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# Pollen Germinability and Cross-Pollination Success in Persian Cyclamen (*Cyclamen persicum* Mill.)

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Abstract

Low seed yield is a limiting factor for cross breeding and hybrid seed production in cyclamen. This study was performed to investigate pollen germination and its relation to cross-pollination success and fruit set in this plant. In order to achieve a high level of pollen germination, the effect of different concentrations of chemical compounds were examined on *in vitro* pollen germination of cyclamen in modified Brewbaker and Kwack medium, containing sucrose (10 and 20%), calcium nitrate (0, 200 and 300 mg $l^{-1}$), and boric acid (0, 100 and 200mg $l^{-1}$) at two pH levels (5.5 and 6.5). Maximum pollen germination was obtained in media containing higher concentration of calcium and boron regardless of sucrose concentration and pH level. Pollen germination percentage was genotype-dependent. Cross-pollination was performed among four different genotypes characterized by various pollen germination percentages. There was a direct correlation between cross-pollination success and pollen germination percentage. Genotypes with 30% higher pollen germination led to 10% increase in fruit set.

**Keywords:** Cross-pollination success, Cyclamen, Medium composition, Pollen germination.

## INTRODUCTION

Cyclamen is a commercially valuable potted flowering plant (Tanaka *et al*., 2013b), which is typically propagated by seed. Cyclamen seeds are expensive [up to 0.20 € per seed] (Schwenkel, 2001) that are usually sold as single seeds (Dole and Wilkins, 2005). The high price is primarily because of the laborious work for emasculation and hand pollination, which are essential for hybrid seeds production (Takamura, 2007). Cross-pollination success is extremely important not only for F1 seed production, but also for cyclamen breeding. However, in controlled crosses, there are some problems such as low pollen viability and germination (Ewald and Schwenkel, 1997; Nan, 2008) and inflorescence abortion (Ewald and Schwenkel, 1997), leading to loss of a large number of crosses, thereby reducing fruit and seed yields.

Emasculation and artificial pollination are required for producing cyclamen hybrid seeds. The stigma dries 3–5 days after anthesis (Reinhardt *et al.*, 2007); hence, the best time for emasculation is a few days before and after anthesis. Takamura (2007) has suggested flower bud emasculation to be done seven days before anthesis, and then the emasculated flower be pollinated at the expected time of anthesis. This would increase seed set percentage. Temperatures above 30°C have detrimental effects on cyclamen pollen germination; 15–25°C has been reported to be the optimal range (Takamura *et al.*, 1996). Higher relative humidity (RH) allows pollens to absorb water easily (Ferrari *et al.*, 1981). Naturally, petals abscise without drying after fertilization, which is catalyzed by ethylene (Halvey *et al.*, 1984), and ovaries will continue to grow for creating seed capsules after 3 months (Naderi, 2000).

High pollen germination percentage and rapid growth of the pollen tube are very important for sufficient seed yield. One of the methods to assess pollen viability is *in vitro* germination. In *in vitro* condition, beside the genetic factor, medium elements and compounds, pH, and incubation temperature affect pollen germination and pollen tube growth (Abdul-Baki, 1992; Sawidis and Reiss, 1995; Taylor and Hepler, 1997). Once the stored nutrients in pollen grain run out, external nutrients are required for further growth (Cruzan, 1986; Mulcahy and Mulcahy, 1982; Herrero and Hormaza, 1996). For example, sugars are required in medium. Pollen consumes sugars (especially sucrose) as respiration substrates for rapid synthesis of cell wall material and elongation (Baker and Baker, 1979; Mascarenhas, 1993; Schlupmann *et al.,* 1994; Derksen *et al*., 1995; Okusaka and Hiratsuka, 2009). Sucrose level in medium depends on plant species and nutrient reserves of the pollens (Vasil, 1960; Sahar and Spiegel, 1984; Golan-Goldhirh *et al.*, 1991; Mortazavi *et al*., 2007). Plant metabolic processes are cytoplasmic-pH-dependent (Tupy and Rhova, 1984). Some studies have reported proton and ion gradients roles for polarized pollen tube growth) Malho *et al.*, 1994; Pierson *et al*., 1994; Malho and Trewavas, 1996; Feijó *et al.*, 1999; Hepler *et al*., 2006; Michard *et al*., 2008).

