Pollen and Pistil Features Involved in the Reproductive Biology of the Olive (*Olea europaea* L.)

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Abstract

The structural and cellular features involved in pollen-pistil interaction in the olive and how these features might affect the system of compatibility/incompatibility in this species have been studied in this work. We have paid special attention to establish the chronogram and the stages of the sexual reproductive cycle of the olive and to analyze the mature pollen and pistil features involved in pollen-pistil interaction.

INTRODUCTION

In general the sexual reproductive cycle of angiosperm species is well known specially in model plants like *Nicotiana*, *Mays*, *Petunia* or *Arabidopsis*. The reproductive biology of many fruit species (like almond, apricot, peach, kiwifruit, pear and others) has also been well studied (Egea et al., 1991; Gonzalez et al., 1995; Herrero, 1992; Herrero and Arbeloa, 1989; Yi et al., 2006). In spite of the enormous agronomical implications of fertilization in the olive for fruit formation, relatively little attention has been paid to the study of the reproductive biology in this species (Ateyyeh et al., 2000; Rapoport, 2001; Reale et al., 2006). Self-incompatibility in the olive is still an open question and a matter of discussion (Cuevas, 2005; Díaz et al., 2006). In our opinion a deep knowledge of the reproductive processes that takes place in this species is necessary to elucidate the current discussion about self-compatibility or incompatibility on the olive.

The aim of this study was to decipher the role of pollen-pistil interactions on the success of the olive fruit formation. However, to answer this open question, it is essential to have a good knowledge on the sexual reproductive cycle, the structures, cellular organization and behavior of the male and female organs on the flower during the progamic phase. These data will help us to proceed to a molecular and proteomic approach that will assist us to address this issue.

MATERIAL AND METHODS

Perfect flowers from olive trees (*Olea europaea* L.) 'Picual', grown in the province of Granada (Spain) were gathered periodically during the months of April to June, between 2004 and 2010. To characterize the different developmental stages of the flower a binocular microscope was used and the visualized images were captured as photomicrographs with a digital camera. We have defined successive floral stages based on external appearance, and correlated anther and pistil morphogenesis with those stages. Since flowering is a continuous process it was necessary to set up morphological markers to discriminate the different steps of the course time studied. The selected markers were easy to be observed and discerned in the different flower stages, such as the position and color of petals, sepals, turgidity and dehiscence of the anthers, presence of pollen on the stigma, and petals falling.

Isolated anthers and pistils were fixed in 4% paraformaldehyde and 2% glutaraldehyde 0.25% with 0.1 M cacodylate buffer at pH 7.5 overnight at 4°C, dehydrated and embedded in Unycril or Epon resin to be studied by LM after toluidine blue staining or specific staining to identify carbohydrates or lipids and also to analyse at transmission electron microscope (TEM) level. Photomicrographs were captured with a digital camera from images visualized with a Zeiss Standard microscope (Carl Zeiss, Oberkochen, Germany) or with a TEM JEM-1011 (JEOL, Japan). Immunocytochemistry

techniques were used to localize pectins and AGPs. For this purpose, several antibodies, (JIM 5, JIM 7, and JIM 13) which are able to recognize specific epitopes, have been used.

RESULTS AND DISCUSSION

The chronogram of the olive sexual reproduction cycle was studied (Fig. 1). The gathering of the samples started when it was possible to manipulate the small green buds to separate the anthers (the tiny floral green buds were covered by the calyx). The collection continued every 2 days until the petals fell. The collection of the pistils was possible when the anthers were just at the tetrad microspore stage. Overall the reproductive cycle lasted for about 5 to 6 weeks depending on the year; the longer period of time is budding development (about a month), coinciding with the formation of the reproductive organs. Anther development begins first followed by pistil formation, once the meiosis of the pollen mother cells is completed. This may be the reason why the imperfect flowers show always anthers and no female organs. The anthesis or flower opening is the shortest period and lasts only between 3 to 5 days, like the post-anthesis stage.

The mature olive pollen grains (Fig. 2) are covered by a reticulated exine with three apertures, type colpus (Fernández and Rodríguez-García, 1988); they are bicellular and contain a large amount of oil bodies as storage material (Rodríguez-García et al., 2003), which allows withstanding the dehydration caused by high temperatures and to have a long life span. Olive pollen contains a lot of proteins, most of them with an allergenic character but their main function is related to pollen-pistil interaction (Alché et al., 1999; Barral et al., 2005; Morales et al., 2008).

