International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 4, No. 4, p. 207-213, 2014

INNSPUB

RESEARCH PAPER OPEN ACCESS

Genotypic specificity of walnut (*Juglans regia* L.) pollen germination on different germination media

Dragan Jankovic[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*

Article published on April 29, 2014

Key words: *Juglans regia*, genotypes, pollen, germination medium

Abstract

The pollen of three walnut cultivars ('Geisenheim 139', 'Geisenheim 251' and 'Elit') was germinated on the culture media designed by combining different concentrations of agar (0.6 and 0.8%), sucrose (10, 15 and 20%), boric acid (0, 300 and 600 ppm) and calcium chloride (0, 50 and 100 ppm). A total of 54 combinations of these substances for each of the cultivars were tested in order to develop the most suitable medium for *in vitro* pollen germination tests in walnut. Walnut pollen germination was significantly affected by genotype and agar, sucrose, boric acid and calcium chloride concentrations in the medium. The average pollen germination percentage was the highest (13.4%) in cv. 'Geisenheim 251' and the lowest (6.8%) in cv. 'Geisenheim 139'. Significant interactions were observed between the germination medium components, as well as between cultivars and germination medium. The optimum germination medium for 'Geisenheim 139' pollen contained 0.6% agar, 20% sucrose, 600 ppm boric acid and 0% calcium chloride. The pollen of cvs. 'Elit' and 'Geisenheim 251' gave maximum germination on the medium containing 0.6% agar, 15% sucrose, 300 ppm boric acid and 50 ppm calcium chloride.

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

Introduction

In vitro pollen germination is the most widely used method of pollen viability testing in breeding programs (Marcellán and Camadro, 1996) and it determines the actual germination ability of pollen under suitable conditions (Shivanna *et al.*, 1991; Dantas *et al.*, 2005; Tuinstra and Wedel, 2000). *In vitro* pollen germination is affected by several factors, such as species, culture medium, temperature and time of incubation, and flower development stage at the time of sampling, in addition to storage conditions (Stanley and Linskens 1974). 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).

There is still no general agreement as to the optimum content of ingredients in pollen germination media for walnuts. In an experiment conducted by Luza and Polito (1985), maximum walnut 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), the 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 *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.

Pfahler (1967), Koncalova (1975), Luza *et al.* (1987), Cheng and McComb (1992), Bolat and Pirlak (1999), Rosell *et al.* (1999), Franzon *et al.* (2005) and Ercisli (2007) proved that cultivars within species respond differently to both the composition of the germination medium and germination conditions. Vergano *et al.* (1990) found that calcium chloride supplementation increased pollen germination in some walnut cultivars, but decreased it in others. Hall and Farmer (1971) observed that the addition of boric acid to the agar-based medium did not improve pollen germination in all black walnut genotypes analyzed (*Juglans nigra* L.). Using the hanging drop method to test walnut pollen germination, Sütyemez (2007) obtained higher germination rates at 15% sucrose concentration than at 10% sucrose in 30 cultivars, whereas the opposite result was reported for two cultivars.

Apart from being quite inconsistent, these results do not provide an explanation of the importance and nature of the interactions occurring between genotype, germinating pollen and culture medium components. Therefore, a trial including all combinations of different concentrations of agar, sucrose, boric acid and calcium chloride should necessarily assess important aspects of the effect of these ingredients and their interactions on pollen germination in a number of walnut cultivars.

The objective of this study was to evaluate the effect of different concentrations of agar, sucrose, boric acid and calcium chloride and their interactions on pollen germination in three walnut cultivars, and determine the optimum composition of the germination medium.

Materials and methods

Plant material

This study was carried out in 2013 in a walnut orchard near Lazac (Central Serbia). The experiment involved *two German cultivars,* 'Geisenheim 139' ('G-139') and 'Geisenheim 286' ('G-286'), and the Slovenian cultivar 'Elit'. 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 experiment was laid out as a 2 x 3 x 3 x 3 x 3 factorial design, with agar, sucrose, boric acid and calcium chloride concentrations and cultivar as independent variables. The following concentrations were used: agar – 0.6%, 0.8%; sucrose - 10%, 15% and 20%; H_3BO_3 - 0 ppm, 300 ppm and 600 ppm; and $CaCl_2$ – 0 ppm, 50 ppm and 100 ppm. A total of 54 combinations of germination media were tested for each of the three cultivars.

