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Viability of eggplant pollen

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ABSTRACT - *This study aimed to test methodologies to evaluate the pollen viability of eggplant. The pollen germination was tested in vitro, incubating the pollen during 0.5 and 16 hours in different sucrose concentrations (0; 2.5; 5.0; 7.5 and 10.0 g* L^{-1}*). For the in vivo germination test, the pollen was placed during 4 and 24 hours in the flower stigma and after that stained with aniline blue and evaluated in an ultraviolet light microscope. The viability of the pollen grains was also evaluated in tetrazolium solutions (at the concentrations of 1%; 0.75% and 0.5%) and the staining was evaluated after 24 hours. The highest in vitro pollen germination was 10.8%, showing the necessity for a better calibration of the culture medium. The in vivo pollen germination was 66%, showing that this is an adequate methodology to estimate pollen viability in eggplant. The methodologies involving tetrazolium solution weren't efficient to estimate pollen viability evaluation, because of the deficient pollen staining.*

Key words: *Solanum melongena,* pollen germination, tetrazolium, *in vivo, in vitro*.

INTRODUCTION

Currently, the demand for eggplant (*Solanum melongena* L.) in the world has increased. As well as being a healthy product it has some properties that reduce the level of cholesterol. The hybrids of eggplant have many advantages compared with open-pollinated cultivars in terms of yield and diseases resistance. Furthermore, the production of hybrid seeds is facilitated by the size of the flower in this species.

The evaluation of the germination capacity (viability) during the storage of pollen grains from the male parent is crucial in the process of artificial hybridization. During the plant maturation, the pollen viability can be affected by several endogenous and exogenous factors, such as the stage of flower development (Lacerda et al. 1994), high temperatures (40 °C) (Giordano et al. 2003) and low temperatures (15°C) (Chira 1963), nutritional status of the plant (Howlett 1936), luminosity (Goss 1971), agricultural pesticides and other chemicals (MacDaniels and Hildebrand 1939, Dubey and Mall 1972).

According to Sari-Gorla et al. (1995), the diameter of pollen grain and the ability of the pollen tube to be emitted may be considered as indicators of the pollen quality, independent of the environment in which the flowers are pollinated, but the performance of pollen varies according to the genetic combination of the pollen.

The pollen grain viability, a measure of male fertility, may be determined by different techniques (Dafni 1992, Kearns and Inouye 1993). These can be grouped into direct methods, such as the induction of *in vitro* (Dutra et al. 2000, Gomes et al. 2003, Pio et al. 2007) and *in vivo* (Oliveira et al. 2001) germination or indirect methods based on cytological parameters such as color (Shivanna and Johri 1985, Dafni 1992, Shivanna and Rangaswamy 1992, Kearns and Inouye 1993).

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The stain techniques including acetic carmine, aniline blue, cotton blue, iodide potassium (Sharma and Sharma 1994, Stanley and Linskens 1974), trifeniltetrazolium chloride and red tetrazolium (Shivanna and Rangaswamy 1992), which promote differences in the staining of pollen grains, provide results quickly and with a low cost. However, there is no report in literature of a universal technique to evaluate pollen eggplant. The nuclear stains, according to Alexander (1969, 1980), have a limited application among species because they only stain functional pollen grains, while those identified by unviable are not stained. They are therefore not suitable for species whose pollen grains have thick walls, are mucilaginous and have spikes present, as they impede the penetration and prevent staining. In this condition, viable pollen grains may not have stained and been mistakenly classified as non-viable. To avoid this, an alternative would be used to stain with malachite green and acid fuchsin, which due to its acidic and basic chemical properties, respectively, stains viable pollen grains and non viable and is effective for several species.