Boron and calcium are required for growth and development of vascular plants (Cakmak *et al.,* 1995; Stangoulis *et al.*, 2001; Zhang *et al.,* 2007). The essential and supplementary roles of these elements in pollen germination have been observed in both *in vitro* and in vivo conditions (Brewbaker and Kwack, 1963; Robbertse *et al.*, 1990; Loomis and Durst, 1992; Feijó *et al.*, 1995; Nyomora *et al.*, 2000; Wang *et al.*, 2003; Mortazavi *et al*., 2007; Vaknin *et al*., 2008; Lee *et al.*, 2009).

Supplemental calcium contributes to pollen tube guidance and enters the tube via the tip (Vaknin *et al.,* 2008; Steinhorst and Kudla, 2013). Furthermore, it affects mechanical properties of the pollen tube wall (Steer and Steer, 1989) and pollen tube sensitivity to tropic trigger (Bou Daher and Geitmann, 2011). In many plants, besides calcium, boron plays critical role in sexual reproduction (Brown *et al.*, 2002). Boron is required for normal pollen germination and tube growth. Some of genes related to B transporter are especially expressed in pollens (Tanaka *et al.*, 2013a). Boron deficiency affects growing points, such as pollen tube tips (Loomis and Durst, 1992).

Improved hybrid cyclamen seed production is beneficial for cross breeding and hybrid seed production, for which, higher cross-pollination success is required, however. For this reason, we

conducted an experiment to investigate the relationship between pollen germination and cross-pollination success. Moreover, to assess pollen viability, the effects of different concentrations of chemical compounds were examined on *in vitro* pollen germination.

## MATERIALS AND METHODS

### *In vitro* germination test

In order to avoid possible discrepancies, the pollen grains were collected from a single flower four days after anthesis in the morning. The pollen grains were cultured in modified Brewbaker and Kwack liquid medium (Brewbaker and Kwack, 1963) containing sucrose (10 and 20%), $Ca(NO_3)_2$ (0, 200 and 300 mg $l^{-1}$) and $H_3BO_3$ (0, 100 and 200 mg $l^{-1}$). pH of the media was adjusted to 5.5 or 6.5 using 0.1 M HCl/NaOH. Considering these factors, 36 types of medium were prepared. The cultures were maintained in germinator at 15°C and in dark. Germinated pollens were counted after 24 h. A pollen grain was considered to be germinated if the length of the pollen tube was equal to or longer than the diameter of the grain (Abdul-Baki, 1992). For each germination test, 800-1200 grains from four microscopic fields were counted. The best medium in this experiment was used for determining pollen germination percentage of other genotypes. Tube length was measured on 30 randomly chosen pollen tubes per genotype after 48 h.

### Cross-pollination

Flowering cyclamens from four inbred diploid genotypes (G1, G2, G3 and G4) were used, which had been growing in greenhouse conditions. Cross-pollination was done among these genotypes as shown in Fig. 1 (approximately 100 times for each direct cross). By removing stamens together with petals, the flowers were emasculated. They were then covered by a paper bags. Next, fresh pollens that were gathered from flowers of the male parent were placed on the stigma 5–7 days after emasculation. Finally, the flowers were covered again. Temperature and relative humidity of the greenhouse were 15–20°C and 60–70%.

Data of medium composition were analyzed in a factorial design using SAS software 9.3, and the means were compared using Duncan's multiple range test. For pollen germination percentage in different genotypes and cross-pollination success in crosses among genotypes, data were analyzed in a completely randomized design using SAS software 9.3, and Duncan's multiple range test was used to compare the means.