Pollen germination procedure

The germination tests were conducted in 35-mm sterile Petri dishes, each containing 3 ml of the germination medium. A fine paint brush was used to deposit the pollen on the surface of the agar in order to ensure uniform distribution of the material. The Petri dishes containing the pollen were incubated at 22°C under dark conditions. The pollen germination was terminated after 24 h by freezing the dishes at –18°C *in order to preserve the samples until further evaluation*. The day before microscopic observation, the frozen Petri dishes were thawed at 4°C.

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. Each field of view was considered as one replication. About 400-600 pollen grains were observed per dish.

Statistical analysis

Data were subjected to a five-way 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 was 10.6%. Pollen germination was significantly affected not only by cultivar, but also by agar, sucrose, boric acid and calcium chloride concentrations in the germination medium. Significant interactions were observed between all independent variables tested (Tab. 1).

Table 1. Effect of agar, boric acid, sucrose and calcium chloride on *in vitro* pollen germination in walnut cultivars 'G-139', 'G-251' and 'Elit'.

Ingredient	Concentration	Germination (%)
Agar (A)	0.6%	10.0 a
	0.8%	12.4 b
H_3BO_3 (B)	0 ppm	5.5 a
	300 ppm	15.3 b
	600 ppm	11.0 c
$CaCl_2$ (C)	0 ppm	6.8 a
	50 ppm	14.4 b
	100 ppm	10.6 c
Sucrose (D)	10%	10.0 a
	15%	13.7 b
	20%	8.2 a
Cultivar (E)	G-139	6.8 a
	G-251	13.4 b
	Elit	11.5 b
Average		10.6
	ANOVA	
Factor		*p*
Agar (A)		0.003
H_3BO_3 (B)		0.000
$CaCl_2$ (C)		0.000
Sucrose (D)		0.000
Cultivar (E)		0.000
A*B		0.002
A*C		0.000
A*D		0.000
A*E		0.018
B*C		0.000
B*D		0.000
B*E		0.000
C*D		0.000
C*E		0.004
D*E		0.000
A*B*C		0.015
A*B*D		0.000
A*B*E		0.000
A*C*D		0.000
A*C*E		0.003
A*D*E		0.119
B*C*D		0.000
B*C*E		0.000
B*D*E		0.000
C*D*E		0.000
A*B*C*D		0.000
A*B*C*E		0.000
A*B*D*E		0.000
A*C*D*E		0.308
B*C*D*E		0.000
A*B*C*D*E		0,000

*Means followed by the same letter are not significantly different

Effect of cultivar

The pollen of cv. 'G-139' showed the highest germination percentage (21.2%) on the medium containing 0.6% agar, 20% sucrose, 600 ppm boric acid and no addition of calcium chloride. A similar germination percentage (20.2%) was obtained on the medium supplemented with 0.8% agar, 15% sucrose, 600 ppm boric acid and 50 ppm calcium chloride. The highest pollen germination percentage in cv. 'G-251' (42.1%) resulted from 0.6% agar, 15% sucrose, 300 ppm boric acid and 50 ppm calcium chloride, whereas the medium containing 0.8% agar, 15% sucrose, 300 ppm boric acid and 100 ppm calcium chloride gave a somewhat lower germination percentage (39.2%). The maximum pollen germination of 51.4% in cv. 'Elit' was obtained on the medium supplemented with 0.6% agar, 15% sucrose, 300 ppm boric acid and 50 ppm calcium chloride. Sütyemez (2007) reported that the pollen germination rate in 32 different walnut cultivars ranged from 33.28% to 50.19%. Cerović *et al.* (1992) tested pollen germination in six English walnut cultivars on the agar-solidified medium supplemented with sucrose, boric acid and calcium chloride, and reported a range of 73.5%-80.9% for pollen germination across the cultivars.

