Methods for Observing Chestnut Pollen Viability, Germinability and Pollen Tube Growth

Giancarlo Bounous, Roberto Paglietta and Cristiana Peano

Istituto di Coltivazionl Arboree dell'Universita dI Torino, Via Pietro Muria 15, 10126 Torino, ITALY

ABSTRACT. This paper describes methods of examining pollen of *Castanea crenata, C. sativa* and their hybrids for *in vitro* viability using tetrazolium salts and fluorescent microscopy, and for *in vitro* germinability on liquid and solid media. All of the methods used to test pollen germinability gave satisfactory results. In general, the Japanese cultivars germinated best at low sugar concentrations (1-2%), while hybrid and European cultivars required higher concentrations (5-10%). Pollen tube growth in styles was observed with fluorescent microscopy. Some difficulty was encountered in the visualization of the pollen germ tubes within the stylar tissues. Part of the problem may be due to the presence of tannins in the styles. Methods for *in vitro* pollen germination and pollen tube growth would allow for further investigation of the self-incompatibility system in the genus, *Castanea.*

Viability or germination testing may be useful in any chestnut pollen handling protocol, whether the pollen is to be used immediately for controlled pollinations, for breeding purposes, for studies of the floral biology, or if the pollen is to be stored for some time after collection as germplasm for the conservation of genetic resources. *In vitro* methods may allow the rapid appraisal of pollen vitality or germinability. However, they should always be considered in relation to the functional capacity of the pollen to produce viable seed when used in controlled pollinations in the field. The present work describes some of the methods used in our laboratory to test the viability and germinability of pollen of *Castanea crenata* Sieb. and Zucc., *C. sativa* Mill. and their hybrids.

MATERIALS AND METHODS

Before testing for viability or germinability, pollen was often pretreated by placing it in a humidity chamber for 12 h, and then in a humid incubator at 30 C for 0.5 h.

Pollen viability. The vitality of the pollen was tested by staining with tetrazolium salts (MTT) that gives a measure of the respiratory activity of the pollen. Vital cells reduce the MTT to an intense violet color. Petri dishes were lined with moistened filter paper. Glass slides were placed on the filter paper and a drop of liquid culture medium in which the pollen was suspended and a drop of MTT solution placed on the slides. Observations were made at the optical microscope after 3-5 h, depending on the cultivar.

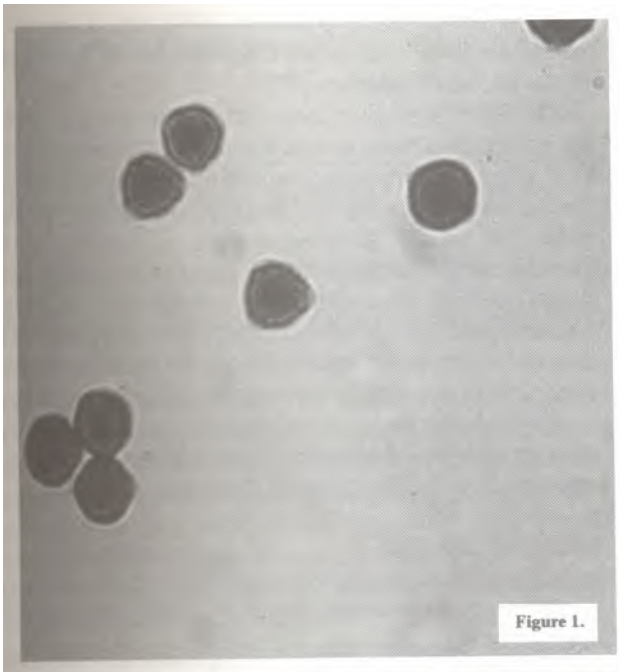
Pollen germinability. The germinability of the pollen was examined using several different methods and culture media. Both optical and fluorescence microscopy were used to observe the germinating pollen, and both liquid and solid culture media were tried.

