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# Effect of different concentrations of agar, sucrose, boric acid and calcium chloride on pollen germination in english walnut cultivar 'geisenheim 251'

**Dragan Janković[1*], Slađana Janković[1], Svetlana Paunović[2], Bratislav Ćirković[1], Zoran Nikolić[1]**

[1]*Faculty of Agriculture, University of Priština, Lešak, Serbia*

[2]*Fruit Research Institute, Čačak, Serbia*

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**Abstract**

The pollen of walnut cultivar 'Geisenheim 251' was germinated on media designed by combining different concentrations of sucrose (10, 15 and 20%), agar (0.6 and 0.8%), boric acid (0, 300 and 600 ppm) and calcium chloride (0, 50 and 100 ppm). A total of 54 combinations of these substances were tested in order to develop the most suitable medium for *in vitro* pollen germination tests in walnut. Significant differences in pollen germination rate in walnut cultivar 'G-251' were the result of varying concentrations of agar, sucrose, boric acid and calcium chloride in the germination medium. Strong and complex interactions were observed between the tested substances. Germination rate was maximized (42%) on the medium containing 0.6% agar, 15% sucrose, 300 ppm $H_3BO_3$ and 50 ppm $CaCl_2$.

* **Corresponding Author:** Dragan Janković ✉ draganjankovickv@gmail.com

## Introduction

English walnut (*Juglans regia* L.) is a wind-pollinated monoecious, herkogamous plant. Walnut trees are genetically self-fertile species, but in most walnut genotypes the period of pollen shedding from anthers does not completely overlap with the period of female flower receptivity (Polito, 1998). This is the main reason why a few selected genotypes should necessarily be placed in commercial walnut orchards to allow cross-pollination. In order to identify suitable pollinators, it is important to define reliable pollen viability testing methods (Cerović *et al.*, 1992). Moreover, understanding the functional ability of the pollen is of great significance to reproductive biology research and walnut breeding.

Among the different methods used to test pollen germination in plants, the following are of utmost importance: 1) *in vivo* test, involving monitoring pollen tube growth on the stigma and in the style; 2) *in vitro* test on an adequate germination medium; 3) staining the pollen with fluorescent dyes; 4) testing pollen for enzyme activity; 5) staining the pollen grain vegetative cell (Galletta, 1983; Kearns and Inouye, 1993). *In vivo* pollen germination test is considered the most authentic pollen viability test; however, it is time-consuming and requires the availability of adequate equipment. Different staining techniques yield rapid results, but they do not determine the actual germination of pollen, but rather the enzyme activity of pollen grains or their membrane integrity. *In vitro* test is the most commonly used pollen viability test in plant breeding programs, as it provides a rapid and simple estimation of actual pollen germination under defined conditions (Shivanna *et al.,* 1991; Tuinstra and Wedel, 2000; Dantas *et al.*, 2005).

The composition of the medium used for pollen germination can significantly affect pollen metabolism (Taylor and Hepler, 1997). Walnut pollen exhibits poor performance on artificial media; therefore, it requires boric acid and calcium, in addition to agar and sucrose (Griggs *et al.*, 1971).

In a experiment conducted by Luza and Polito (1985) the maximum pollen germination was obtained on a culture medium solidified with 0.65% agar. Increasing agar concentration in the medium resulted in a decline in pollen germination rate, with minimum germination occurring at 1.0% agar. Conversely, Sağlam and Gülcan (1995) and Blidariu *et al.* (2009) observed that 1% agar in the medium provided the optimum concentration for germination. In an experiment by QI Guo-hui *et al.* (2007), percentage germination of pollen from four walnut cultivars on germination media containing a low concentration of sucrose (5%) and a high concentration of boric acid (0.5%) was higher than at 5% sucrose and 1% boric acid. In a test conducted at 5%, 10%, and 15% sucrose in 1% agar media, Sütyemez (2007) obtained the highest germination rate at 10% sucrose concentration. Hall *et al.* (1971) found that media consisting of 0.5% agar, 20% sucrose and 100 ppm boric acid supported the highest percent germination, whereas WU Kai-zhi *et al.* (2008) reported optimum germination at 100 g $L^{-1}$ sucrose, 10 mg $L^{-1}$ boric acid and 40 mg $L^{-1}$ calcium chloride. These results are inconsistent and, therefore, they do not provide grounds for reliable conclusions as regards the optimum proportion of these components in pollen germination media for walnut. Hence the need to test various combinations of different concentrations of agar, sucrose, boric acid and calcium chloride in an attempt to analyze key aspects of the effects of these components and their interactions on walnut pollen germination.

