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In Vitro GERMINATION OF TOBACCO (*Nicotiana tabacum* L.) POLLEN

Germinación *in vitro* del polen de tabaco (*Nicotiana tabacum* L.)

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ABSTRACT. Knowing the tobacco genotypes pollen viability during breeding programs is important. Culture media for *in vitro* pollen germination allows determining the germinative ability in a short period of time. To contribute to this knowledge, the chemical composition of a simple culture medium for *in vitro* germination of pollen grain was determined. The pollen grains *in vitro* germinations from seven tobacco genotypes: F_1 hybrids 'C-1', 'C-2'; 'Habana 2000' cv., 'Habana 92' cv., 'Corojo 99' cv., 'NC 1071' cv. and the breeding line 'Wz' were evaluated in the culture medium Brewbacker and Kwack (BK), SaCaBor(SCB), SaCaBorMag (SCBM) and SacaBorMag-PEG (SCBMP) through a random block experimental design with two replicates. The analysis of univariate factorial variance shows the highest general germination percentages were achieved in the solid medium SCB containing 150 mg L^{-1} H_3BO_3, 400 mg L^{-1} $CaCl_2$ and 15 % sucrose. No pollen grain and pollen tube destruction in any medium due to osmotic potential alterations were observed. The presence of PEG-6000 in the medium SCBMP did not have any significant effect on the germination whether comparing with the SCB medium. The effect of the tobacco genotypes and the culture media chemical composition over the *in vitro* pollen germination was evidentiated.

Key words: boron, calcium, culture medium, viability

RESUMEN. Es importante conocer la viabilidad del polen de los genotipos de tabaco durante los programas de mejora. Los medios de cultivo para la germinación *in vitro* de polen permiten determinar la capacidad germinativa de los mismos en un corto período de tiempo. Para contribuir a este conocimiento se determinó la composición química de un medio de cultivo simple para la germinación *in vitro* de granos de polen de tabaco. Se evaluó la germinación *in vitro* del polen de genotipos de tabaco: híbridos F_1 'C-1', 'C-2'; cultivares 'Habana 2000', 'Habana 92', 'Corojo 99', 'NC 1071' y la línea 'Wz' en los medios de cultivo Brewbaker y Kwack (BK), SaCaBor(SCB), SaCaBorMag(SCBM) y SaCaBorMag-PEG(SCBMP), mediante un diseño experimental completamente aleatorizado con dos repeticiones. El análisis de varianza factorial univariante de los resultados mostró que los mayores porcentajes generales de germinación se lograron en el medio sólido SCB que contiene 150 mg L^{-1} de H_3BO_3, 400 mg L^{-1} de $CaCl_2$ y 15 % de sacarosa. No se observó destrucción de los granos de polen o de los tubos polínicos por alteraciones del potencial osmótico en ninguno de los medios que se analizaron. La presencia de PEG-6000 en el medio SCBMP no tuvo un efecto significativo en la germinación con respecto al medio SCB o SCBM pero si en comparación con el medio BK. Se evidenció el efecto de los genotipos de tabaco y de la composición química de los medios de cultivo sobre la germinación *in vitro* de los granos de polen.

Palabras clave: boro, calcio, medio de cultivo, viabilidad

INTRODUCTION

In tobacco improvement programs (*Nicotiana tabacum* L.) in Cuba, it is necessary to know the viability and germination capacity of the pollen of the selected parents during the first stages of the program, on this, the occurrence of successful pollination events depends and therefore the obtaining of viable hybrid seeds for the tobacco production. Similarly, it is very important to evaluate in advance the pollen germination of the male parent in the programs to obtain seeds of commercial F1 hybrids of tobacco in the country. This ensures the highest production of quality seeds during the annual tobacco campaign to avoid delays in the sowing schedule according to the established tobacco policy.

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In the Institute of Tobacco Research (IIT), Cuba, tobacco pollen germination tests are carried out with the culture medium developed by Brewbaker and Kwack in 1963 for the *in vitro* germination of pollen from different species including *N. tabacum* (1). Sometimes it is difficult to have all the elements that make up the chemical composition of this culture medium, which means that many genotypes of tobacco are not determined their germinative capacity of pollen. Therefore, it is necessary to develop a culture method with a simple chemical composition, which allows reliably estimating the germinative capacity of tobacco pollen grains in the shortest possible time.

