pollen (**D**; plant 2), and mostly starchy pollen (**E**; plant 5). Starch is evidenced as dark spots. **F.** Pollen tubes after 1.5 h of *in vitro* culture. Scale bars – 20 μm (A, C–E), 10 μm (B), 40 μm (F).

regular trend of initial pollen viability decrease, there were unusual increments at different stages in plants 1 (D7) and 4 (D10), although viability dropped abruptly after that (Figure 3).

In vitro *pollen germination*

After 90 minutes of *in vitro* pollen culture, pollen tubes developed up to 100–120 μm in length (Figure 2F). Pollen germinability was variable among individuals,

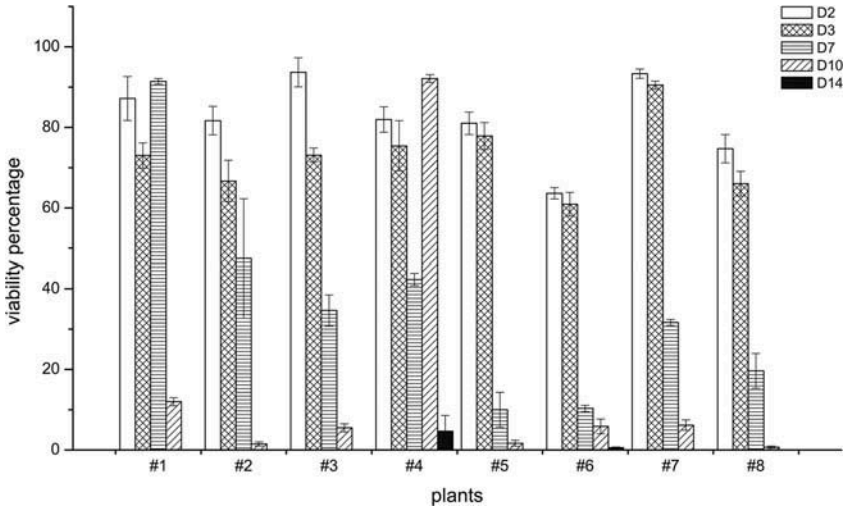

Figure 3. Pollen longevity in different individuals of *Capsicum pubescens* (pollen viability tested with MTT; D: days after anthesis).

ranging between 40 and 55% in most plants, except in plant 6 (Table I). There was no significant correlation either between the percentages of pollen viability and germinability ($r = 0.51$), or between the percentage of germinability and the type of pollen, according to the starch content ($r = -0.15$).

Discussion

Pollen morphology

Palynological studies are in general scarce for *Capsicum*. However, there is a detailed record for *C. pubescens* (Halbritter 2000), which is consistent with the observations made for the Argentinian samples analysed in this work. By contrast, in a previous record for the species, the exine was described as reticulate based on light microscope observations, although such sculpture cannot be distinguished in the figure shown (Murry & Eshbaugh 1971, figure 5).

The average diameter of the hydrated pollen of *Capsicum pubescens* is below the range mentioned for swollen pollen in *Capsicum* (27–33 µm; Quagliotti 1979). However, pollen grains can be even smaller in some wild *Capsicum* species (e.g. *c.* 15 µm in *Capsicum rhomboideum* (Dunal) Kuntze; CCG personal observation, August 2012). Although pollen grain diameter can vary significantly among *C. pubescens* plants, the differences were small (*c.* 9%, which is *c.* 2 µm). With regards to pollen shape, it has been described as sub-prolate to prolate for *C. pubescens* (Murry & Eshbaugh 1971), most probably in dehydrated condition, as it was observed in this work. Tricolporate pollen grains, as those of *C. pubescens*, usually acquire a prolate shape when dehydrated due to the infolding of the furrows (Pacini & Hesse 2004).

Our results agree with findings of Yaqub and Smith (1971), who described binucleate ripe pollen grains for *Capsicum pubescens*. According to the authors, this feature agrees with the hypothesis that species with binucleate pollen grains would have gametophytic systems of self-incompatibility, as suggested for *C. pubescens* (Yaqub & Smith 1971; Saborío & Da Costa 1992).

Pollen performance

The study of fruit and seed set requires determining the fertility of a species. In this work, male

Table I. Pollen features in *Capsicum pubescens*.

Plants	Pollen diameter (µm)	Starchy pollen (%)	Non-aborted pollen (%)	Viable pollen (%)	Pollen germinability (%)
1	23.06 ± 1.12^{a}	0	88.00 ± 1.73^{a}	87.17 ± 5.48^{a}	54.67 ± 5.86^{a}
2	21.99 ± 1.39^{b}	6.67 ± 2.08	94.00 ± 1.73^{a}	$81.67 \pm 3.55^{a,c}$	$41.94 \pm 7.53^{a,b}$
3	22.76 ± 1.39^{a}	72.00 ± 3.61	94.33 ± 1.53^{a}	93.67 ± 3.62^{a}	40.61 ± 4.16^{b}
4	22.69 ± 1.50^{a}	0	93.67 ± 3.21^{a}	$81.94 \pm 3.15^{a,c}$	$44.67 \pm 3.21^{a,b}$
5	21.74 ± 1.28^{b}	75.67 ± 3.06	94.33 ± 1.54^{a}	$81.00 \pm 2.78^{a,c}$	$49.50 \pm 3.04^{a,b}$
6	24.29 ± 1.54^{c}	61.67 ± 0.58	79.25 ± 4.99^{b}	63.61 ± 1.40^{b}	30.50 ± 3.56^{b}
7	23.04 ± 1.27^{a}	93.00 ± 1.73	95.33 ± 1.53^{a}	93.33 ± 1.15^{a}	$49.17 \pm 4.86^{a,b}$
8	22.12 ± 1.38^{b}	48.33 ± 3.21	$83.00 \pm 2.65^{a,b}$	74.67 ± 3.51^{c}	$50.67 \pm 2.02^{a,b}$

