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The effect of different germination media on pollen germination in vitro of the walnut (*Juglans regia* L.) cultivar 'Ibar'

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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 *in vitro pollen germination capacity* of the Serbian walnut cultivar 'Ibar'. 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* (38.5%) when the germination medium contained 0.6% of agar, 15% of sucrose, 200 ppm of H_3BO_3 and 50 ppm of $CaCl_2$. Significant differences in pollen germination were observed in response to changing concentrations of agar, sucrose, boric acid and calcium *chloride. Significant* interactions were detected *b*etween most of these substances.

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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 improvement 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; 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 and 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). 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). There is no reliable information about the *ideal* culture medium for *in vitro* testing of the walnut pollen viability. *According to literature,* optimal agar content for the walnut pollen germination varied from 0.65% (Luza and Polito, 1985*) to 1% (Galetta, 1983;* Parihar and Bajpai, 1992), sucrose content from 10% (Wu *et al*., 2008) to 20% (Luza and Polito, 1985), boric acid content from 10 ppm (Wu *et al*., 2008*) to 200 ppm (*Sağlam and Gülcan, 1995) and calcium chloride content from 40 ppm (Wu *et al*., 2008) to 111 ppm (Cerovic *et al*., 1992).

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 pollen germination *in vitro* of the walnut cultivar 'Ibar'.

Materials and Methods

Plant material

This study was carried out in the year 2010 on Serbian walnut cultivar 'Ibar'. Samples of pollen were collected near Čačak (West 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 design

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.

Pollen germination procedure

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°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 (Hedhly *et al*., 2005).

Pollen germination determination

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 replication. 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 and multiplying by 100.

Statistical analysis

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 'Ibar' walnut pollen* was 15.5%. The g*ermination rate was maximized* (38,5%) when the germination medium contained 0.6% of agar, 15% of sucrose, 200 ppm of H_3BO_3 and 50 ppm of $CaCl_2$. The analysis of variance of experimental data showed the significant effects on pollen germination of agar, sucrose, boric acid, calcium chloride and most of their interactions (Table 1).

Effect of agar

Pollen of walnut cultivar 'Ibar' germinated significantly better on the media containing 0.6% of agar than on the media with 0.8% and 1% of that ingredient (Table 1). There were no significant differences between the media with 0.8% and 1% of agar. *These results are consistent to* the findings by Luza and Polito (1985), according to whom the medium containing 0.65% of agar was suitable for freshly collected pollen for each of the 21 tested walnut clones. However, Cerović *et al.* (1992) obtained the best germination of the walnut pollen on the medium with 0.75% of agar. According to Luza and Polito (1985), the agar content in germination media appears to be important in providing the necessary conditions for good hydration and germination of the walnut pollen.

Table 1. Effect of agar, boric acid, sucrose and calcium chloride on pollen germination in vitro of the walnut cultivar 'Ibar'.

Ingredient	Concentration	Germination* (%)
Agar (A)	0.6%	17.7 a
	0.8%	13.7 b
	1%	14.9 b
H_3BO_3 (B)	0 ppm	11.1 a
	200 ppm	17.7b
	400 ppm	16.6 c
$CaCl_2$ (C)	0 ppm	13.2 a
	50 ppm	17.7b
Sucrose (S)	10%	16.0 a
	15%	22.7b
	20%	7.6 c
Average		15.4

ANOVA	
Factor	*p*
Agar (A)	0.000
H_3BO_3 (B)	0.000
$CaCl_2$ (C)	0.006
Sucrose (S)	0.000
A*B	0.001
A*C	0.795
A*S	0.001
B*C	0.247
B*S	0.000
C*S	0.000
A*B*C	0.015
A*B*S	0.000
A*C*S	0.337
B*C*S	0.004
A*B*C*S	0.003

*Means with the same letter are not significantly different

Effect of sucrose

The germination of the pollen was affected by sucrose concentrations, and media containing a 15% of sucrose concentration had the highest average germination rate, and the media with 20% of sucrose had the lowest (Table 1). Sütyemez (2007) also 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 in the media with the lowest level of sucrose

(10%) the germination rate was lower in combination with the higher contents of agar (0.8% and 1%) than with 0.6% of it (Fig. 1-b).

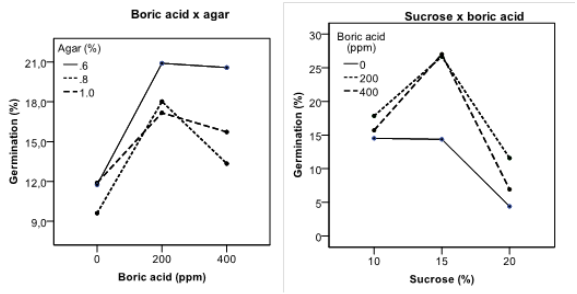

Fig. 1. Effects of some interactions between agar, sucrose, boric acid and calcium chloride on pollen germination in vitro of the walnut cultivar 'Ibar'.

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. An excess or deficiency of any of these components could cause the breaking of the pollen grains. In our study pollen germination in media not containing boric acid was hindered by a highest concentration of sucrose (20%), but in presence of boric acid, the middle concentration of sucrose (15%) *resulted in high* germination rates (Fig. 1-c).

Effect of boric acid

The germination of the pollen was affected by boric acid concentrations, and the average germination rate was the highest in the media containing 200 ppm of H_3BO_3, and the lowest in the media without it (Table 1). Positive effect of boric acid to the pollen germination was higher in the media with the lowest content of agar (0.6%) than in the media with a higher content of it (Figure 1–a). 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 substrate generally has a significant positive effect on pollen germination. Addition of calcium chloride to the media containing 10% or 15% of sucrose stimulated pollen germination, but in the media with highest contenet of sucrose (20%) and 50 ppm of $CaCl_2$ pollen germination was decreased (Figure 1-d). Wu *et al.* (2008) noted that optimal culture medium contained 40 mg/L of $CaCl_2$. 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 pollen of walnut cultivar 'Ibar' were observed in response to changing concentrations of agar, sucrose, boric acid and calcium *chloride* in the germination medium. Strong and complex interactions between tested substances were identified. The g*ermination rate was maximized* (38.5%) when the germination medium contained 0.6% of agar, 15% of sucrose, 200 ppm of H_3BO_3 and 50 ppm of $CaCl_2$.

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