Effect of agar

The total average pollen germination on the media supplemented with 0.8% agar was significantly higher than on 0.6% agar solidified media. The higher agar concentration in the medium promoted pollen germination in cvs. 'G-251' and 'Elit'. Conversely, pollen germination in cv. 'G-139' was somewhat higher on 0.6% agar based medium. 'G-251' pollen showed the strongest response to varying agar concentrations in the medium (Fig. 1-a). Luza and Polito (1985) obtained the highest pollen germination on 0.65% agar medium. According to Cerović *et al.* (1992), pollen germination declined with increasing agar concentration, and was minimum at 1% agar.

Effect of sucrose

The highest pollen germination percentage in each cultivar was observed on the media containing 15% sucrose. Differences in pollen germination rate in cvs. 'G-139' and 'G-251' on 10% and 20% sucrose media were not significant, whereas cv. 'Elit' exhibited significantly higher pollen germination on 10% sucrose media than on the media containing the highest sucrose concentrations (Fig. 1-b). Sutyemez (2007) obtained a higher percentage of pollen germination on 1% agar medium supplemented with 15% sucrose than at 5% and 10% concentrations, and suggested that sucrose concentration in the medium is an important factor contributing to pollen germination. In a study by Vergano *et al.* (1990), the sucrose content (20-25%) of the media used for germination studies did not affect germinability. Mert (2009) reported different effects of sucrose concentrations on pollen germination percentage, but the differences were non-significant among the cultivars. Sugar is used to provide osmotic equilibrium between pollen and the germination medium, as well as an energy source to assist the pollen development process (Stanley and Linskens, 1974).

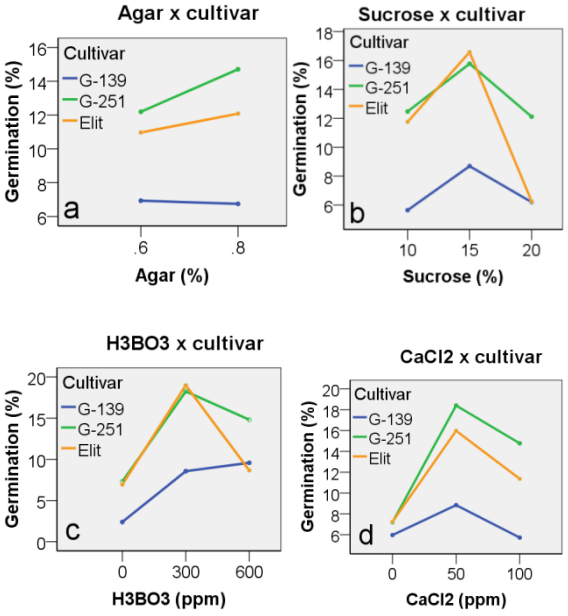

Fig. 1. Effects of some two-way interactions between culture medium ingredients and cultivar on *in vitro* pollen germination in walnut.

Effect of boric acid

The average pollen germination rate was significantly higher on the media containing boric acid than on those without it. The boric acid concentration of 300 ppm was the most suitable for pollen germination in cvs. 'Elit' and 'G-251', and that of 600 ppm gave maximum pollen germination in cv. 'G-251'. The high boric acid concentration was the least suitable for

'Elit' pollen (Fig. 1-c). According to Luza and Polito, (1985), small amounts of boron added to the culture medium improve germination, pollen tube growth and reduce the probability of their disruption. Janković *et al.* (2013) reported significantly higher germination rates of walnut cv. 'Ibar' at 200 ppm boric acid concentration than on media containing 400 ppm or no addition of boric acid. Wu *et al.* (2008) found that the optimal culture medium for 'Yunxin' walnut pollen contained 10 mg/L boric acid. Hall and Farmer (1971) observed an increase in germination percentage in two genotypes of *Juglans nigra* after addition of 100 ppm boric acid to the agar-based medium, while the performance of another genotype was independent of boron presence or absence. Adding boron to the culture medium stimulates pollen tube growth, giving rise to a sugar-borate complex, which can act more rapidly on the cell membranes (Pfahler 1967).

