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Betulaceae pollen germination using different culture medium and L-Proline and sucrose levels

Germinação de pólen de Betulaceae utilizando diferentes meios de cultura e níveis de L-Prolina e sacarose

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Resumo
A avaliação da fertilidade do pólen é importante para vários aspectos relacionados com a produtividade da planta, como programas de melhoramento e seleção das plantas. Neste trabalho foram testados dezesseis meios de cultura para germinação *in vitro* de pólen da *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth, *Alnus glutinosa* L. e *Corylus avellana* L. para encontrar o meio mais adequado. Os meios testados para germinação continham duas concentrações diferentes de sacarose, ácido bórico (H_3BO_3) e cálcio (na forma de nitrato de cálcio ($Ca(NO_3)_2$) ou cloreto ($CaCl_2$). Todos os meios foram testados com e sem L-prolina. O tempo de incubação foi de 24h a 25ºC. Os resultados mostram que as melhores taxas de germinação foram obtidas nos meios contendo L-prolina. Em *Betula pendula* Roth, a germinação de pólen e o crescimento do tubo polínico aumentaram pela adição de uma fonte de cálcio ao meio.
Palavras-chave: Betulaceae, Pólen, Germinação *in vitro*, L-prolina.

Abstract
Evaluation of pollen fertility is important for various aspects related with plant productivity such as plant breeding programmes or in cultivar selection. In this work sixteen culture medium were tested for in vitro germination of *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth, *Alnus glutinosa* L. and *Corylus avellana* L. pollen in order to find the most suitable one. The media tested for germination contained two different concentrations of sucrose, boric acid (H_3BO_3) and calcium (in the form of calcium nitrate ($Ca(NO_3)_2$) or chloride ($CaCl_2$). All media were tested with and without L-proline. The incubation time was 24 h to 25º C. The results show that the best germination rates were obtained in the media containing L-proline. In *Betula pendula* Roth, pollen germination and pollen tube growth increased by the addition of a source of calcium to the medium.
Keywords: Betulaceae, Pollen, *In vitro* germination, L-Proline.

INTRODUCTION

Germination leads to the formation and growth of pollen tube that carries the male gametes to the embryo sac to generate the double fertilization in flowering plants. The methodologies usually used to evaluate pollen fertility can be diverse such as staining techniques, germination or seed set percentage (Dafni & Firmage 2000; Heslop-Harrison et al., 1984; Stanley & Linskens, 1974).

The stigma possesses the optimal medium for pollen germination to occur, which comprises several stages of development of the pollen tube and the time taken for each stage varies greatly from species to species depending on the type of reserve material present in pollen as well as external factors (Feijo et al., 2001).

Generally, for in vitro germination to occur it is necessary to prepare, under laboratory conditions, a medium with similar composition to the stigma for the pollen germinate. Usually, in vitro pollen germination requires hydration, a source of carbohydrates and boron.

Additionally, the germination medium can also include other components such as calcium, magnesium and potassium (Brewbaker & Kwack, 1963; Fan et al., 2001). All these elements can be combined at different concentrations according to plant species tested (Feijo et al., 1995; Linskens, 1964). Pollen hydration is required once, at the time of dehiscence, the pollen is released into the atmosphere dehydrated, suffering rehydration on the stigma (Wilsen & Hepler, 2007).

Sucrose is normally used as the source of carbon necessary for energy supply and carbohydrate skeleton formation. Boron plays an important role in pollen germination and tube growth by the construction of the wall during the pollinic tube elongation (Feijo et al., 1995; Obermeyer & Blatt, 1995; Taylor & Hepler, 1997). Some studies point out for a specific role of calcium in the regulation of polarized growth of pollen tubes (Hepler, 2005; Michard et al., 2011). This substance may be added to the germination medium in the form of chloride or nitrate ($CaCl_2$ and $Ca(NO_3)_2$). Furthermore, incubation temperature and time are also important parameters to control (Fu et al., 2001; Kelen & Demirtas, 2003).

Several studies have been developed along the years in order to determine qualitatively and quantitatively the best germination medium for a tested species (Heslop-Harrison et al., 1984; Steer, 1989). These studies provide the basis for establishing of germination medium for species not tested in the literature.