The *in vitro* germination test is a methodology to germinate a small sample of grains in an appropriated culture medium. Through this technique, it is possible to observe in a microscope the percentage of pollen grains that developed pollen tubes after a certain period. The composition and pH of the medium are among the factors that affect pollen germination. The angiosperms pollen requires a carbon source, boron, and often other nutrients to promote their germination (Galleta 1983). According to Pfahler (1967), the addition of boron is important, and their responses vary according to species. Its mechanism of action occurs through its interaction with the sugar, forming a complex sugar-borate, which reacts more rapidly with the cell membranes. Thompson and Batjes (1950) found that the addition of boron to the medium markedly increased the percentage of germination and pollen tube length of various fruit species of temperate climates. Calcium added in a culture medium for germination provides physiological characteristics to the pollen tube and pollen grain with less sensitivity to changes in the basic medium, lower permeability, growth in a linear and rigid appearance of pollen tube (Bhojwani and Bhatnagar 1974). In the absence of calcium there is increased permeability of the pollen tube membrane, causing the release of internal metabolites to the external environment (Stanley and Linskens 1974). Beyoung (1965) observed in 46 vegetable species, that the addition of calcium promoted the germination of pollen grain and pollen tube growth in all species studied. Brewbaker and Kwack (1963), working with 86 species of 39 families, showed that the addition of calcium and boron acts as a primary factor controlling the germination of the pollen tube *in vitro*.

Sugar is used in the culture medium to provide the osmotic balance between the solution and pollen germination, and provides energy to assist the development process of the pollen tube (Stanley and Linskens 1974, Galleta 1983, Miranda and Clement 1990). Among sugars, sucrose is the most suitable for the ovule culture of *Lycopersicum peruvianum*, because it is crucial for *in vitro* ovary growth and seed formation (Torres and Murashige 1985). For the tomato culture (Torres and Murashige 1985), tobacco (Loguercio 2002) and *Acacia mearnsii* (Stiehl-Alves and Martins-Corder 2007), the culture medium of Brewbaker and Kwack (1963) should be calibrated with 10 g L^{-1} of sucrose.

The *in vitro* germination test tries, through the culture medium, to simulate the plant ovary, providing the balanced environment for the development of the pollen tube. The *in vivo* germination consists in placing the pollen grains in the receptive stigma of the flower, and evaluating the developing pollen tube under the microscope (Stanley and Linskens 1974). An indirect way of evaluating the percentage of pollen germination is by fruit (Galleta 1983) or the seed production (Stanley and Linskens 1974, Akihama et al. 1978). Galleta (1983) considered that the stain method overestimates the percentage of pollen grain germination, while the *in vitro* test underestimates it. However, samples of pollen that don't seem viable when tested *in vitro* germination tests can produce a high percentage of seeds *in vivo* (Einhard et al. 2006). The staining methods, although simple and low cost, don't provide information about the germination capacity of pollen, which can be obtained by testing the *in vitro* (Techio et al. 2006) or *in vivo* germination (Einhard et al. 2006).

The objective of this study was to test some methodologies to evaluate the pollen viability of eggplant.

MATERIAL AND METHODS

In vitro germination test

The first trial aimed to evaluate the viability of eggplant pollen grains by *in vitro* germination

treatments. The trial was carried out in the Laboratório de Biologia Celular of Embrapa Hortaliças, Brasília-DF, Brazil, in January 2006. Pollen grains from male parents of the eggplant cultivar 'Ciça' were collected from flowers at the anthesis using a small electric vibrator. In order to germinate the pollen grains, germination tests were carried out using the culture medium of Brewbaker and Kwack (1963), which consisted of KNO_3 (100 g L^{-1}), $MgSO_4.7H_2O$ (200 g L^{-1}), H_3BO_3 (100 g L^{-1}) and $Ca(NO_3)_2.4H_2O$ (300 g L^{-1}).