Lipids and proteins are present in the pollen coat (Fig. 3), which together with the lipid components of the stigmatic exudates has been suggested to be involved in the adhesion, hydration and germination of pollen grains on the stigma (Lord, 2003; Murphy, 2006; Wolters-Ars et al., 2002). They are also germinated easily in vitro, which allows to analyze the dynamics and behaviour of pollen tube germination (M‘rani-Alaoui, 2000; Majewska-Sawka et al., 2002). Pectins and AGPs have also been immunolocalized on the pollen grain and the pollen tube, showing a specific localization at the pollen cell wall, the aperture and the apex of the pollen tube (Suarez, 2009).

Light and transmission electron microscopy was used to provide the first comprehensive description of the tissue and cellular modifications of the pistil from pre-anthesis through the anthesis and post-anthesis events in the olive tree. The olive pistil structures cannot be considered completely mature and ready for pollination and fertilization until just at the onset of anthesis. The olive pistil is composed of a bilobed wet stigma coated by multicellular papillae, a solid style and a bilocular ovary containing four ovules (Serrano et al., 2008). A high amount of stigmatic exudates is observed during anthesis. Stigmatic receptivity is considered optimal when the olive flower is completely open and anthers are turgid and still not dehiscent. The stigmatic secretion of the olive has a heterogeneous composition including carbohydrates, lipids and proteins. Some of these proteins correspond to non specific esterases (esterase activity has been determined on the stigma, data not published). The funnel-shaped and the continuity of the subpapilar stigmatic tissue and the style transmitting tissue (Fig. 4) should play a role to regulate the passing of the numerous pollen grains germinating and growing through the stigma towards the style: many of the germinated pollen grains stop their growth in the funnel region and a few of them reach the style; finally only one or two pollen tubes fertilise the ovules. The access from the transmitting tissue to the ovary does not present any specialized structure (e.g. obturator, ponticulus) regulating the pollen tube entrance as described in other species (Martínez-Pallé and Herrero, 1995; Herrero and Hormaza, 1996).

After pollination we observe degradation of the papillae on the top of the stigma. The stigma senescence progresses with petal abscission, and, finally, the style is also degraded (Fig. 4). The stigma and style senescence occurred always regardless of the fertilization process being successful or not.

According to the scientific literature (Frankling-Tong et al., 1994; Cheung, 1996) the pollen and pistil features that have been associated with the gametophytic self-incompatibility system (GSS) are: bicellular pollen grains with a high amount of storage substances, easy to germinate in vitro and with a long life span, and the pistil should have a humid stigma coated by extracellular exudates, a solid funnel-shaped style and continuity between the subpapilar stigmatic tissue and the style transmitting tissue. Our results show that pollen grains and pistils of olive present all these features and, therefore, we postulate the existence of a GSS in the olive. The next step is to obtain molecular evidence (e.g. the identification and characterization of S-RNases) to prove this hypothesis. For this purpose, we have initiated a research program to identify proteins from the stigmatic exudates on a large scale using proteomic techniques. Another goal, in which we are interested, is the study of macromolecules involved in the signaling processes taking place during the pollen-pistil interactions.

ACKNOWLEDGEMENTS

This work was supported by the "Consejería de Innovación, Ciencia y Tecnología de la Junta de Andalucía" and Fondo Europeo de Desarrollo Regional (FEDER) in the frame of the Proyecto P06-AGR-01791 and Grupo de Investigación BIO283. The authors would like to thank Dr Hava Rapoport for her helpful comments and discussion concerning the female organs, and to Conchita Martínez for her skillful technical assistance.