The pollen germination assessment is important in the study of various aspects related with plant productivity such as pollen vigour during storage conditions for controlled pollination, for the choice of pollinizers, in the evaluation of cultivar compatibility, in plant breeding programmes or in cultivar selection (Cheung et al., 2010; Dafni & Firmage 2000). Pollen germination in several fruit trees, are closely related to a greater or lesser ability to produce fruit and consequently higher or lower yields (Shukla et al., 1998).

The trees of the Betulaceae family comprise several genus that are usually used for timber and fruit production and also have been spread out for ornamental purposes (Chen et al., 1999). They are anemophilous trees, producing great amounts of pollen and so, have been recorded in aerobiological and allergenic studies. Furthermore, in the literature, information on in vitro germination media for all these species is scarse and the existing studies describe different methodologies and different germination media, making it difficult to compare the obtained results.

OBJECTIVES

The pollen germination capacity of five cultivated plant was compared in different medium and L-Proline and sucrose concentration levels.

MATERIAL AND METHODS

Anthers of *Ostrya carpinifolia* Scop., *Carpinus betulus* L., *Betula pendula* Roth., *Alnus glutinosa* L. and *Corylus avellana* L. were collected during the flowering season (February and March) in the botanic garden. They were dried at 27°C, gently crushed and the pollen thus released was passed through different grades of sieves to obtain pure pollen. Pollen samples were then stored at –20°C.

Pollen germination was assayed in eight different germination medium at two sucrose concentration levels (15% and 25%). All medium contained boric acid (H_3BO_3), but several have also Ca in form the nitrate ($Ca(NO_3)_2$) or chloride($CaCl_2$). Comparison between different concentrations of boron and calcium were tested (a total of 16 different combinations). These elements were selected based on literature.

Pollen grains were distributed in petri dishes with medium and maintained in a thermocontrolled dryer at 25 °C in the dark. The germination was scored after 24 hours of incubation and pollen was considered germinated when the tube has at least twice the diameter of pollen. In order to calculate the germination percentage, two replications per plant were performed and in each one five fields per sample (each one containing 100 pollen grains) were counted using a light microscope (Leica DM LB).

RESULTS AND DISCUSSION

In this work, the comparison of the pollen germination capacity of five species of trees belonging to the Betulaceae family, namely *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth, *Alnus glutinosa* L. and *Corylus avellana* L. was performed. A total of 16 solid media were used and tested with and without L-proline. The greater values of the germination were obtained with *Ostrya carpinifolia* pollen around 43.3% in a medium containing 200 ppm H_3BO_3, 200 ppm $CaCl_2$, 400 ppm L-proline and 25% sucrose; *Carpinus betulus* pollen around 36% in the medium with 200 ppm H_3BO_3, 400 ppm $Ca(NO_3)_2$, 400 ppm de L-proline and 15% sucrose; *Betula pendula* pollen with 39.9% in the medium containing 200 ppm H_3BO_3, 400 ppm L-proline and 15% sucrose. *Alnus glutinosa* and *Corylus avellana* pollen had germination of 31.6% and 28.4% respectively in a medium with 100 ppm H_3BO_3, 100 ppm $CaCl_2$, 400 ppm L-proline and 15% sucrose (Table 1).

Table 1. Pollen germination in different culture medium, L-Proline and sucrose levels.
Tabela 1. Germinação de pólen em diferentes meios de cultura e níveis de L-Prolina e sacarose.

Germination percentage	medium	H3BO3 (ppm)	200				200				100				200				
		CaCl2 (ppm)	x				x				100				200				
		Ca(NO3)2 (ppm)	400				x				x				x				
		L-proline (ppm)	x	x	400	400	x	x	400	400	x	x	400	400	x	x	400	400	
		Sacarose (%)	15	25	15	25	15	25	15	25	15	25	15	25	15	25	15	25	
		Betula pendula	3.6	1.0	1.6	1.2	15.0	ng	***39.9***	4.0	9.0	3.2	6.8	4.8	2.0	ng	4.4	1.6	
		Alnus glutinosa	2.4	4.8	6.4	4.8	ng	12.8	10.0	11.4	23.2	9.8	***31.6***	9.0	24.6	10.0	28.8	12.6	
		Corylus avellana	8.0	ng	4.6	4.2	ng	ng	19.0	3.0	25.6	ng	***28.4***	6.0	8.8	4.6	4.6	3.6	
		Ostrya carpinifolia	10.2	4.0	2.8	5.8	2.0	10.0	ng	5.8	8.6	12.6	10.0	13.4	10.0	19.6	6.2	***43.3***	
		Carpinus betulus	15.8	ng	***36.0***	11.6	ng	2.6	ng	2.4	3.6	4.2	2.4	8.2	9.8	7.0	5.6	9.2	