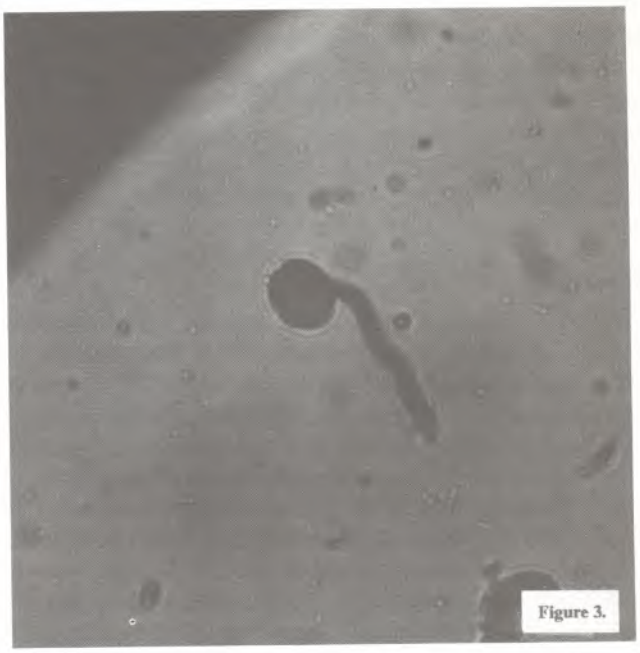
The liquid culture media was based on that of Hoestra and Bruisma who proposed a very general medium for observation of bi- and trinucleate pollens (6). Their medium was composed of Ca(NO3)2*4H20, H3B03, and sucrose. The sugar concentration may vary from 0.94 M to 1.32 M. In the specific case of chestnut, it was observed that the presence of calcium and boron did not substantially modify the percentage of germinated pollen grains. Twice distilled water was used to prepare 20 sugar solutions at concentrations from 5 to 100 g/l. Germinated pollen were counted and the pollen tube growth was measured after 5, 10, 15 and 24 h incubation at 30 C.

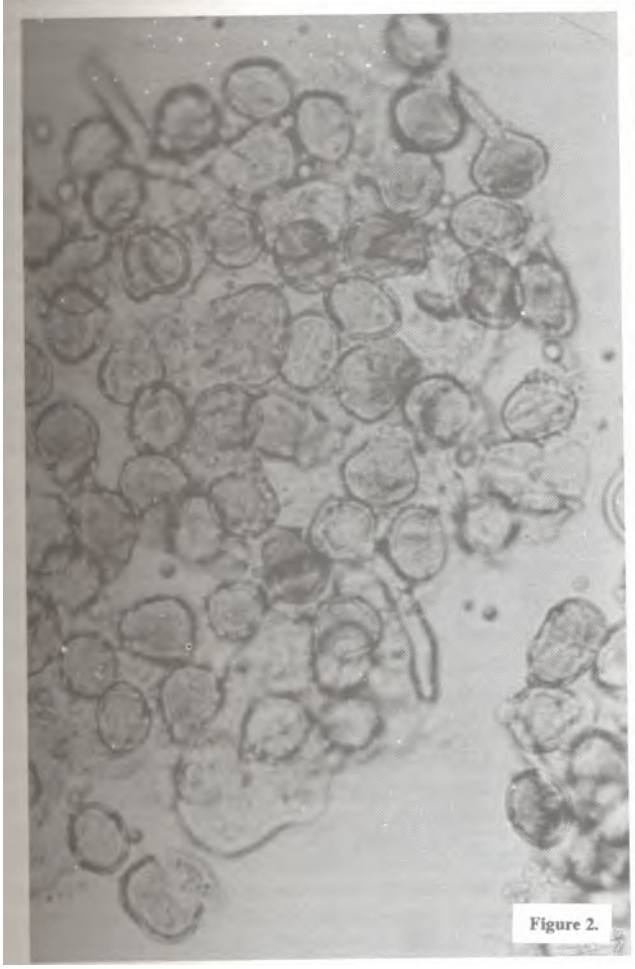
Permanent slide mounts. Jona's method, used to study apple pollen, was modified to evaluate chestnut pollen germinability (8). Amore complex agar medium was used, which allowed the preparation of permanent slide mounts. The pollen was spread onto a dry fragment of autoclaved Millipore membrane (Millipore Corp., Bedford, Mass.) that had been placed on a nutrient medium solidified with 6 g/I agar, containing 5 to 100 g/1 sucrose. In order to observe the germination under the light microscope, the membrane was diaphanated and transferred to a glass slide.