The objective of this study was to examine the effects of different concentrations of agar, sucrose, boric acid and calcium chloride and their interactions on pollen germination in English walnut, and identify the optimum proportion of these components in germination media.

## Materials and methods

*Plant material*

This research was conducted in spring 2012 using pollen obtained from the German cultivar 'Geisenheim 251' (G-251) near Lazac, Western Serbia.

Pollen samples were collected between 8:00 and 10:00 a.m. at the time the catkins started to shed pollen. Catkin samples were collected from a number of trees, from different parts of the crown. Then, under laboratory conditions, they were placed on a piece of black paper to release pollen for three to four hours.

*Experimental design*

The pollen was germinated in Petri dishes 35 mm in diameter, each containing 3 ml agar-based germination medium. Apart from agar, the medium was supplemented with sucrose, boric acid and calcium chloride. The experiment was laid out in a four-factor design, with sucrose, agar, boric acid and calcium chloride as independent variables used at concentrations of 10, 15 and 20% for sucrose; 0.6 and 0.8% for agar; 0, 300 and 600 ppm for boric acid; and 0, 50 and 100 ppm for calcium chloride. Pollen germination was tested on 54 different media.

*Pollen germination procedure*

As recommended by Taylor (1972), prior to pollen culture, the Petri dishes containing the medium were kept for 24 hours at 4°C to allow the agar to solidify. This prevented the pollen from sinking into the medium, which otherwise may have hindered germination or led to pollen tube breakage due to overhydration. The pollen was deposited by a fine brush to ensure even distribution of pollen grains on the surface of the agar. The importance of even distribution of pollen over the agar was brought to attention by Giulivo and Ramina (1974), who found that agglomeration of pollen grains leads to higher pollen germination. Pollen was incubated at 22°C in the dark for 24 hours. Upon incubation, the Petri dishes were frozen at -18°C to hold back pollen tube growth and preserve the material until further evaluation. The day before microscopic observation, the dishes were thawed at 4°C (Hedhly *et al.*, 2005).

*Determination of pollen germination*

The dishes containing the cultured pollen were examined under a light microscope at 100x magnification to count germinated and non-germinated pollen grains. The pollen grains were considered germinated when the pollen tube length was greater than the grain diameter. Fifteen fields of view randomly selected from different parts of the Petri dish were examined per dish, with 20-50 pollen grains per field of view per replicate. About 400-600 pollen grains were observed per dish.

*Statistical analysis*

Data were subjected to analysis of variance, and differences between means were determined by Tukey's test at a probability level of 0.05.

**Results and discussion**

The total average pollen germination percentage in 'G-251' walnut was 13.5%. The germination rate was maximized (42.0%) on the medium composed of 0.6% agar, 15% sucrose, 300 ppm $H_3BO_3$ and 50 ppm $CaCl_2$. The analysis of variance for the experimental data revealed a significant effect of agar, sucrose, boric acid and calcium chloride and most of their interactions on pollen germination (Table 1).

*Effect of agar*

The average pollen germination of cv. 'G-251' was significantly higher on the media supplemented with 0.8% agar than on 0.6% agar solidified media. There is inconsistency in the related literature data on optimum agar concentrations in germination media for walnuts. Luza and Polito (1985) found that the agar concentration of 0.65% was the most suitable for pollen germination in 21 walnut cultivars. Cerović *et al.* (1992) reported the highest pollen germination on the medium containing 0.75% agar. Galletta (1983) obtained the highest percent germination on 1% agar medium. Agar content in a germination medium contributes to ensuring good hydration and germination of pollen grains in walnuts (Luza and Polito, 1985).

In the present experiment, the increase in agar concentration from 0.6% to 0.8 % promoted pollen germination on the media containing 10% and 15% sucrose, respectively, but had the opposite effect on those supplemented with 20% sucrose (Fig. 1-a).

Increasing agar concentrations were found to adversely affect pollen germination on the media without calcium chloride, while increasing pollen germination on the media containing calcium chloride (Fig. 1-b).