Different concentrations of sucrose were added to the Brewbaker and Kwack culture medium (0, 2.5, 5, 10, 15 and 20 g L^{-1}). Two drops of culture medium with the respective levels of sucrose were applied on slides, and then the pollen grains were distributed on the culture medium. The slides were incubated for 30 minutes at ambient temperature. The germinated pollen grains were not counting, and the development of pollen tubes were only assessed visually in an optical microscope with a 10x objective lens. Based on the results of the preliminary test, the trial was repeated, but added the following concentrations of sucrose to the culture medium: 0, 5.0, 7.5 and 10.0 g L^{-1}. Two drops of the culture medium with the respective levels of sucrose were applied on each slide, which was followed by the addition of the pollen grains. Each plot consisted of a single slide and each treatment was replicated 3 times. The slides were placed in moist chambers (petri dishes lined with moistened filter paper) and incubated at 25 °C under fluorescent light for 16 hours. After the incubation period, germinated and non germinated pollen grains were photographed with a digital camera in an optical microscope with 10x objective, in order to increase the images. Three areas of each image were evaluated, where germinated and non germinated pollen grains were counted. The emission of the pollen tube was considered as the germination criteria. The experimental design was completely randomized, with three replications, using the Sisvar (Ferreira 2000) as the statistical program for the analysis. Previously to the analysis, the experimental data was transformed using the formula $(x)^{1/2}$ and the averages were compared by polinominal regression.

In vivo germination test

To assess the viability of eggplant pollen grains through an *in vivo* germination test by fluorescence, an experiment was carried out in the Laboratório de Biologia Celular and in the experimental area of Embrapa Hortaliças, Brasília, DF, in January 2006.

Flower buds of the female parent were emasculate and pollinated with fresh pollen grains, newly extracted. Four and 24 hours after pollination, the flower buds were harvested. The pistil of these flower buds were extracted and submitted to a fixation in ethanol 30% (3 parts of ethyl alcohol and 1 part acetic acid) for 10 minutes, which the function was to maintain the pollen grains on stage as they were when removed from the plant. Then, the pistils were washed during 30 minutes in ethyl alcohol 70%, in order to depigment carpel tissues and facilitate analysis. Subsequently, the pistils were washed in distilled water followed by sodium hydroxide (NaOH) for 20 minutes to soften the pistil tissues. The pistil was washed again in distilled water and macerated in a slide with two drops of blue pigment from aniline at 0.1% in 0.1 N potassium phosphate (O'Brien and McCully 1981, Torres and Murashige 1985), for 5 minutes. After this treatment, the pistil was examined using an ultraviolet light microscope with 10x objective lens, in which the development of the pollen tube was observed through fluorescence. Pollen grains which emitted a bright and/or green tube were considered germinated, those ones that emitted a brown color were considered non germinated (Figure 1).

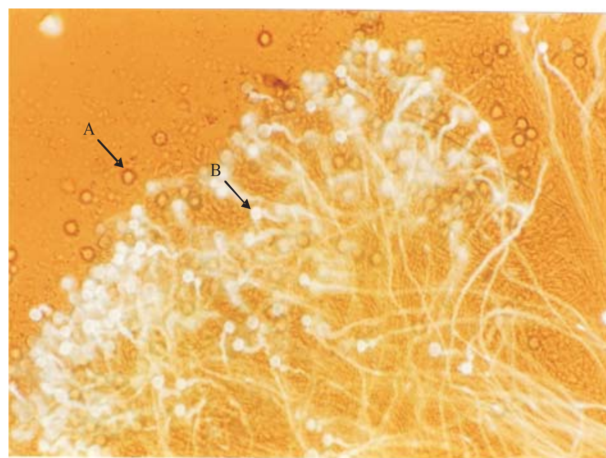

Figure 1. *In vivo* germination of eggplant pollen grains, showing unviable (A) and viable (B) pollen grains

One flower button was used for the period of 4 hours as a preliminary test. Beyond this period there was no emission of the pollen tube. For the period of 24 hours, each slide with a stigma was considered as a plot, which was replicated three times. In order to evaluate a considerable amount of pollen grains, three randomized areas were evaluated in each plot, representing the amount of 5 mm². In the evaluated area, the germinated and non-pigmented pollen grains were counted, and the germination was expressed as a percentage of the total number. The pigmentation is obtained by the reaction of aniline blue solution with the calosis, which is present in mature grains ready to emit the pollen tube (Currier 1957, Stanley and Linskens 1974), that confers the grains a fluorescent aspect, which is an indication that pollen grains are viable (Linskens and Esser 1957, Stanley and Linskens 1974). This analysis was not performed using photos, because only the viable grains are visible.