In this study, the media presenting higher germination percentages for each of the five Betulaceae studied contained L-proline. Some studies showed that levels of proline in the pollen are very high, being the highest among floral organs (Chiang & Dandekar, 1995; Krogaard & Andersen, 1983; Lansac et al., 1996; Schwacke et al., 1999). Nevertheless, the reason for this accumulation has been associated with multiple functions such as free radical scavenger, protector of membranes and cellular structures, energy source or as metabolic precursor for pollen tube elongation (revised in Mattioli et al., 2012). This amino acid is therefore an important component for the successful male fertility (Mattioli et al., 2012; Zhang & Croes, 1983).

All Betulaceae species tested required boron to germinate although in different concentration. Boric acid is known to be crucial for pollen

germination and tube growth. Boron deficiency can lead to a reduce in pollen germination rate, retardation of pollen tube growth, pollen tube anomalies such as the swelling at the tip of the pollen tube or tube bursting (Acar et al., 2010; Holdaway-Clarke et al., 2003; Wang et al., 2003; Yang et al., 1999).

It is reported a concentration of 100 ppm to be required for successful pollen germination for most species (Brewbaker and Majumder, 1961), whereas higher concentrations can inhibited pollen grain germination and pollen tube elongation (Potts & Marsden-Smedley, 1989; Wang et al., 2003). However, in our study only *Alnus glutinosa* L. and *Corylus avellana* L. pollen germination was favored in the presence of this level of boric acid while the pollen germination of the other three species was best in the presence of higher boric acid concentration (200 ppm). Yet, the best germination medium for *Ostrya carpinifolia* Scop, *Carpinus betulus* L., *Betula pendula* Roth, differed in the other constituents, the presence of calcium for the first two species and only the presence of boron in the last one.

Our results showed that the need of calcium for successful germination varied between the tested species. Also, when necessary, the form of how calcium is added to the germination medium and its concentration varied between the species. *Betula pendula* Roth. was the only species where pollen germination percentage was higher in the absence of calcium. This is in accordance with the results of Käpylä (1991) and Pasonen & M. Kapyla (1998) that working with *Betula pendula* pollen, reported germination percentages over 50% using a medium containing only 100 ppm of boric acid and 0.5 M sucrose. Also, these authors showed that an incubation medium containing also $Ca(NO_3)_2$ did not increased the pollen germination. Nonetheless, it has been admitted that the calcium is an important element in in the growth medium of several plant species (Brewbaker & Kwack, 1963) known to stimulate the growth of pollen tube (Hepler, 2005; Michard et al., 2011) and required for maintenance of membrane integrity (Kell & Donath, 1990; Van Stkveninck, 1965). Also, in *Arabidopsis thaliana* pollen was observed that the absence of calcium did not inhibited pollen germination, however its presence improved germination percentage (Daher et al., 2009). These authors suggest that this may be related to the presence in or on the surface of the pollen of a stock of calcium.

In any pollen germination media, a source of energy is a key element for successful germination since pollen tube does not perform photosynthesis and therefore needs a carbon source, usually in the form of sucrose, for energy supply and Carbohydrate skeleton formation (Daher et al., 2009). In the literature the optimal sucrose percentage varies from species to species. A study performed in *Betula luminifera* H.J.P.Winkl. pollen was observed that excess of sucrose inhibit their germination, being 10% sucrose the optimal percentage (Bao et al., 2009). The same was observed in *Corylus heterophylla* Farnch. × *C. avellana* L. where was shown that different concentrations of sucrose had significant impact on pollen germination being the optimal concentration 15% (Zhai et al., 2009). In our work only *Ostrya carpinifolia* Scop. germinated best in higher values of sucrose, 25%, the other Betulaceae species achieved higher pollen germination percentages in a medium containing 15% of sucrose.

CONCLUSION

To sum up, in our study was observed that the five Betulaceae species tested differed in their pattern of response to boric acid, calcium and sucrose. Sucrose and boric acid were essential for pollen germination while the need for calcium and its form was species dependent. In terms of the optimal level of boric acid, *Alnus glutinosa* L. and *Corylus avellana* L. pollen required low concentration than the other species. Appart from *Betula pendula* Roth. Pollen, calcium improved the germination percentage.

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