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A STUDY OF DIFFERENT GERMINATION MEDIA FOR THE 'ŠAMPION' WALNUT CULTIVAR POLLEN

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Abstract

Different culture media were tested in an attempt to improve the knowledge of the most suitable germination media for studying the in vitro pollen germination capacity of 'Šampion', a Serbian walnut cultivar. The research was conducted on agar media, as a four-factorial experiment, with different concentrations of agar (0.6%, 0.8% and 1%), sucrose (10%, 15% and 20%), H_3BO_3 (0 ppm, 200 ppm and 400 ppm) and $CaCl_2$ (0 and 50 ppm). Pollen germination was maximized (39%) when the germination medium contained 0.8% agar, 15% sucrose, 400 ppm H_3BO_3 and 50 ppm $CaCl_2$. Large and significant differences in pollen germination were observed in response to changing concentrations of agar, sucrose, boric acid and calcium chloride, and strong interaction was identified between all substances used.
Keywords: walnut, pollen, germination medium.

Introduction

Information on pollen viability and germination is important for the study of the reproductive biology of the walnut and for the development of its genetic crop program. Various methods can be used for estimation of pollen viability and germinability in horticultural crops. Two basically different approaches can be taken to estimate pollen viability: staining pollen with dyes and *in vitro* germination assay. Staining techniques aim to determine pollen enzymatic activity and membrane integrity. *In vitro* germination determines the actual germination ability of pollen under suitable conditions (Shivanna et al., 1991.; Dantas et al., 2005; and Tuinstra and Wedel, 2000), and it is the most widely used method of testing pollen viability in breeding programs (Marcellán and Camadro, 1996). The composition of the germination medium can dramatically affect pollen metabolism (Taylor, Hepler 1997). Different culture media for the *in vitro* germination of pollen grains have been reported for a large number of species, with considerable variations within and among species (Pfahler et al. 1997). However, there is no reliable information about the ideal culture medium for *in vitro* testing of the walnut pollen viability. Pollen of walnut has been considered difficult to germinate *in vitro* and it requires sucrose, boron and calcium as the necessary components of the culture medium for germination and pollen tube growth (Griggs et al. 1971).

The objective of this study was to test the effect of different contents of agar, sucrose, boric acid and $CaCl_2$ in germination medium on 'Šampion' walnut pollen germination *in vitro*.

Material and Methods

This study was carried out in the year 2010 on walnut cultivar ‘Šampion’. Samples of pollen were collected near Kraljevo (central Serbia) in the morning, between 8:00 and 9:00 a.m., at the time when the first staminate flowers of the catkins had begun to shed their pollen grains. Catkins were brought into the laboratory and laid on clean black paper. The catkins were kept under the laboratory conditions to shed their pollens for 1-2 hours. The experiment was set up as a 3 x 3 x 3 x 2 factorial design with concentrations of agar, sucrose, boric acid and calcium chloride as independent variables. Concentration ranges investigated were: agar – 0,6%, 0,8% and 1%, sucrose - 10%, 15% and 20%, H_3BO_3 - 0 ppm, 200 ppm and 400 ppm and $CaCl_2$ - 0 and 50 ppm). A total of 54 combinations of germination media were tested. The pollen samples were germinated in 35-mm sterile Petri dishes, each containing three ml of prepared germination medium. Before the deposition of the pollen onto the agar, the Petri dishes with agar are needed to be aged for at least 24 hours. If this is not done, the pollen grains tend either to sink into the agar, where it will not germinate, or to take up excessive moisture and rupture (Taylor, 1972). A fine paint brush was used to deposit the pollen on the surface of the agar in a Petri dish in order to promote a uniform distribution of the material. This is important, as agglomeration of pollen grains results in higher pollen germination (Giulivo and Ramina, 1974). The Petri dishes planted with pollen were incubated at 22^{o}C in dark conditions. Pollen germination was arrested after 24 h by immediate freezing at −20°C. This procedure has been shown to be a highly efficient method to arrest pollen germination while preserving the material for further evaluation. One day before observation under the microscope, the frozen Petri dishes were thawed at 4°C (Hendley et al., 2005). Pollen germination was observed using an optical microscope at a 100x magnification, with approximately 20-50 pollen grains per field. The number of pollen grains counted per dish was approximately 400-600. A pollen grain was considered to be germinated when the length of pollen tube was equal to or exceeded its diameter. Fifteen different fields of vision were examined per dish. Each count was considered as one replicate. Germination percentage was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen grains per field of view. A four-way analysis of variance was performed. Means were separated by Tukey’s multiple range test at $P \leq 0,05$.