## RESULTS AND DISCUSSION

### *In vitro* germination

Variation in pollen germination among the treatments was high. Sucrose 10% was better than sucrose 20% (Fig. 2a). Takamura (1996) already reported low concentrations of sucrose (between 5-15%) to be proper for pollen germination in cyclamen. High concentration of sucrose can

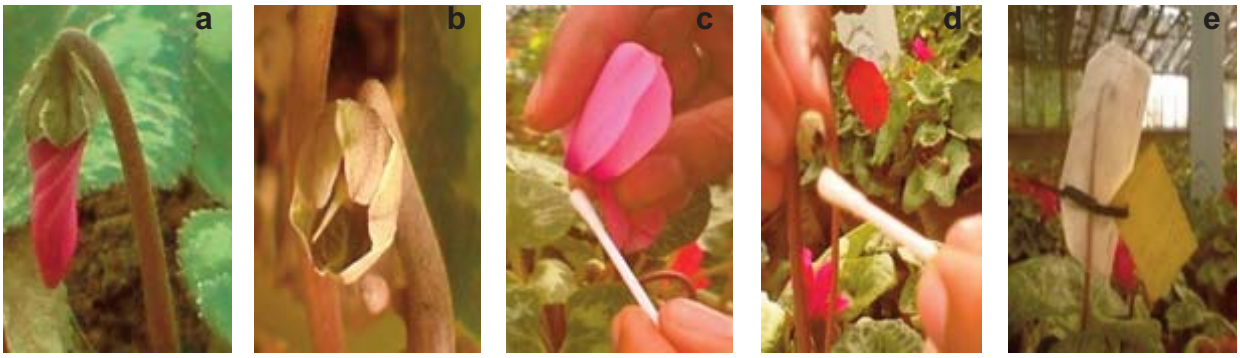


Fig. 1. Different stages of emasculation and cross-pollination in cyclamen. a: Appropriate time for removing stamens (bud stage); b: Emasculated female parent; c: Releasing pollens of male parents on ear cleaner or nail, d: Placing pollens on stigma of an emasculated flower; e: Packing.

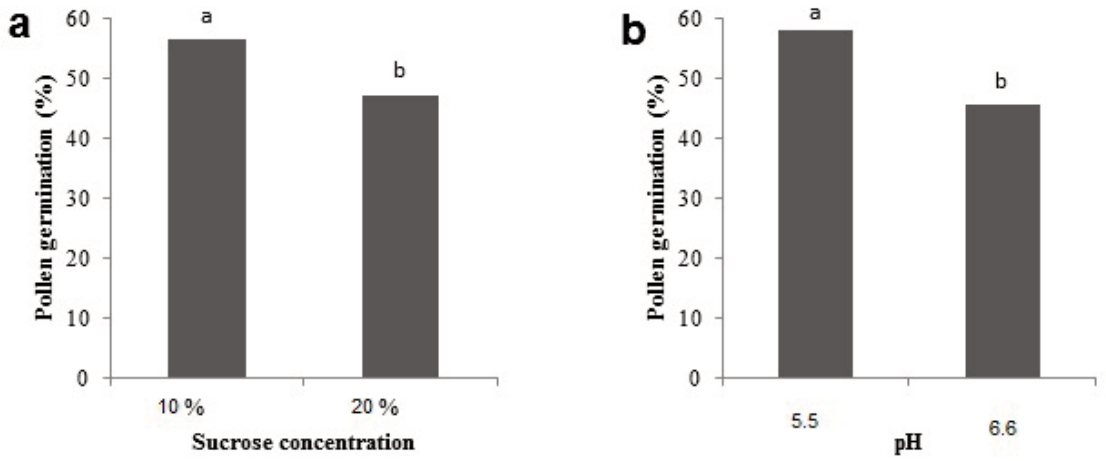


Fig. 2. Pollen germination percentage of cyclamen. (a) In two sucrose concentrations. (b) At two pH levels. Values with different letter are significantly (P<0.01) different.

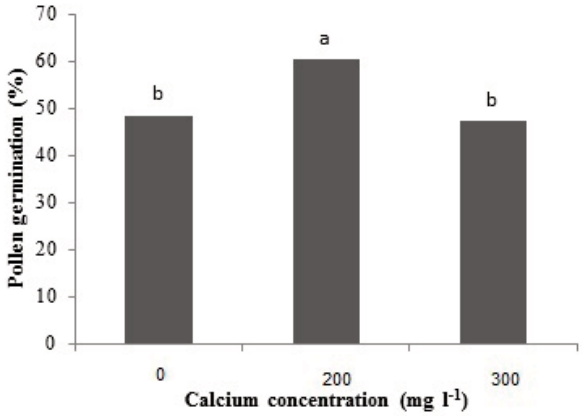


Fig. 3. Pollen germination percentage of cyclamen in different concentrations of calcium. Values with different letter are significantly (P<0.01) different.

decrease water absorption and pollen germination, which depends on the species, nevertheless (Stone, 2003).