Note: Values within each variable with a different letter are different at the 95% level.

performance was analysed as a first step of a comprehensive study of the reproduction of *Capsicum pubescens*; the data obtained will allow us to make reliable inferences, because pollen quality can always limit reproductive success (Tangmitcharoen & Owens 1997). All the plants analysed, a small sample of the germplasm cultivated in Argentina, were male fertile. A very low percentage of pollen was aborted (i.e. without cytoplasm, less than 10% in most cases), which means that pollen development has been mostly normal in all the plants. Moreover, active cytoplasm was detected in over 75% of pollen grains, except in one plant that had a lower percentage; therefore, vital pollen was a high proportion of the non-aborted pollen in all cases.

The analysis of pollen germinability showed that a high proportion of the vital pollen grains were capable of germinating *in vitro*, although results were significantly variable among individuals. Even though a significant correlation between vital pollen and germinability may be intuitively expected, this may not be always true (e.g. Słomka et al. 2010), since there are variables that may affect pollen germination. For instance, controlled rehydration at high relative humidity increased germinability in *Epilobium angustifolium* L. (Heslop-Harrison & Heslop-Harrison 1993), while different times and temperatures of rehydration affected the percentage of pollen germination in pecan (Yates & Spark 1993). The pollen of *Capsicum pubescens* was prehydrated before *in vitro* culture, and then slight differences in the pollen capability to rehydrate may have influenced the percentages of germinability.

The percentages of pollen germinability reached in *Capsicum pubescens* are similar to those recorded in other *Capsicum* species such as *C. frutescens* (Juntawong & Deepralad 1996) and *C. annuum* (Mercado et al. 1994; Carrizo García 2010). Higher values (above 60%) were recorded when solid culture media were used in species of the *C. annuum* complex, in *C. chacoense* Hunz. and also in *C. pubescens* (Avetikyan & Stepanyan 1973; Reddy & Kakani 2007; Kafizadeh et al. 2008). By contrast, lower values (20–30%) were recorded in a different group of *C. annuum* cultivars (Aloni et al. 2001; Karni & Aloni 2002). A very particular case was detected in *C. annuum*, in which a higher level of germination was obtained in a culture medium lacking boron, a substance that was found to inhibit pollen germination (Vasil 1964). That record is in contrast with the others mentioned before as well as with the general conditions recommended for pollen culture, since boron is regarded as a fundamental component of culture media (Shivanna 2003). Anyhow, germination percentages do not seem comparable among species or cultivars in *Capsicum* because different growing conditions are usually used; however, *in vitro* germinability is a useful tool to test pollen functioning.

Pollen longevity was longer than the flower lifespan in every plant of *Capsicum pubescens* analysed; this feature could favour allogamy (Dafni & Firmage 2000). It would be interesting to evaluate conditions for long-term pollen storage and the effect on pollen viability to facilitate germplasm exchange and to make selection based on male gametophyte performance, considering the expression of self-incompatibility in the species.

A significant increment of pollen viability, after an initial diminution, was a striking phenomenon recorded in two individuals of *Capsicum pubescens*. About that, pollen viability in rosemary (*Rosmarinus officinalis* L.) can be raised after treatment with different environmental temperatures and humidities (Aronne et al. 2006). Therefore, a similar effect may not be discarded in the cases registered in *C. pubescens*, since pollen was exposed under laboratory conditions and, although the environmental humidity was regular at the time of our measurements, it was not controlled by any device along the tests performed and it may have fluctuated along the day/s. The phenomenon observed could mean that the pollen cytoplasm may be inactive or dormant (and then unable to metabolise the tetrazolium salts found in the medium), but not dead, at least for a while, and that it could be eventually reactivated with time.

Pollen starch reserves

Pollen starch content was not quantified in *Capsicum pubescens*, but there were noticeable qualitative differences among plants. The differences observed in the ripe pollen may be due to variations in the processes of amylolysis and/or amylogenesis during pollen development. For instance, in *C. annuum* pollen, there is a wave of amylogenesis during pollen development and then a sharp decline in starch content before anther opening (Aloni et al. 2001), and both processes may be subjected to changes. Besides, both processes may be asynchronous in some individuals of *C. pubescens* (to be determined if within a flower or between flowers of the same plant), as it has been stated in other cases (Pacini & Viegi 1995; Clément & Pacini 2001). In the end, these variations would turn out in different amounts of starch stored in the ripe pollen as well as in different percentages of starchy pollen grains. Even though the differences in pollen starch content were not correlated to the functional variables tested in *C. pubescens* (pollen viability and germinability), they may have some

impact on other vital functions, for which carbohydrate metabolism has proven to be critical, such as pollen desiccation tolerance or germination speed (Franchi et al. 2011).

Conclusions

Some pollen features were uniform among the studied *Capsicum pubescens* plants such as exine sculpture and number of nuclei at maturity. However, even though all the individuals were male fertile, there were significant variations in pollen performance. Therefore, as it has been recorded for the compatibility level, the male function can also be variable among plants. It is worth mentioning that all the plants used in this study were obtained in a small area of cultivation, and later on kept together under the same conditions. This could mean that the differences registered would not be caused by the environmental conditions under which the plants were grown. Further research is necessary to determine whether the functional variations are related to the reproductive strategy of each individual (if it is actually variable). Finally, whether the differences in pollen performance are genotype-dependant or just an evidence of some plasticity that may ensure fertilisation in the species remains to be elucidated. If the former is true, there could be post-pollination pollen genotype selection, according to its performance, and it would be important to determine pollen performance for selection of superior genotypes.

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