Tetrazolium test

This trial was carried out at the Laboratório de Cultura de Tecidos of Embrapa Recursos Genéticos e Biotecnologia, in Brasília, DF, in June 2006. The tetrazolium solutions (at concentrations of 1.0%, 0.75% and 0.5%) were placed in microtube eppendorfs with a volume of 1.5 mL, containing a small portion of fresh pollen grains. The microtubes were covered with an aluminum sheet and agitated in order to allow a better contact between the pollen grains and the tetrazolium solution. Then, the microtubes were placed in a BOD incubator at 25 °C for 24 hours. After this period, the pollen grains were observed in three ways: a) the color of the pollen mass in the microtube visualized with naked eyes; b) staining of pollen grains on slides, observed under an optical microscope at 10x objective lenses and c) color of pollen grains on filter paper, using magnifying lenses.

Based on the results of the previous test, two lots of eggplant pollen grains were placed in microtubes containing a tetrazolium solution with a concentration of 0.75%: a fresh one and a two year stored one. The microtubes were covered with an aluminum sheet, agitated and then placed in a BOD incubator at 25 °C for 24 hours. The pollen grains were evaluated using the same "a" and "b" methods described above.

RESULTS AND DISCUSSION

In vitro germination test

In vitro germination tests are considered suitable to estimate pollen viability of many species (Sari-Gorla et al. 1995). The culture medium simulates the stigma environment of the plant where the pollen germination usually takes place. In the test in which the pollen grains were incubated for 30 minutes, the addition of sucrose (2.5, 5, 10, 15 and 20 g L^{-1}) or not (0 g L^{-1}) to the culture medium allowed the beginning of the emission of pollen tubes, although these were not so developed. Apparently, the germination of pollen grains was higher at concentrations of 5 g L^{-1} and 10 g L^{-1} of sucrose, compared with the other concentrations of sucrose, although it was not possible to quantify precisely the number of germinated grains with the method used. However, the germination of pollen grains in this trial was at a general low. Probably, the short incubation period, as stated by Lacerda et al. (1995), was a much more important factor affecting the low germination rates than the sucrose concentration. For this reason, in the second test the pollen grains were incubated for a longer period, 16 hours.

When incubated for 16 hours, the pollen grains germinated in the culture medium at all concentrations of sucrose tested (0, 2.5, 5, 7.5 and 10 g L^{-1}), however, when incubated at 5.0 g L^{-1}, the rate of broken pollen grains was higher (Figure 2). According to Akamine and Girolami (1959) and Salles et al. (2006), pollen tube ruptures happen, among other factors, because of the high humidity, changes in the environment caused by the increase in the osmotic pressure and the low resistance of the cell wall. Among the three factors mentioned above, the pollen grain moisture incubated in sucrose at 5 g L^{-1} was probably higher compared to the moisture grains incubated in other concentrations, since the cell walls of the grains have similar resistance because all grains were from the same material, and as the elevation of osmotic pressure that probably was not the reason for the rupture of the tubes emerged in the medium with 5 g L^{-1} sucrose, because the tubes of grains incubated in a medium with 7.5 g L^{-1} and 10 g L^{-1} also had been broken and that was not observed.

The level of sucrose in the culture medium affected the pollen germination of eggplant, although the germination rates were generally low. Pollen grains incubated in the culture medium with a 7.5 g L^{-1} of

sucrose level showed higher germination (10.80%), while at the concentrations of 0, 2.5, 5 and 10 g L^{-1} of sucrose the germination was 6.38%, 6.30%, 6.45% and 7.11%, respectively (Figure 3). The observed pollen *in vitro* germination rates of eggplant pollen grain, using the current technique, were low comparing to some studies *in vitro* with other species. For instance, Gomes et al. (2003) observed 49.8% germination in onion pollen grains, while Dutra et al. (2000) obtained 82.0% germination in pepper pollen and Franzon et al. (2005) 79.7% in beans.