There are different methods to evaluate the viability of pollen. Among the most rapid and precise are staining with vital dyes and germination in artificial media. Staining tests have advantages as indicators of pollen viability, since they are faster and easier than pollen germination; however, they tend to overestimate the viability and the actual germinative power of pollen grains because they can stain non-viable pollen grains due to the presence of enzymes, starch and other substances. On the other hand, *in vitro* germination depends on the genotype, environmental conditions, pollen maturity, composition and pH of the culture medium, so it is necessary to determine the optimal conditions for pollen germination (2,3) .

In vitro germination allows reliable estimates of fertility (3). This technique simulates the development of the pollen tube in the stylar tissues, since the culture medium used resembles the mucilage of the stigma (4). The pollen viability study is used to improve plants of several species due to the ease, speed, low cost and reliability of the technique (4). The genotype of an individual comes from a male and a female gamete; therefore, the more viable the pollen, the greater the probability of obtaining different combinations of alleles and genetic variability (5).

Taking all the above into consideration, the present work proposed the following objectives: (a) to determine the chemical composition of a simple culture medium for the *in vitro* germination of tobacco pollen and, (b) to evaluate the *in vitro* germination of grains of pollen of tobacco genotypes that are grown in Cuba for use in genetic improvement programs.

MATERIALS AND METHODS

Vegetal material

They were selected 25 plants of five Black tobacco genotypes, the Cuban commercial cultivars: 'Corojo-99' (C-99), 'Habana-92' (H-92) and 'Habana-2000' (H-2000) and hybrids F1: 'C-1' and 'C-2'; and two of tobacco Virginia, the line 'Wz' (Wz) and the cultivar 'NC 1071' (NC).

All the plants were free of diseases and with the presence of prominent inflorescences at the beginning of flowering. At the time of the collection (8:00 a.m.-9: 00 a.m.), 80 flowers of each genotype that did not present any open anthers were chosen. These flowers were deposited in dry glass jars and immediately transferred to the laboratory to monitor the dehiscence of the anthers and collect the pollen. Soft bristle brushes were used to remove all the anther content and place it in Petri dishes, which were stored at 4 ^{0}C, in a dry and cool place until use.

In vitro germination of pollen

It was decided to evaluate the germination of tobacco pollen grains *in vitro* in four culture media. Three culture media developed in the IIT Genetics laboratory on the basis of the importance of the Ca^{2+}, B^{3+}, Mg^{2+} ions and of sucrose in the development of the pollen tube, the solid media: SaCaBor (SCB) and SaCaBorMag (SCBM) and the liquid medium SaCaBorMag-PEG 6000 (SCBMP). The culture medium of Brewbaker and Kwack (BK) (1) developed in 1963, of which its capacity for the germination of tobacco pollen is known, was taken as Control. The pH of the culture media was adjusted to a value of 5,52 ± 0,02 and its chemical compositions are shown in the Table. The media was sterilized in an autoclave at 121 °C for 15 minutes.

Table 1. Composition of culture media (n=4) to evaluate *in vitro* germination of pollen grains of genotypes (n=7) of tobacco (*Nicotiana tabacum* L.)

Medium	H_3BO_3 (mg L^{-1})	$Ca(NO_3)_2 \cdot 4H_2O$ (mg L^{-1})	$MgSO_4 \cdot 7H_2O$ (mg L^{-1})	$CaCl_2$ (mg L^{-1})	KNO_3 (mg L^{-1})	$C_{12}H_{22}O_{11}$ (%)	PEG-6000 (%)
BK*	100	300	200	--	100	10	--
SCB*	150	--	--	400	--	15	--
SCBM*	100	--	300	450	--	20	--
SCBMP	100	--	300	450	--	20	12,5

* For the solid media of Brewbaker and Kwack (BK), SaCaBor (SCB) and SaCaBorMag (SCBM) was added 1g of agar per 0,5 L of culture medium

The pollen grains were dispersed in Petri dishes containing 0,01 L of the culture media, capped and incubated in the light during 20 hours / 22 ^{0}C to maximize the exposure time of the pollen to the components of the culture media. Two replications per tobacco genotype for each one *in vitro* germination medium were performed. After this time, the germination of 200 ± 2 pollen grains was evaluated, which were randomly selected, with an inverted microscope (Zeiss, Germany), in different fields of observation. The percentage of germination was calculated as:

Germination (%)= germinated pollen grains / total selected grains

Germinated pollen grain was considered to be one whose pollen tube was twice as large as the diameter of the pollen grain (6).