Media pH when was 5.5 increased pollen germination in comparison to when it was 6.5 (Fig. 2b). Acidification results in cell membrane loosening (Franklin-Tong, 1999), which is necessary for pollen germination and pollen tube growth. Proton efflux and proton-sugar co-transport has been seen in growing pollen tubes. It shows H+ exchanges with the medium for cytoplasmic pH regulation of pollen tube (Tupy and Rhova, 1984). Cyclamen is an acidophilic plant (Dole and Wilkins, 2005). Therefore, the better pollen germination in low pH could be a response to its acidophilic nature.

Calcium nitrate in medium had a positive effect on pollen germination; 200 mg $l^{-1}$ was optimum (Fig. 3). Many researchers have confirmed the essential role of $Ca^{2+}$ for pollen germination (Steinhorst and Kudla, 2013). Cell structure is directly and indirectly affected by $Ca^{2+}$ concentration (Zhang *et al.,* 2007). The tip growth of pollen is correlated with $Ca^{2+}$ gradient. Extracellular $Ca^{2+}$ ions establish this gradient in combination with internal stores (Steinhorst and Kudla, 2013). Calcium also has signal transduction role in pollen germination (Zhang *et al.*, 2007). Proper concentration of $Ca^{2+}$ for pollen germination varies with different species. Similarly, many other studies reported that high or low Ca concentrations had decreased pollen germination (Voyiatzis and Paraskevopoulou-Paroussi, 2002; Lee *et al*., 2009). With high Ca concentration, the unbalanced Ca gradient in the pollen tube tip could suppress pollen tube growth (Lee *et al.,* 2009).

Without calcium, boric acid decreased pollen germination percentage. Reduction in pollen

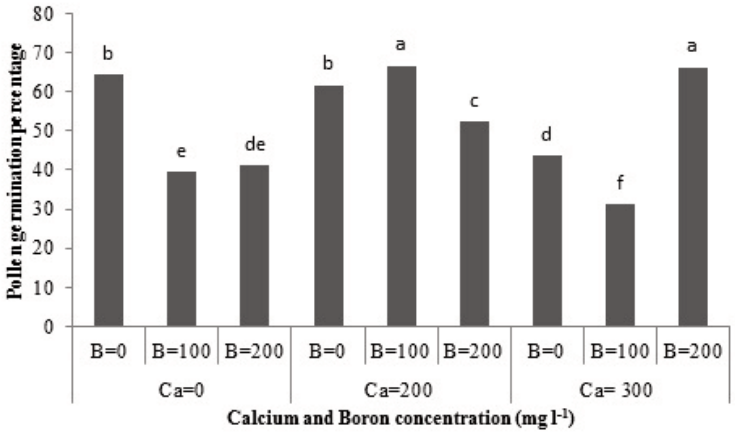


Fig. 4. Pollen germination percentage of cyclamen in different concentrations of calcium and boron. Values with different letter(s) are significantly (P<0.01) different.

germination may be attributed to the higherB combination with rhamnogalacturonan II in pollen tube wall, which results in pollen cell wall stability (Hu and Brown, 1994; Kaneko *et al*., 1997). The reduction of pollen germination by boron in absence of calcium was already reported (Bruyn, 1966; Pfahler, 1967).

There are interactions between calcium and boron in plant tissues (Brewbaker and Kwack, 1963). The highest pollen germination percentage was obtained in media containing the highest concentration of boron and calcium (Fig. 4). Some researchers have demonstrated that boric acid is effective on pollen germination and pollen tube elongation in medium (Nyomora *et al.,* 2000; Wang *et al.*, 2003). Pollen tube precursors form strong complexes with B (Loomis and Durst, 1992). Some authors suggested B to improve pollen germination by promoting H+-ATPase activity (Feijó *et al.*, 1995; Obermeyer and Blatt, 1995). Boron is needed also for cell wall expanding (Fleischer *et al.*, 1998). In our study, B had positive effect on pollen germination provided that Ca was present. Synergistic interaction between Ca and B in increasing pollen germination has already been reported (Bruyn, 1966; Pfahler, 1968).