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Figures

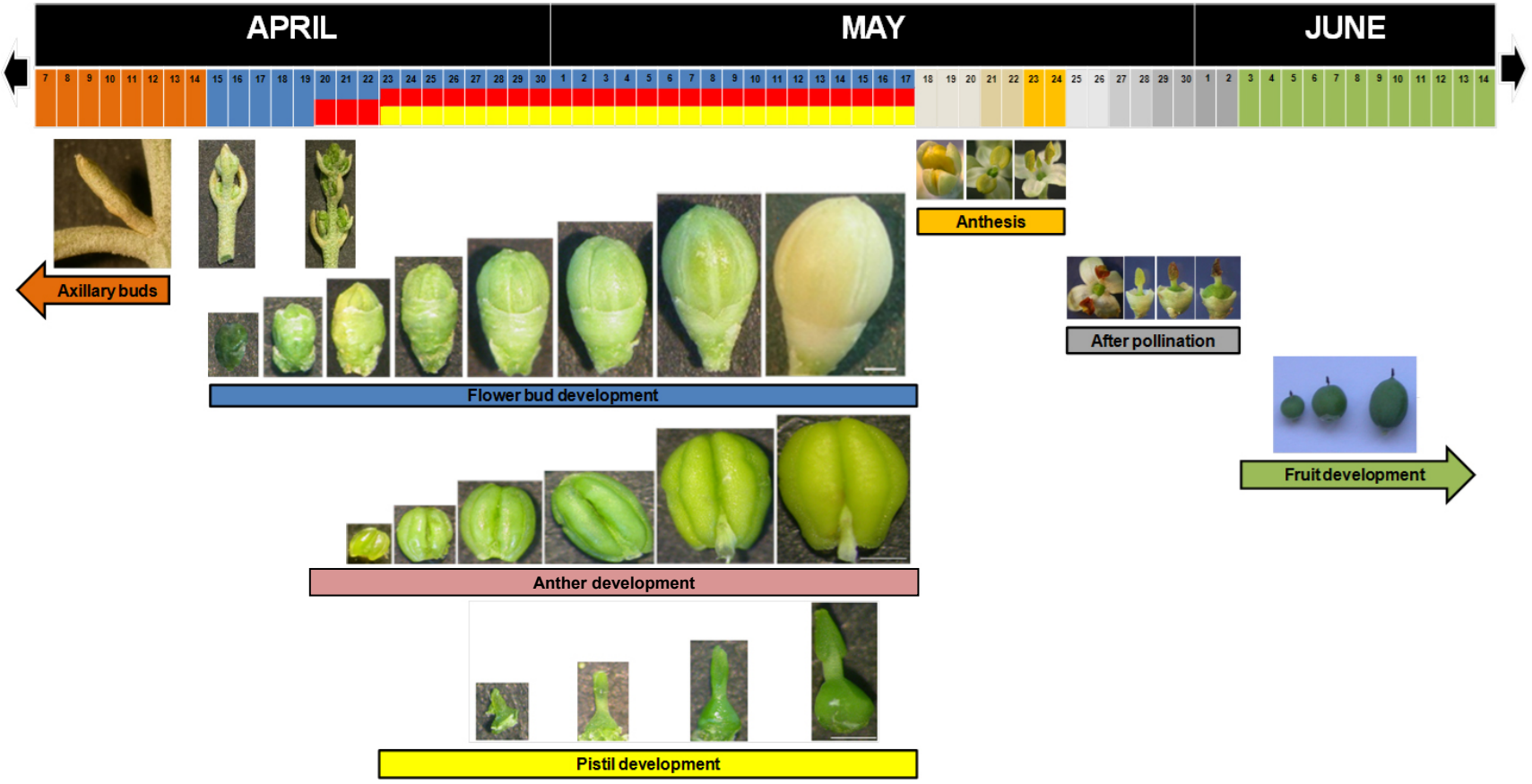

Fig. 1. Chronogram of the sexual reproductive cycle in the olive tree.

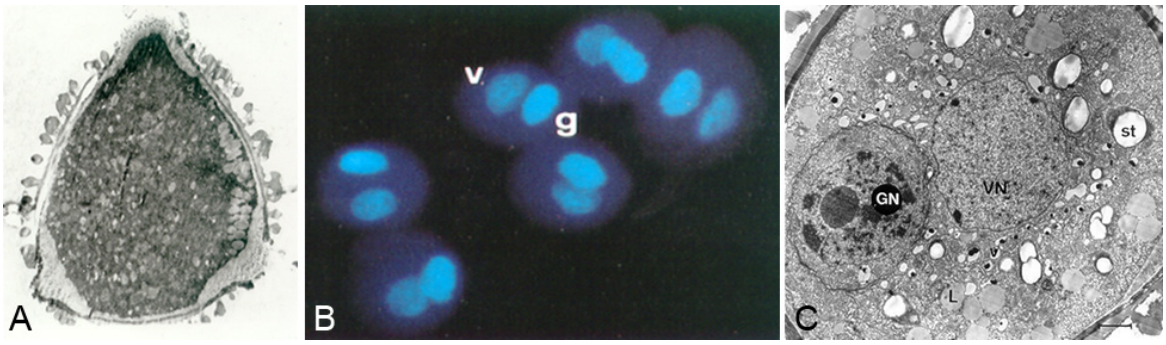

Fig. 2. Bicellular pollen grains of olive. A) The pollen wall is composed by a thick, well structured exine with 3 apertures; B) after staining with DAPI, two nuclei are observed, the brighter corresponding to the generative (g) nucleus; C) section of a bicellular pollen grain at the TEM where it is possible to distinguish both nuclei. The generative nucleus presents large patches of heterochromatin. GN=generative nucleus; VN=vegetative nucleus; st = starch.