Results and discussion

The total average germination percentage of the ‘Šampion’ walnut pollen was 16,4%. The germination rate was maximized (39%) when the germination medium contained 0.8% agar, 15% sucrose, 400 ppm H_3BO_3 and 50 ppm $CaCl_2$. The analysis of variance of experimental data showed the significative effects on pollen germination of agar, sucrose, boric acid, calcium chloride and their interactions (Table 1).

Effect of agar

Pollen of walnut cultivar ’Šampion’ germinated significantly better on the media containing 0.8% agar (19,1%) than on the media with 0,6% and 1% agar (15,0% and 15,1%, respectively) (Table 1). There were no significant germination differences between the media with 0,6% and 1% agar. These results are consistent to the findings by Cerović et al. (1992), who obtained the best germination of the walnut pollen on the medium with 0,75% agar. According to Luza and Polito (1985) the medium containing 0,65% agar was suitable for freshly collected pollen for each of the 21 tested walnut clones. The agar content appears to be important in providing the necessary conditions for good hydration and germination of the

walnut pollen (Luza and Polito, 1985). The concentration of agar, by changing the physical characteristics of the medium, determines the embedding degree of the grains into the surface which may affect the amount of oxygen absorbed by the grain (Visser, 1955). When lower concentrations of agar are used, the pollen tends to sink into the surface of the medium where it will not germinate.