**Table 1.** Effect of agar, boric acid, sucrose and calcium chloride on in vitro pollen germination of English walnut cultivar *'G-251'*.

| Ingredient | Concentration | Germination (%) |
|---|---|---|
| Agar (A) | 0.6% | 12.2 a |
| | 0.8% | 14.7 b |
| $H_3BO_3$ (B) | 0 ppm | 7.3 a |
| | 300 ppm | 18.2 b |
| | 600 ppm | 14.8 c |
| $CaCl_2$ (C) | 0 ppm | 7.2 a |
| | 50 ppm | 18.4 b |
| | 100 ppm | 14.8 c |
| Sucrose (D) | 10% | 12.5 a |
| | 15% | 15.8 b |
| | 20% | 12.1 a |
| Average | | 13.5 |
| | ANOVA | |
| Factor | *p* | |
| Agar (A) | 0.003 | |
| $H_3BO_3$ (B) | 0.000 | |
| $CaCl_2$ (C) | 0.000 | |
| Sucrose (D) | 0.001 | |
| A×B | 0.994 | |
| A×C | 0.000 | |
| A×D | 0.014 | |
| B×C | 0.000 | |
| B×D | 0.000 | |
| C×D | 0.000 | |
| A×B×C | 0.652 | |
| A×B×D | 0.092 | |
| A×C×D | 0.022 | |
| B×C×D | 0.000 | |
| A×B×C×D | 0.001 | |

Mean values followed by the same letter within a column do not differ significantly according to Tukey's test at $P \leq 0.05$.

*Effect of boric acid*

Pollen germination rate was significantly higher on the media containing boric acid than on those without it. In terms of the average germination rate, the media containing 300 ppm boric acid were significantly superior to 600 ppm boric acid media. Wu *et al.* (2008) observed that the optimum boric acid concentration for the germination medium for cv. 'Yunxin' was 10 ppm. In contrast, Cerović *et al.* (1992) found no significant positive effect of supplementing germination media with boric acid on pollen germination in six cultivars tested.

The addition of boric acid had a significantly higher positive effect on pollen germination on the media containing 15% sucrose than on 10% and 20% sucrose media (Fig. 1 –c). Pollen germination on the media without calcium chloride was not significantly affected by boric acid, whereas the media supplemented with both boric acid and calcium chloride significantly improved pollen germination (Fig. 1-d). Pfahler (1967) observed that boron

combines with sucrose to form a sucrose-borate complex, which enhances sugar activation on membranes. Boron plays a major role in the synthesis of pectin as an essential component in pollen tube growth (Richards, 1986).

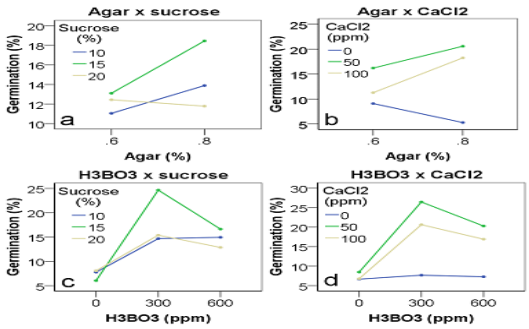


**Fig. 1.** Effects of agar x sucrose (a), agar x $CaCl_2$ (b), $H_3BO_3$ x sucrose (c) and $H_3BO_3$ x $CaCl_2$ (d) interactions in culture medium on *in vitro* pollen germination in English walnut cultivar *'G-251'*.

*Effect of calcium chloride*

Pollen germination was significantly improved on the media containing 50 ppm calcium chloride than on those containing 100 ppm or no addition of calcium chloride. The significantly lowest average pollen germination was obtained on the media without calcium chloride. These results are in agreement with the findings of Wu *et al.* (2008) who determined an optimum calcium chloride concentration of 40 ppm for pollen germination in cv. 'Yunxin'.

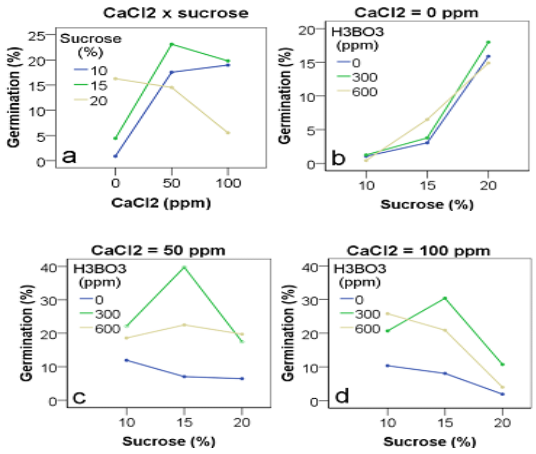


**Fig. 2.** Effects of $CaCl_2$ x sucrose (b) and sucrose x CaCl2 x $H_3BO_3$ (b, c, d) interactions in culture medium on *in vitro* pollen germination in English walnut cultivar *'G-251'*.