Effect of calcium chloride

Pollen germination in all cultivars was the highest on the media containing 50 ppm calcium chloride. In cvs. 'Elit' and 'G-251', pollen germination was significantly improved on the media supplemented with 100 ppm calcium chloride than on those without calcium chloride. The pollen of cv. 'G-139' exhibited slightly lower germination at 100 ppm calcium chloride concentration than on calcium chloride-deficient media (Fig. 1-d). Wu *et al.* (2008) noted that optimal culture medium contained 40 mg/L calcium chloride. Janković *et al.* (2013) reported that adding calcium chloride to the substrate generally has a significant positive effect on pollen germination, but Vergano et al. (1990) found that calcium chloride increased pollen germination in some walnut cultivars, while decreasing it in others.

Agar x sucrose x cultivar interaction

The high agar concentration in 10% sucrose media enhanced pollen germination in all cultivars, but the germination percentage decreased on 20% sucrose media. The increase in agar concentration from 0.6% to 0.8% on 15% sucrose media had a positive effect on pollen germination in cvs. 'G-251' and 'Elit', and an adverse effect in cv. 'G-139' (Fig. 2-a,b,c).

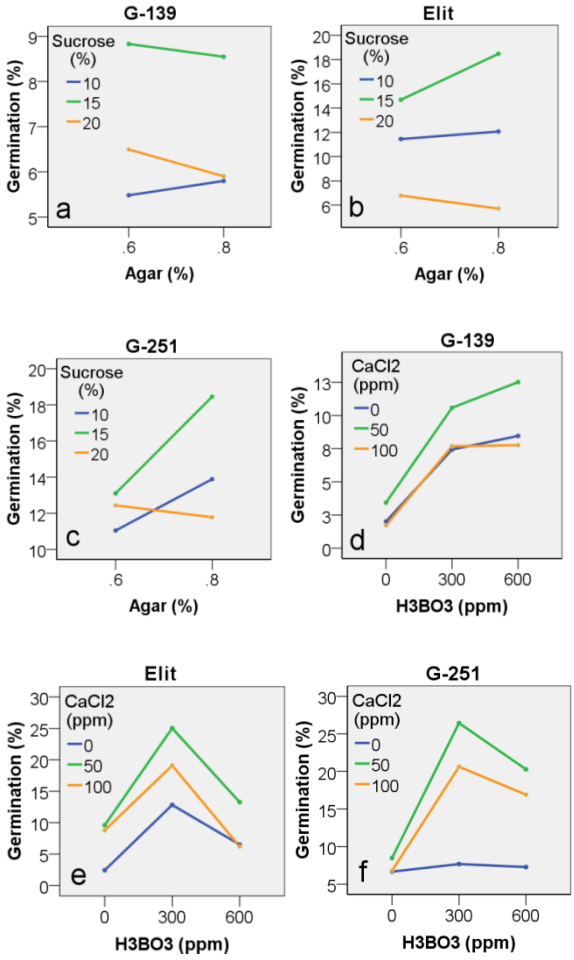

Fig. 2. Effects of three-way interactions between agar, sucrose and cultivar (a, b, c) and between boric acid, calcium chloride and cultivar (d, e, f) on *in vitro* pollen germination in walnut.

Boric acid x calcium chloride x cultivar interaction

The pollen of cvs. 'G-251' and 'Elit' exhibited the highest germination rate on 300 ppm boric acid media regardless of the calcium chloride content. The change in boric acid concentration in the medium without calcium chloride addition had a significantly lower effect on pollen germination in cv. 'G-251' than in the other two cultivars. Pollen germination in cv. 'G-139' was maximum on 600 ppm boric acid media, regardless of calcium chloride concentration. In this cultivar, the change in the boric acid content of the media containing the highest concentration of calcium chloride (100 ppm) produced an effect on pollen germination very similar to that obtained on the media without calcium chloride.