Statistical analysis

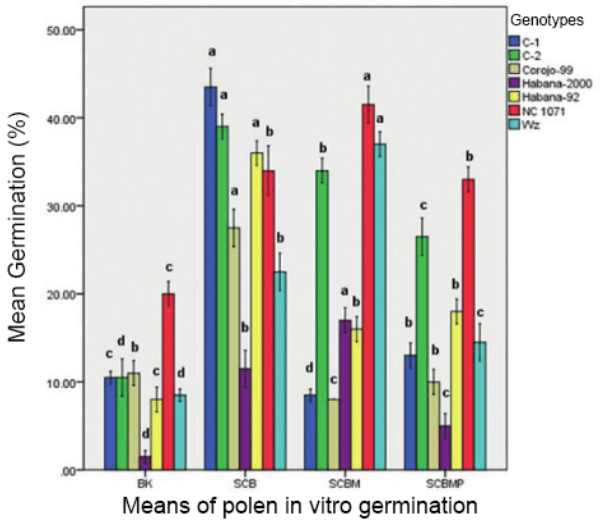
The study of *in vitro* germination was carried out by means of a completely randomized experimental design with two repetitions by means of culture. The Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to check the fit of the data to a normal distribution. The data were subjected to a univariate factorial variance analysis. The comparison of the means of the values obtained was done by the Kruskal-Wallis test.

Afterwards, not assuming homogeneity of variances, the post-hoc test of Games-Howell was carried out. The IBM® SPSS® Statistics Version 19 program (SPSS Inc., USA, 2010) was used to analyze the data.

RESULTS AND DISCUSSION

The *in vitro* germination results of the pollen grains for each culture medium, as well as for each tobacco genotype that was evaluated, showed a great variation. Figure 1 shows the results. A general analysis of germination makes it evident that the three culture media developed for this study allowed the *in vitro* germination of pollen in the greatest number of genotypes when compared to the BK culture medium.

The highest percentage of germination for a medium was achieved with the SCB culture medium, where the 'C-1' hybrid was the genotype with the highest germination (43,50 %); however, the SCB medium did not favor the pollen germination of the cultivars 'Habana-2000' and 'Wz'. One possible explanation for the greater germination of some tobacco genotypes in the SCB medium is that this culture medium offers sufficient consistency, concentration and supply of minerals for some genotypes, but not for the 'Habana-2000' and 'Wz' genotypes. This culture medium contains only Ca^{2+} and B^{3+} as mineral elements and sucrose as a nutritive element. Brewbacker and Kwack (1), demonstrated the importance of calcium in the process of development of the pollen tube *in vitro* to verify that the absence of this ion in the culture medium completely inhibits the germination of pollen grains of 86 species of 39 different families, including the genus *Nicotiana*.



In vitro germination media for pollen
The bars represent ME ± DV. In the graph: BK (Brewbaker-Kwack culture medium); SCB (SaCaBor culture medium); SCBM (SaCaBorMag culture medium) and SCBMP (SaCaBorMag-PEG culture medium). Different letters for each culture medium indicate significant differences ($p < 0.05$) between genotypes evaluated according to Games-Howell post-hoc test

Figure 1. Percentage of average germination of the genotypes (n=7) of tobacco (*Nicotiana tabacum* L.) after 20 hours of incubation in four culture media

The development of the pollen tube is dependent on the concentration of Ca^{2+}. An inhibition of the germination, as well as the elongation of the pollen tube is observed in Ca^{2+} concentrations less than 10 mg L^{-1} and above 1000/10,000 mg L^{-1} depending on the species (1,7). It is known that in the pollen grain the cytoplasmic concentration of Ca^{2+} increases in the potential germination site and remains high until the emergence of the pollen tube (8,9). Calcium is able to bind to the pectins of the walls of the pollen tube, an event that increases the rigidity of the cell wall and regulates the permeability of pollen cells and thus enhances the growth of the pollen tube without provoking a rupture of the membrane (10).

The SCB culture medium contains the highest concentration of H_3BO_3 (150 mg L^{-1}) of the four media analyzed, thus providing the highest amount of B^{3+} ions for germination and subsequent development of the pollen tube.

Boron is directly involved in the synthesis of pectins and helps in the synthesis of calosa (11), in this way it is indirectly involved in the development of the membrane of the pollen tube during its growth. It is believed that boron promotes pollen germination by affecting the activity of the H^+ -ATPase that participates in the process (11,12).