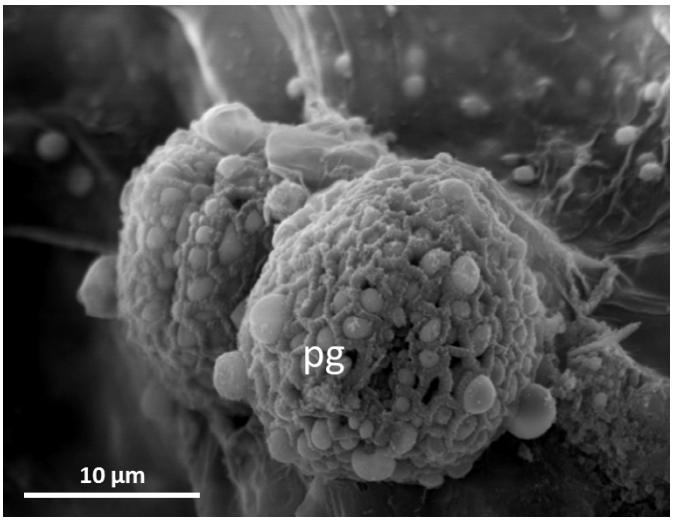

Fig. 3. Scanning electron microscope photomicrography showing the surface of two olive pollen grains (pg). The exine is reticulate and highly sculpted. A pollen coat proceeding from the tapetum is deposited between the exine arcades.

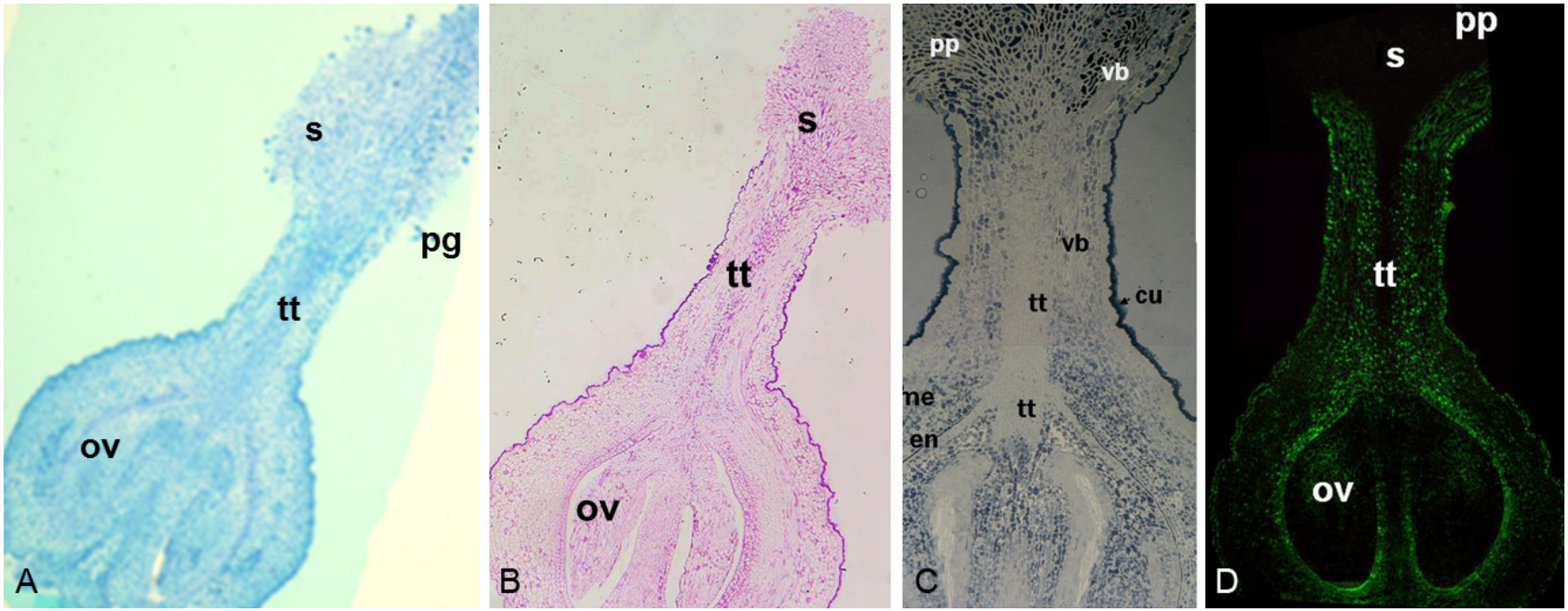

Fig. 4. Pistil sections of olive with different stains showing different tissues of the style: stigma, style and ovary; the inner stigmatic cells are continuous with the central transmitting tissue which terminates in the ovary, without any specialized structure on the path gate of the ovary. A) Toluidine blue staining; B) PAS staining; C) Sudan Black staining; C) Localization of sterified pectins by immunofluorescence. S=stigma; tt=transmitting tissue; ov=ovary; pp=papillae; vb=vascular bound; cu=cuticle; me=mesocarp; en=endocarp.