Conclusion

Pollen germination in walnut was significantly affected by genotype and agar, sucrose, boric acid and calcium chloride concentrations in the germination medium. The average pollen germination percentage was the highest (13.4%) in cv. 'Geisenheim 251' and the lowest (6.8%) in cv. 'Geisenheim 139'. Significant interactions were observed between the medium components and the cultivars. The optimum germination medium for 'Geisenheim 139' pollen contained 0.6% agar, 20% sucrose, 600 ppm boric acid and 0% calcium chloride. The pollen of cvs. 'Elit' and 'Geisenheim 251' gave maximum germination on the medium containing 0.6% agar, 15% sucrose, 300 ppm boric acid and 50 ppm calcium chloride.

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

Bolat I, Pirlak L. 1999. Effects of some chemical substances on pollen germination and tube growth in apricot. Acta horticulturae (ISHS) **488**, 341-344.

Cerović S, Korać M, Ninić-Todorović J. 1992. Germination and storage of English walnut (*Juglans regia* L.) pollen. Jugoslovensko Voćarstvo **26**, 17-22 (in Serbian).

Cheng CH, McComb JA. 1992. In vitro germination of wheat pollen on raffinose medium. New Phytologist **120**:459–462.

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.

Ercisli S. 2007. Determination of pollen viability and *in vitro* pollen germination of *Rosa dumalis* and *Rosa villosa*. Bangladesh Journal of Botany **36**, 185–187.

Franzon RC, Corrêa ER, Raseira MC B. 2005. In vitro pollen germination of feijoa (Acca sellowiana (Berg) Burret). Crop Breeding and Applied Biotechnology **5**, 229-233.

Griggs WH, Forde HL, Iwakiri BT, Asay RN. 1971. Effect of subfreezing temperature on the viability of Persian walnut pollen. HortScience **6**, 235-237.

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

Janković D, Janković S, Ćirković B, Paunović G, Paunović S. 2013. The effect of different germination media on pollen germination in vitro of the walnut (*Juglans regia* L.) cultivar 'Ibar'. International Journal of Agronomy and Agricultural Research **12**, 61-66.

Koncalova MN. 1975. Studies in *Rosa* pollen. I. *In vitro* germination of pollen grains of *Rosa hugonsis*. Preslia **47**, 22-25.

Luza JG, Polito VS. 1985. In vitro germination and storage of English walnut pollen. Scientia Horticulturae **27**, 303–316.

Luza JG, Polito VS, Weinbaum SA. 1987. Staminate bloom date and temperature responses of pollen germination and tube growth in two walnut (*Juglans*) species. American Journal of Botany **74,** 1898–1903.

Marcellán ON, Camadro EL. 1996. The viability of *Asparagus* pollen after storage at low temperatures. Scientia Horticulturae **67**, 101-104.

Mert C. 2009. Temperature Responses of Pollen Germination in Walnut (*Juglans regia* L.). *Journal of Biological & Environmental Sciences* **3**, 37-43.

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.

Qi Guo-hui, Zhang Jing-lan, Guo Jun, Zheng Hui, Li Bao-guo, Guo Su-ping, Liu Li-hua. 2007. Study on pollen vitality of different cultivars of walnut (*Juglans regia* L.). Hebei Journal of Forestry and Orchard Research **22**, 54-56.

Rosell P, Herrero M, Galán Saúco V. 1999. Pollen germination of cherimoya (*Annona cherimola* Mill.). *In vivo* characterization and optimization of *in vitro* germination. Scientia Horticulturae **81,** 251-265.

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.

Stanley RG, Linskens HF. 1974. Pollen: biology, biochemistry, management. Springer Verlag, Berlin, Heidelberg, New York, p. 307.

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 PL, Hepler PK. 1997. Pollen germination and tube growth. Annual review of plant physiology and plant molecular biology **48**, 461–491.

Tuinstra MR, Wedel J. 2000. Estimation of pollen viability in grain sorghum. Crop Science **40**, 968-970.

Vergano G, Radicati L, Martino I. 1990. Investigations on viability and germinability of English walnut pollen. Acta Horticulturae (ISHS) **284**, 285-296.

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