These results indicate the necessity for more studies concerning the development of a more suitable culture medium to test the viability of *in vitro* eggplant pollen grains. There is a high possibility of increasing *in vitro* pollen germination rates through the calibration of macro and/or microelements, such as calcium and boron, in the Brewbaker and Kwack (1963) culture medium, since these elements are essential for the extension of the intine and consequent formation of the pollen tube (Brewbaker and Kwack 1963, Pio et al. 2004, Marcos Filho 2005). High doses of calcium and the absence of boron can increase the amount of broken pollen grains, with the release of cytoplasmic contents to the external environment in addition to a low rate of pollen grain germination (Pio et al. 2004). Thus, the calibration of these elements in the culture medium can bring it closer to the stigma conditions of the plant and can increase the percentage of *in vitro* germination. Alternatively, although the protocol of Brewbaker and Kwack (1963) is efficient for the germination of pollen grains of tomatoes (Torres and Murashige 1985) it may not be for the germination of eggplant pollen, and in this case other protocols should be tested to improve the *in vitro* pollen germination in this species.

In vivo germination test

Initially, the pollen grains remained in contact with the stigma during 4 hours, however, this time was not enough for the emission of the pollen tube. The period required for the germination of the pollen in the stigma varies according to the species. Eucalipto, for instance, requires only 6 hours (Pereira et al 2002), while peach requires 4 days (Einhardt et al. 2006). In eggplant, the period of 24 hours apparently is enough for the evaluation of *in vivo* pollen germination, once after this period an average of 66% of pollen germination was observed, with well development tubes. According to Scorza and Sherman (1995), a good percentage of pollen germination varies between 50% and 80%. In this way, the period of 24 hours of contact between the pollen grains and the stigma can be considered suitable for the assessment of pollen viability in eggplant.

Tetrazolium test

The results of the trial to test concentrations of the tetrazolium solution showed that the best staining (red carmine) of the pollen grains occurred with a concentration of 0.75%. At 1.0%, the pollen grains were too dark, while at 0.5% the staining was not strong enough, which led in both cases to an inadequate evaluation. However, although the mass of pollen grains

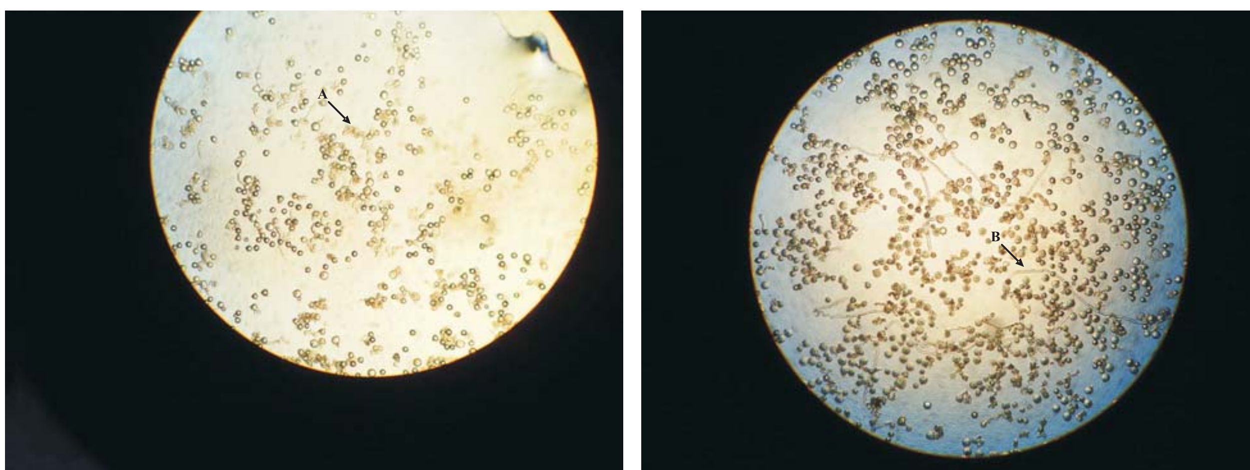

Figure 2. *In vitro* germination of eggplant pollen grains in the culture medium of Brewbaker and Kwack. A – Pollen grains tube broken in sucrose concentration of 5.0 g L^{-1}. B - Well developed tube in sucrose concentration of 7.5 g L^{-1}