Fluorescence microscopy. Use of the fluorescence microscope permitted visualization of growing pollen tubes within the stylar tissues. To do this, female inflorescence were isolated in bags on emasculated branches before the appearance of the styles. The isolated flowers were then hand pollinated and observed in the laboratory, after several days. The procedure was based on the secondary fluorescence of Kho and Baer (9), and depends on the selective absorbance of fluorescent substances known as fluorochromes. In our case, the goal was to make evident callose, known to be one of the constituents of pollen germ tubes since described by Mangin (10). There are many methods for staining, based on the work of Currier (4), yet the only one that gave satisfactory results in our lab for chestnut, as shown earlier for hazelnut (17), was that of Martin (11), used also by Kho and Baer (9) and Velguth (21). The technique is based on the selective fluorescence complexed with aniline blue. The complex gives off a fluorescent light when struck by the ultraviolet radiation of a mercury lamp.

The styles to be observed were fixed in formalin:acetic acid (FAA) for at least 24 h. After thorough rinsing to completely remove the fixative, the styles were soaked in 8 N NaOH for 24 h to clear and soften the tissues and permit an adequate penetration of the stain. Then, the







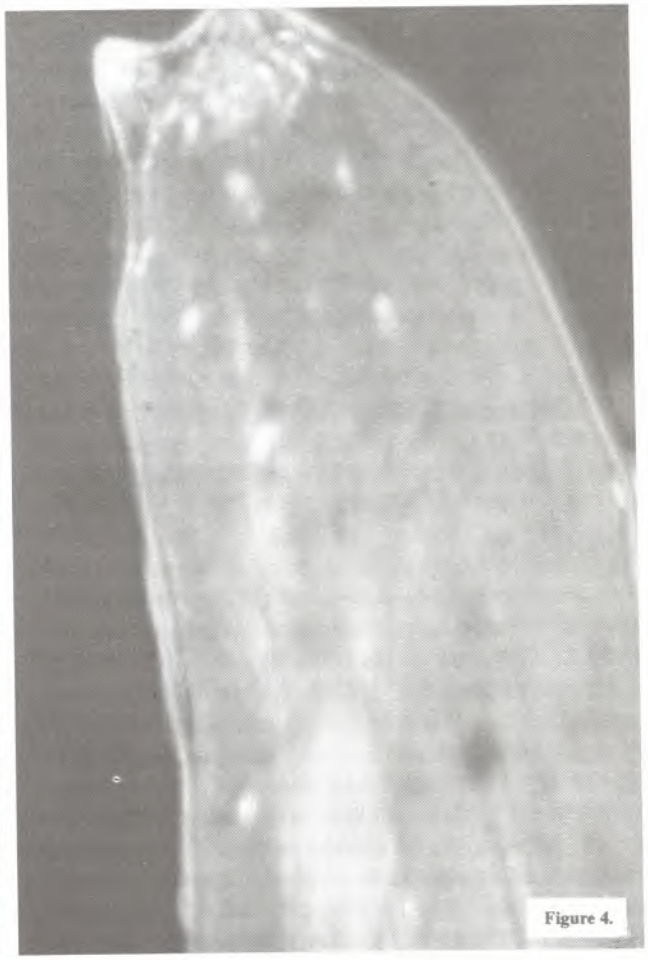


Figure 1. Pollen of the Japanese chestnut cultivar `Gianyose' viewed under the light microscope without staining.
Figure 2. Group of pollen grains with pollen germ tubes of the Japanese cultivar `Tanzawa' viewed under the light microscope. Germination medium is a simple 1% sucrose solution in twice distilled water.
Figure 3. `Tanzawa' pollen tube, no staining, in 1.5% sucrose solution.
Figure 4. Receptive style of the cultivar `Garrone Rosso' viewed under fluorescence microscopy.

NaOH was completely removed by rinsing in running water for 1 h or more, prior to staining with 1% aniline blue in an aqueous solution of tribasic potassium phosphate. Observation was done by placing the styles, thus prepared, in a drop of stain on a microscope slide. Under these conditions the callose of the pollen germ tubes fluoresced yellow or yellow-green while the remaining tissues were blue or grey.

RESULTS

The pollen grains, when collected directly from the field from catkins in full bloom or from flowers forced in the laboratory, have an oblong shape and yellowish color (Figure 1). After staining (19), three germinative pores may be distinguished as well as the smooth or slightly granular exine and three grooves passing through the pores. The dimensions of the grains varied according to the cultivar. The length varied from 14-18 ,um and the width from 10-14 ,um (Figure 1).