The SCBM culture medium favored the germination of the cultivar 'NC 1071' (41,50 %) and the 'Wz' line (37 %), both genotypes of Virginia-type tobacco. The presence of Mg^{2+} in this particular culture medium can contribute to enhance the germination of the Virginia tobacco genotypes over the rest of the Black tobacco genotypes. The specific requirements for the *in vitro* germination of pollen, sucrose, Ca^{2+}, B^{3+} or Mg^{2+} as well as other ions can vary among the tobacco genotypes evaluated, as evidenced in the results.

A means of pollen germination can be considered effective when maximum germination and minimum destruction of the grains is obtained, due to some alteration of the osmotic potential of the medium, for example. In this work, the SCB medium can be considered as an effective medium, although in the culture media BK, SCBM and SCBMP the destruction of the grains did not occur, according to observations under the microscope, the percentage of general germination in these was not greater than in the SCB culture medium. Furthermore, it is possible that the SCB culture medium provides germination conditions similar to the *in vivo* conditions of pollen grain germination and the development and elongation of the pollen tube in tobacco flowers for most of the genotypes that were evaluated.

Although the BK, SCBM and SCBMP media provide other important ions such as potassium, nitrates and magnesium, the essential role of the Ca2 + and B3 + ions is evident, as well as the presence of sucrose as a carbon source in the SCB culture medium. The culture media analyzed may not accurately reflect the environment surrounding the pollen in the stigma during the pollination event; however, the concentrations of nutritive elements in the SCB medium that are required to stimulate pollen germination may be similar or close to the concentrations under *in vivo* conditions for most of the tobacco genotypes that were evaluated.

An important aspect to be analyzed is the influence of the genotype per se on the *in vitro* germination of pollen grains. There are no relations of kinship among the set of genotypes, only between 'C-1' and 'C-2' and between 'Havana-2000' and 'Corojo-99'. The genotypes 'C-1' and 'C-2' correspond to F1 hybrids that were obtained through a reciprocal cross between their parents and it would be expected that their percentages of germination in the same culture medium would have similar values. However, the behavior of the hybrids in terms of germination is not uniform, with significant differences for the culture media SCB, SCBM and SCBMP. In general, the differences in pollen germination and pollen tube growth that were found in this study may be due to real differences in the intrinsic vigor of the pollen among the tobacco genotypes, as a consequence of the different genetic origin of the pollen each genotype. In particular, the high degree of heterozygosity of 'C-1' and 'C-2', a characteristic element of the F1 hybrids, may be the possible cause of variation between both genotypes.

Sucrose plays a nutritious role for pollen and variations in the effect of different concentrations of sucrose are associated with different osmotic potentials. This double role of sucrose is currently well accepted (3,13).

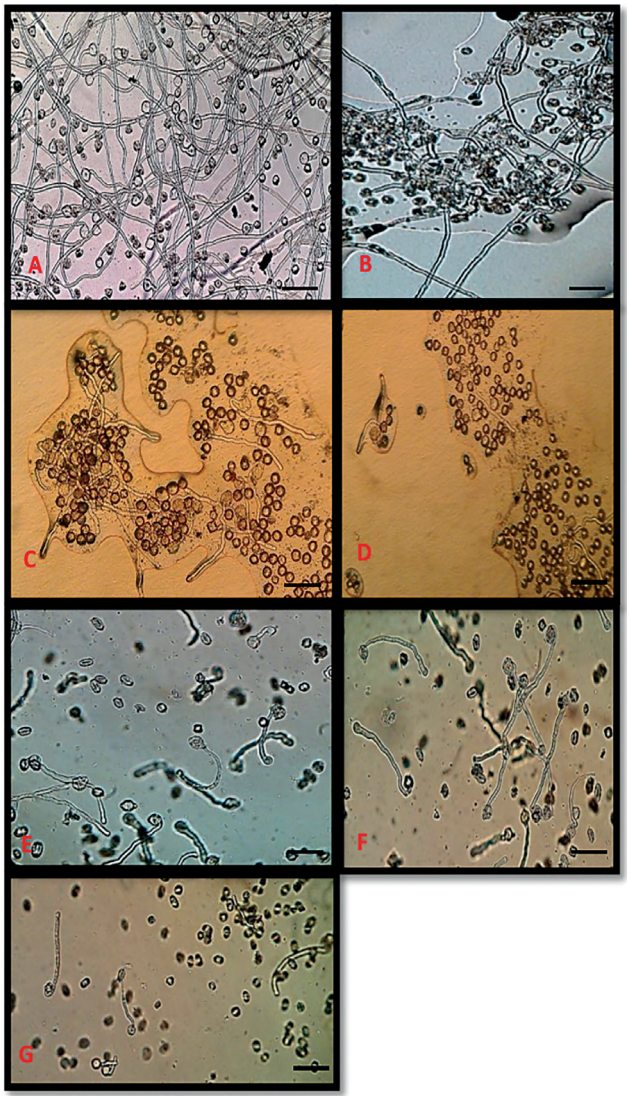
The concentrations of the different culture media ranged between 10-20 % and did not adversely affect the germination of the pollen, with an optimal concentration of 15 % for the SCB. Sucrose (15 %) in combination with boric acid (150 mg L^{-1}) in the SCB culture medium showed good results for germination. Boron can create a complex with sucrose and this borate-sugar complex is able to translocate through the membranes better than non-ionized sugar molecules (11).