The results of the present study showed statistically significant interactions between calcium chloride and sucrose concentrations, as well as between calcium chloride, sucrose and boric acid concentrations. The addition of calcium chloride to 10% and 15% sucrose media resulted in a high pollen germination rate, whereas increasing calcium chloride concentration in 20% sucrose media decreased pollen germination (Fig. 2-a). Supplementing the media containing no calcium chloride with sucrose led to a high increase in pollen germination rate, regardless of boric acid concentration (Fig. 2-b), but the positive effect of sucrose addition decreased with increasing calcium chloride concentration in the medium, with sucrose adversely affecting pollen germination at maximum calcium chloride concentrations, particularly in combinations containing a higher content of boric acid (Fig. 2 – c, d).

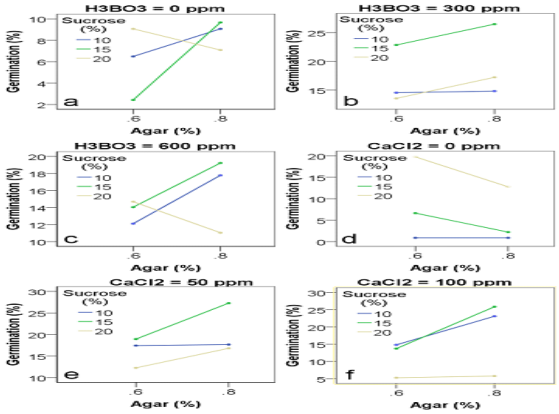


**Fig. 3.** Effects of three-way interactions among agar, sucrose and boric acid (a, b, c) and among agar, sucrose and calcium chloride (d, e, f) in culture medium on *in vitro* pollen germination in English walnut cultivar *'G-251'*.

*Effect of sucrose*

The highest average pollen germination rate was obtained on the media containing 15% sucrose. Differences in pollen germination rate on 10% and 20% sucrose media were not significant (Tab. 1). Sütyemez (2007) also found that a 15% sucrose concentration in germination medium gave the highest germination rates for walnut cultivars, but Wu *et al.* (2008) reported that the pollen germination rate in 'Yunxin' walnut was the highest at a sucrose concentration of 10%.

Osmotic equilibrium between the medium and pollen grain cytoplasm is critical to pollen grain integrity, and is governed by a proper concentration ratio of sucrose to substances such as boric acid and calcium. Excessive concentrations or a deficiency of any of these substances can prevent or hinder pollen grain germination (Silva *et al.*, 1999).

In the media without boric acid and in those containing 600 ppm boric acid, the high sucrose concentration (20%) combined with the high agar concentration (0.8%) gave a lower pollen germination rate than at an agar concentration of 0.6% in the media (Fig. 3 - a, c). The increased agar concentration in the media containing a medium boric acid concentration (300 ppm) had a positive effect on pollen germination even at a high sucrose concentration in the medium (Fig. 3-b).

The increase in agar concentration in the media without calcium chloride had an increasingly depressive effect on pollen germination with increasing sucrose concentration in the medium (Fig. 3-d), whereas a positive effect was observed on the media supplemented with calcium chloride (Fig. 3 – e, f).

**Conclusion**

Significant differences in pollen germination rate in walnut cultivar 'G-251' were the result of varying concentrations of agar, sucrose, boric acid and calcium chloride in the germination medium. Strong and complex interactions were observed between the tested substances. Germination rate was maximized (42%) on the medium containing 0.6% agar, 15% sucrose, 300 ppm $H_3BO_3$ and 50 ppm $CaCl_2$.

**References**

**Blidariu A, Iordanescu OA. Micu RE, Blidariu C.** 2009. Research concerning the pollen germination of some nut tree byotypes in Sannicolau Mare. Journal of Horticulture, Forestry and Biotechnology **13,** 302-305.

**Cerović S, Korać M, Ninić-Todorović J.** 1992. Klijavost i čuvanje polena domaćeg oraha (*Juglans regia* L.). Jugoslovensko Voćarstvo **26,** 17-22.

**Dantas ACDM, Peixoto ML, Nodari RO, Guerra MP.** 2005. Viabilidade do pólen e desenvolvimento do tubo polínico em macieira (*Malus* spp). Revista Brasileira de Fruticultura **27,** 356-359. http://dx.doi.org/10.1590/S010029452005000300005.