Effect of sucrose

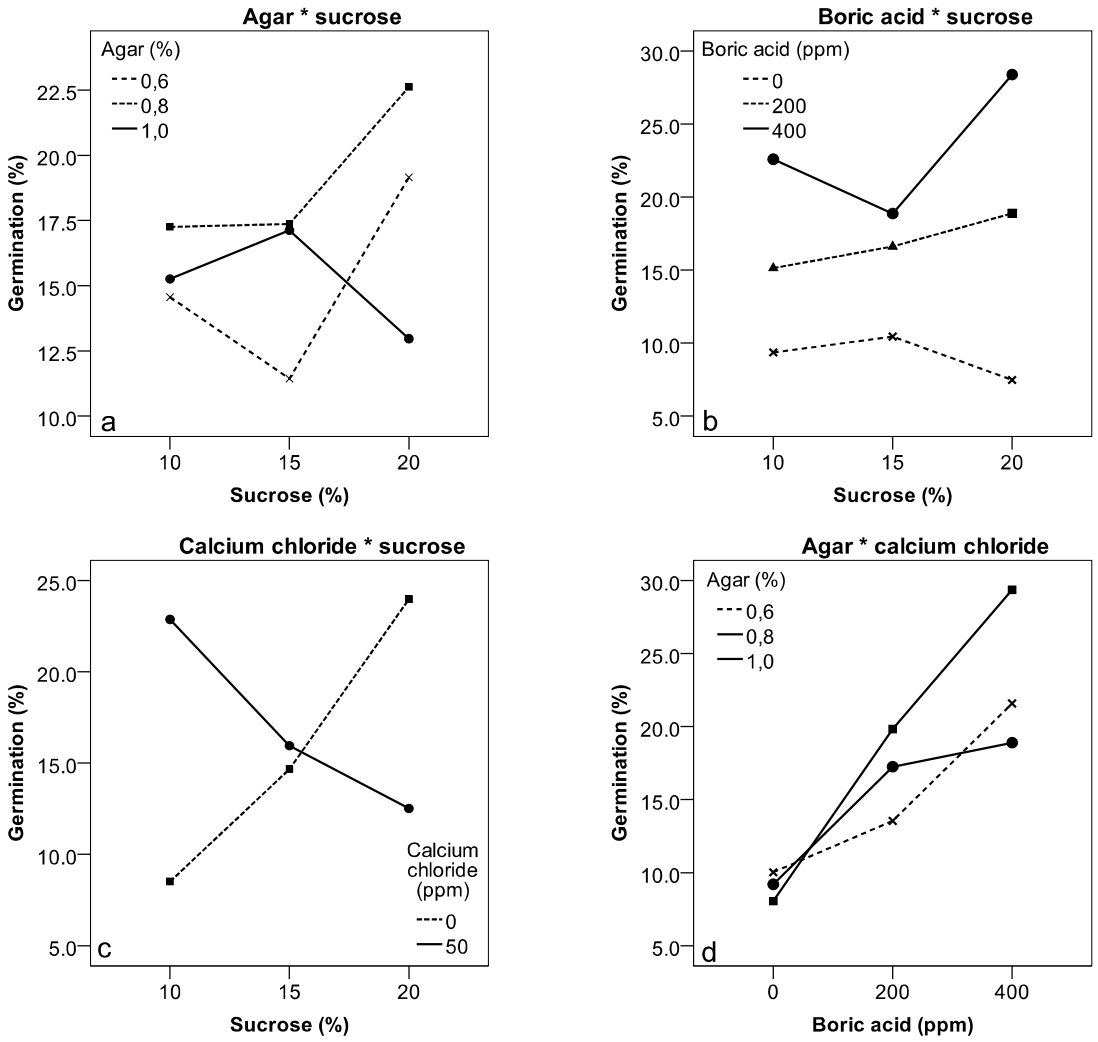
The germination of the pollen was also affected by sucrose concentrations, and media containing a 20% sucrose concentration had the highest average germination rate (18,2%), while there were no significant germination differences between the media with 10% and 15% sucrose (Table 1). Sütyemez (2007) found that a 15% sucrose concentration gave the highest germination rates for walnut cultivars, but according to Wu et al. (2008), the germination rate of 'Yunxin' walnut pollen was the highest when the sucrose concentration in germination medium was 10%. In our study, high concentration of sucrose (20%) in combination with high concentration of agar (1%) exhibited a detrimental effect on pollen germination (Graf. 1-a).

Table 1 – Effect of agar, boric acid, sucrose and calcium chloride on 'Šampion' walnut pollen germination *in vitro*

Factor	Concentration	Pollen germination (%)
Agar (A)	0,6%	15,0 a
	0,8%	19,1 b
	1%	15,1 a
Boric Acid (B)	0 ppm	9,1 a
	200 ppm	16,9 b
	400 ppm	23,2 c
Calcium chloride (C)	0 ppm	15,7 a
	50 ppm	17,1 b
Sucrose (S)	10%	15,7 a
	15%	15,3 a
	20%	18,2 b
Total		16,4
ANOVA		
Factor	*p*	
Agar (A)	0,000	
Boric acid (B)	0,000	
Calcium chloride (C)	0,006	
Sucrose (S)	0,000	
A*B	0,000	
A*C	0,000	
A*S	0,000	
B*C	0,000	
B*S	0,000	
C*S	0,000	
A*B*C	0,003	
A*B*S	0,000	
A*C*S	0,000	

B*C*S	0,000
A*B*C*S	0,000

The sucrose addition to the germination medium has the objective of providing osmotic equilibrium between the pollen and the germination medium, as well as being an energy source to aid the pollen development process (Stanley and Linskens 1974). Silva et al. (1999) stated that osmotic equilibrium between the germmination medium and pollen grain content determines the cell integrity, and this equilibrium can be determined by the relation between the concentration of sucrose and of substances such as boric acid and calcium.