looked well stained when visualized with naked eyes, the majority of individual pollens remained uncolored when observed under an optical microscope, despite the *in vivo* test results. When the pollen grains were placed on filter paper and observed with the aim of a magnifying glass, it was not possible to distinguish the color of pollen grains, possibly because of the difficulty in placing a small amount of grains on a filter paper without interfering with the treatment, or by the small size of grains, which complicates their handling and visualization, or maybe because the magnifying glass does not provide an adequate increase in the observed material.

In the study comparing fresh and stored pollen grains in the tetrazolium solution of 0.75%, it was observed that the fresh pollen mass stained (carmine red color) when observed with the naked eye, which did not occur with the stored pollen mass. However, the quantification of the stained grains, through the use of a microscope, was not possible, because it was difficult to distinguish the colored and the uncolored individual pollen grains.

These results suggest that the mass eggplant pollen grains stain well in the tetrazolium solution at 0.75% when viable, however more studies are necessary to develop a methodology for the evaluation of the color of individual pollen grains through a microscope. A suggestion to improve the viability test of eggplant pollen grains using the tetrazolium test could be by pre-soaking the pollen grains in water before their exposure to the tetrazolium solution, similarly to the prescribed methodology for seed analysis (Brazil 1992). However, the size and the softness of the pollen grains make these treatments not as easy to handle.

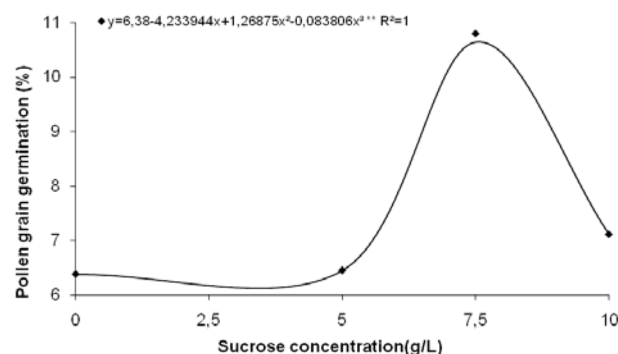

Figure 3. Percentage of *in vitro* germination of pollen grains of eggplant cultivar Ciça submitted to the culture medium of Brewbaker and Kwack with different concentrations of sucrose

Viabilidade polínica em berinjela

RESUMO - *Este estudo objetivou avaliar metodologias de viabilidade polínica em berinjela. Na germinação in vitro o pólen foi incubado (0,5; 16 horas) em diferentes concentrações de sacarose (0; 2,5; 5,0; 7,5 e 10,0 g L^{-1}). Na germinação in vivo o pólen após 4 e 24 horas em contato com o estigma foi pigmentado com azul de anilina e analisado em microscópio ultravioleta. Grãos de pólen foram colocados em diferentes concentrações de soluções de tetrazólio (1%; 0,75% e 0,5%) por 24 horas, analisando a coloração da massa de pólen. Na germinação in vitro foi observado germinação polínica máxima de 10,80%, sendo necessário a calibração do meio de cultura. A germinação polínica com a metodologia in vivo foi de 66% tornando esta metodologia valida para avaliação polínica de berinjela. A metodologia de coloração com tetrazólio não foi eficiente para avaliação polínica devido à dificuldade dos grãos entrarem em contato com a solução de tetrazólio.*

Palavras-chave: *Solanum melongena*, tetrazólio, germinação *in vivo*, *in vitro.*

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