**Galletta GJ.** 1983. Pollen and seed management. In: Moore JN, Janick J, ed. Methods in fruit breeding. West Lafayette, Indiana, USA: Purdue University Press, 23-47 p.

**Giulivo C, Ramina A.** 1974. Effetto di massa ed azione del calcio sulla germinazione del polline di alcune specie arboree da frutto. Rivista Ortoflorofrutticoltura Italiana **58,** 3-13.

**Griggs WH, Forde HL, Iwakiri BT, Asay RN.** (1971): Effect of subfreezing temperature on the viability of Persian walnut pollen. HortScience **6,** 235-237. http://dx.doi.org/101111/j.13653040.1987.tb01826.x

**Hall GC, Farmer RE.** 1971. In vitro germination of black walnut pollen. Canadian Journal of Botany **49,** 799-802.

**Hedhly A, Hormaza JI, Herrero M.** 2005. Influence of genotype–temperature interaction on pollen performance. Journal of Experimental Biology **18,** 494-1502. http://dx.doi.org/10.1111/j.1420-9101.2005.00939.x

**Kearns CA, Inouye DW.** 1993. Techniques for pollination biologists. University Press of Colorado, Niwot, Colorado, USA, 98 p. http://dx.doi.org/10.2307/1312388

**Luza JG, Polito VS.** 1985. In vitro germination and storage of English walnut pollen. Scientia Horticulturae **27**, 303-316. http://dx.doi.org/10.1016/0304-4238(85)90035-4

**Pfahler PL.** 1967. In vitro germination and pollen tube growth of maize (*Zea mays* L.) pollen: I. Calcium and boron effects. Canadian Journal of Botany **45**, 839-845. http://dx.doi.org/10.1007/BF00385108.

**Polito VS.** 1998. Floral biology: flower structure, development, and pollination. In: Ramos DE, ed. Walnut production manual. Oakland, California, USA: University of California, 127-132 p.

**Qi Guo-hui, Zhang Jing-lan, Guo Jun, Zheng Hui, Li Bao-guo, Guo Su-ping, Liu Li-hua.** 2007. Hebei Journal of Forestry and Orchard Research **22,** 54-56. http://dx.doi.org/CNKI:ISSN:1007-4961.0.2007-01-014.

**Richards AJ.** 1986. Plant breeding systems. George Allen & Unwin, London, England.

**Sağlam H, Gülcan R.** 1995. Bazi meyve türlerinde çiçek tozu saklama yöntemleri Turkiye II. Ulusal Bahçe Bitkileri Kongresi, Cilt I, 229-232.

**Shivanna KR, Linskens HF, Cresti M.** 1991. Pollen viability and pollen vigor. Theoretical and Applied Genetics **81**, 38-42. http://dx.doi.org/10.1007/BF00226109.

**Silva MM**, **Brucner CH**, **Picanço M**, **Cruz CD**. 1999. Fatores que afetam a germinação do grão de pólen do maracujá: meios de cultura e tipos de agrotóxicos. Pesquisa Agropecuária Brasileira **34,** 347-352. http://dx.doi.org/10.1590/S0100204X1999000300004

**Stanley RG, Linskens HF.** 1974. Pollen: biology, biochemistry, management. Springer Verlag, Berlin, Heidelberg, New York.

**Sütyemez M.** 2007. Determination of pollen production and quality of some local and foreign walnut genotypes in Turkey. Turkish Journal of Agriculture and Forestry **3,** 109-114.

**Taylor RM.** 1972. Germination of cotton (*Gossypium hirsutum* L.) pollen on the artificial medium. Crop Science **12,** 243-244.

**Taylor PL, Hepler PK.** 1997. Pollen germination and tube growth. Annual review of plant physiology and plant molecular biology **48,** 461-491. http://dx.doi.org/10.1146/annurev.arplant.48.1.461

**Tuinstra MR, Wedel J.** 2000. Estimation of pollen viability in grain sorghum. Crop Science **40,** 968-970. http://dx.doi.org/10.2135/cropsci2000.404968x

**Vergano G, Radicati L, Martino I.** 1990. Investigations on viability and germinability of English walnut pollen. Acta Horticulturae **284,** 285-293.

**Wu KZ, Xiao QW, Liao YH, Zhou LY, Pu GL.** 2008. Study on culture medium for walnut pollen germination in vitro. Journal of Fruit Science **6,** 941-945.