All of the methods used to test pollen germinability, both in liquid and on solid media, gave satisfactory results (Figures 2 and 3). At this preliminary phase of the trial, we were able to observe that the optimal sugar concentrations of the medium varied according to the cultivar under examination. In general, the Japanese cultivars germinated best at low sugar concentrations (1-2%), while the hybrid and European cultivars required higher concentrations (5-10%). All cultivars germinated after 3-5 h incubation at 30 C. Pollen viability may be low for some cultivars.

Some difficulties were encountered in the visualization of the pollen germ tubes within the stylar tissues (Figure 4). Part of the problem, as mentioned by Velguth (21), may the presence of tannins in the styles, typical of the genus, which causes darkening of the tissues upon contact with the NaOH. A longer or different softening process may be necessary although there is the risk of washing the pollen grains off of the stigmatic surface that is located only at the extreme tip of the style (5).

DISCUSSION

An understanding of the floral and fruiting biology is of fundamental importance to achieve high quality and consistently high production in chestnut orchards. Studies by various authors have shown that in the genus *Castanea,* there are two sterility mechanisms at work, a morphological type and a factorial type (7, 12, 15, 19). The morphological type of sterility involves the staminate flowers that may present various kinds of anomalies including the absence of anthers (astaminate catkins) or anthers with shortened filaments with consequent scarce production of often non-functional pollen (brachystaminate and mesostaminate catkins). These types of male sterility may be, in part, under the control of cytoplasmic hereditary factors (7, 20).

Female sterility usually involves only the more distal pistillate inflorescence on the androgynous catkins or it may involve one or both of the lateral flowers in each pistillate inflorescence. Female sterility rarely influences the productivity of the trees. Male sterility, especially in its extreme form, astaminate catkins, may directly affect the productivity of an orchard and be a determinate factor in the choice of cultivars (15).

The floral biology of chestnut is further complicated by the presence of more or less complete autoincompatibility (1, 2, 13, 14, 16). Autoincompatibility in chestnut may be controlled by a polyallelic series (15) as is known for *Nicotiana* (18) and for *Theobroma cacao* L.(3). This type of gametophytic incompatibility is manifest by the failure of union of either the nuclei of the endosperm or of the egg with the generative nucleus of the pollen. The pollen tube may in fact grow normally through the stylar tissues and may even penetrate the embryo sac (12).

Methods for *in vitro* pollen germination and pollen tube growth would allow for further investigation of the self-incompatibility system in *Castanea.*

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