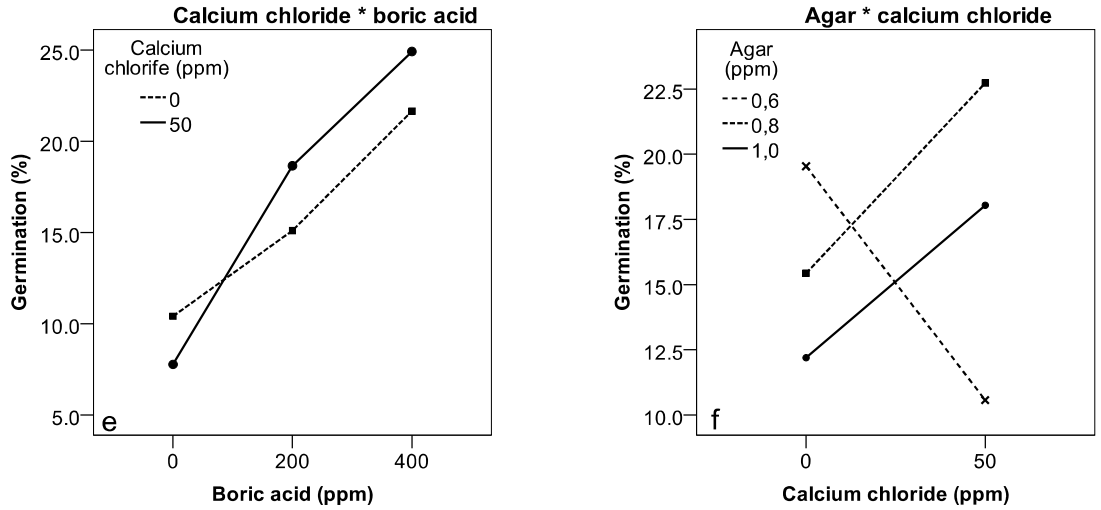


Figure 1 – Graphs showing effect of different concentration of media on pollen germination.

An excess or deficiency of any of these components could cause the breaking of the pollen grains. In our study pollen germination in media containing 20% sucrose was promoted by a high concentration of boric acid (400 ppm), but in absence of boric acid, the highest concentration of sucrose resulted in a significant decline of the germination percentage (Figure 1-b). In the media without calcium chloride pollen germination was stimulated with the increase in sucrose concentration, but in combination with $CaCl_2$ the germination decreased (Figure 1-c).

Effect of boric acid

Adding boric acid to the substrate generally has a significant positive effect on pollen germination, but the germination increase was significantly lower in the media with the highest content of agar (1%) than in the media with a lower content of it (Figure 1–d). Wu et al. (2008) found that the optimal culture medium for ‘Yunxin’ walnut pollen contained 10 mg/L of boric acid. Boron interacts with sugar, giving origin to a sugar-borate complex (Pfahler 1967). Boron facilitates sugar uptake and has a role in pectin production in the pollen tube (Richards, 1986), thus it is indirectly involved in development of pollen tube membrane (Stanley and Loewus, 1964). According to Vasil (1960), the role of boron in pollen germination and pollen tube growth may be 3-fold: (1) it promotes absorption of sugars (2) it increases oxygen uptake and (3) it is involved in the synthesis of pectic material for the wall of actively growing pollen tube.

Effect of calcium chloride

Adding calcium chloride to the media with 0,8% and 1% agar significantly increased the germination capacity of the ‘Šampion’ pollen, while in media containing 0,6% agar the addition of $CaCl_2$ sharply reduced the germination (Figure 1-f). The addition of calcium chloride in the media without boric acid, slightly reduced the pollen germination rate (Figure 1-e). Wu et al. (2008) noted that optimal culture medium contained 40 mg/L $CaCl_2$. According to Steer (1989), calcium ions are essential for pollen tube growth, but they are inhibitory at the concentrations higher than 10^{-2} M.

Conclusion

Significant differences in germination rate of the ‘Šampion walnut pollen were observed in response to changing concentrations of agar, sucrose, boric acid and calcium chloride in the germination medium. Strong interactions were identified between all substances used. The germination rate was maximized (39%) when the germination medium contained 0.8% agar, 15% sucrose, 400 ppm H_3BO_3 and 50 ppm $CaCl_2$.

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