The presence of polyethylene glycol-6000 (PEG-6000) in the SCBMP medium, in its function as an osmotic regulator not metabolized by pollen, it pursued the objective of avoiding the destruction of pollen grains, a very common event when there are alterations of the components of the *in vitro* germination medium (14), to check if its incorporation into the culture medium potentiated the germination of tobacco pollen. The osmotic potential of the medium with an approximate value of 800 mOsm was determined, where the initial concentration of sucrose has the greatest contribution to the osmotic potential (20 %, 584 mM) in relation to the concentration of PEG-6000 (12,5 %, 21 mM).

It was demonstrated that PEG is effective in the germination of pollen and its addition to the culture medium is recommended for different genera, such as *Brassica* (15), *Cicer* (16) and *Nicotiana* (17). The highest percentages of germination in this culture medium were obtained for the cultivar 'NC 1071' (33 %) and the hybrid 'C-2' (26,50 %) even though they were lower than their equivalent values in the medium of SCB crop.

In none of the culture media were observed morphological abnormalities in the pollen grains, destruction of the pollen tube or destruction of the apex of the pollen tube (Figure 2), all these effects are consequences of an osmotic shock due to alterations

or deficiencies in the concentrations of the ions or other osmotically active components in the culture medium (11,12). B3 + deficiency reduce the plastic extensibility of the cell wall and prevent normal cell elongation in growing plant tissues. It can also cause morphological abnormalities such as the destruction of the apices of the pollen tube that can increase the number of pollen grains that are destroyed with the consequent liberation of the cytoplasmic content to the exterior and a decrease of the germination rate (11,18).



A) 'C-1' hybrid; B) hybrid 'C-2'; C) cultivate 'Corojo-99'; D) cultivate 'Havana-2000'; E) cultivate 'Habana-92'; F) cultivate 'NC 1071'; G) line 'Wz'. Note the greater germination and longer length of the pollen tubes in the hybrid 'C-1' and the absence of morphological abnormalities in all genotypes. Magnification: 10X. The scale bars represent 40µm

Figure 2. *In vitro* germination of the genotypes (n=7) of tobacco (*Nicotiana tabacum* L.) in the SaCaBor culture medium after 20 hours of incubation

Concentrations in all culture media were maintained at acceptable and permissible levels for germination of pollen grains and proper development of the pollen tube. Of all genotypes, the 'C-1' hybrid showed the highest germination of the study (43, 50 %) in the SCB medium, with pollen tube lengths (not shown), which exceeded the length of the polar axis of pollen grains by four times the rest of the genotypes (Figure 2)

CONCLUSIONS

None of the three culture media that were developed allowed uniform germination of the tobacco genotypes. The highest general percentage of average germination of the pollen grains was obtained in the SCB culture medium (150 mg L^{-1} of H_3BO_3, 400 mg L^{-1} of $CaCl_2$ and 15 % of sucrose), only for five of the genotypes. The germination *in vitro* pollen grains are dependent on the intrinsic vigor of pollen between tobacco genotypes and the chemical composition of culture media.

BIBLIOGRAPHY

1. Brewbaker JL, Kwack BH. The essential role of calcium ion in pollen germination and pollen tube growth. American Journal of Botany. 1963;50(9):859–65. doi:10.2307/2439772
2. Singh RJ. Plant cytogenetics. EUA: Boca Raton; 2003. 463 p.
3. Chen J-C, Fang S-C. The long pollen tube journey and *in vitro* pollen germination of Phalaenopsis orchids. Plant Reproduction. 2016;29(1–2):179–88. doi:10.1007/s00497-016-0280-z
4. Rodriguez-Enriquez MJ, Mehdi S, Dickinson HG, Grant-Downton RT. A novel method for efficient *in vitro* germination and tube growth of Arabidopsis thaliana pollen. New Phytologist. 2013;197(2):668–79. doi:10.1111/nph.12037
5. Coelho APD, Morais KP, Dail Laughinghouse IV H, Giacomini SJ, Tedesco SB. Viabilidad del grano de polen en accesiones de *Crotalaria juncea* L. (Fabaceae). Agrociencia. 2012;46(5):481–7.
6. Silva LF de O da, Zambon CR, Pio R, Oliveira AF de, Gonçalves ED, Silva LF de O da, *et al*. Establishment of growth medium and quantification of pollen grains of olive cultivars in Brazil's subtropical areas. Bragantia. 2016;75(1):26–32. doi:10.1590/1678-4499.213
7. França LV, Nascimento WM, Carmona R, Freitas RA. Viability of eggplant pollen. Cropp breeding and applied biotechnology. 2009;9(4):320–7. doi:10.12702/1984-7033.v09n04a06
8. Soumitra S, Mondal S. An investigation on *in vitro* pollen germination and tube development of *Jacquinia ruscifolia* Jacq. International Journal of Advanced Research in Biological Sciences (IJARBS). 2016;3(9):71–7. doi:10.22192/ijarbs.2016.03.09.010
9. Vogler F, Schmalzl C, Englhart M, Bircheneder M, Sprunck S. Brassinosteroids promote Arabidopsis pollen germination and growth. Plant Reproduction. 2014;27(3):153–67. doi:10.1007/s00497-014-0247-x

10. Wu J, Qin X, Tao S, Jiang X, Liang Y-K, Zhang S. Long-chain base phosphates modulate pollen tube growth via channel-mediated influx of calcium. The Plant Journal. 2014;79(3):507–16. doi:10.1111/tpj.12576
11. Patel R, Mankad A. *In vitro* pollen germination- A review. International Journal of Science and Research. 2014;3(5):304–7.
12. Yu G-H, Zou J, Feng J, Peng X-B, Wu J-Y, Wu Y-L, *et al*. Exogenous γ-aminobutyric acid (GABA) affects pollen tube growth via modulating putative Ca^{2}+-permeable membrane channels and is coupled to negative regulation on glutamate decarboxylase. Journal of Experimental Botany. 2014;65(12):3235–48. doi:10.1093/jxb/eru171
13. Rottmann T, Zierer W, Subert C, Sauer N, Stadler R. STP10 encodes a high-affinity monosaccharide transporter and is induced under low-glucose conditions in pollen tubes of Arabidopsis. Journal of Experimental Botany. 2016;67(8):2387–99. doi:10.1093/jxb/erw048
14. Ischebeck T, Valledor L, Lyon D, Gingl S, Nagler M, Meijón M, *et al*. Comprehensive cell-specific protein analysis in early and late pollen development from diploid microsporocytes to pollen tube growth. Molecular & Cellular Proteomics. 2014;13(1):295–310. doi:10.1074/mcp.M113.028100
15. Shivanna KR, Sawhney VK. Polyethylene glycol improves the *in vitro* growth of Brassica pollen tubes without loss in germination. Journal of Experimental Botany. 1995;46(11):1771–4. doi:10.1093/jxb/46.11.1771
16. Golan-Goldhirsh A, Schmidhalter U, Müller M, Oertli JJ. Germination of *Pistacia vera* L. pollen in liquid medium. Sexual Plant Reproduction. 1991;4(3):182–7. doi:10.1007/BF00190002
17. Read SM, Clarke AE, Bacic A. Stimulation of growth of cultured *Nicotiana tabacum* W 38 pollen tubes by poly(ethylene glycol) and Cu(II) salts. Protoplasma. 1993;177(1–2):1–14. doi:10.1007/BF01403393
18. Moura CRF, Machado C de A, Lédo A da S. *In vitro* germination and viability of pollen grain of coconut accessions. Revista Ciência Agronômica [Internet]. 2015 [cited 2018 Feb 8];46(2). doi:10.5935/1806-6690.20150022

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