Maximum pollen germination percentage (84%) was obtained in medium containing 10% sucrose, 300 mg $l^{-1}$ Ca and 200 mg $l^{-1}$ B having pH of 5.5. The second medium by 80% germination was containing 20% sucrose, 300 mg $l^{-1}$ Ca and 200 mg $l^{-1}$ B with pH of 6.5. Medium containing 10% sucrose, 200 mg $l^{-1}$ Ca and 200 mg $l^{-1}$ B with pH of 5.5 and by 76% germination was the third.

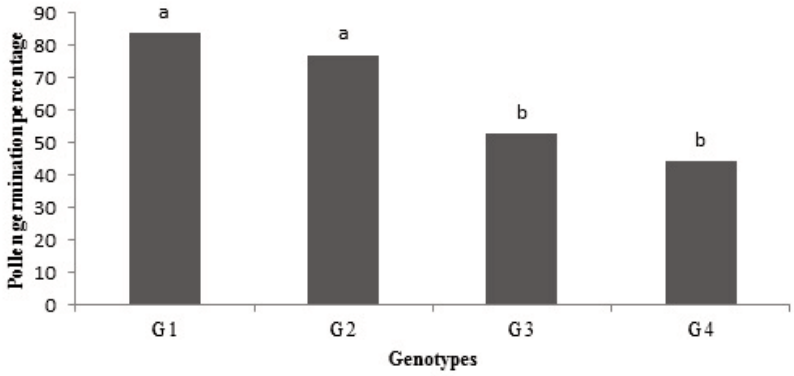


Fig. 4. Pollen germination percentage of cyclamen in different concentrations of calcium and boron. Values with different letter(s) are significantly (P<0.01) different.

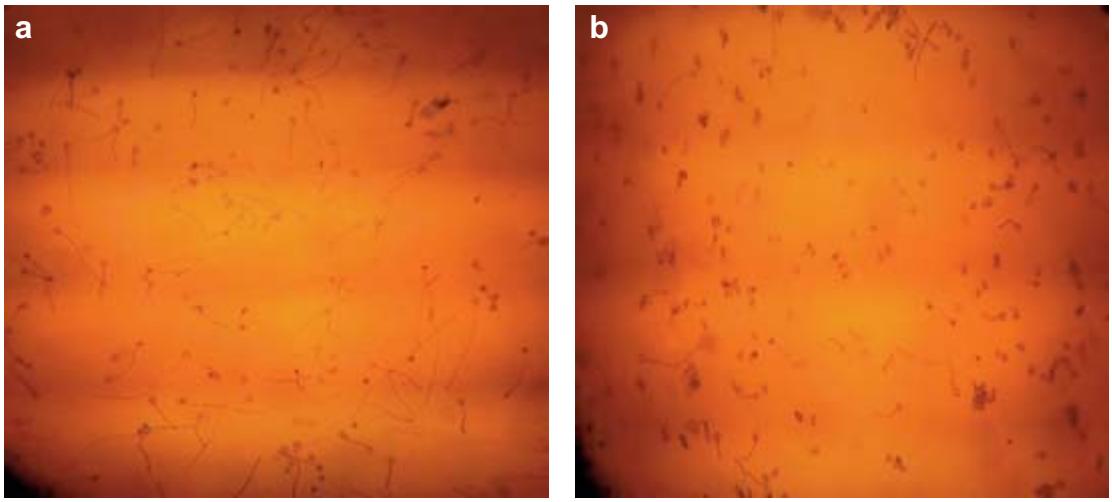


Fig. 6. pollen germination in two cyclamen genotypes: G1(a) and G4 (b).

Table 1. Cross-pollination success (%) in crosses among four cyclamen genotypes with different pollen germination percentage.

| ♀ | ♂ | G1 | G2 | G3 | G4 |
|---|---|---|---|---|---|
| G1 | | – | 34 $^{a}$ | 25 $^{bcd}$ | 19 $^{de}$ |
| G2 | | 35 $^{a}$ | – | 20 $^{de}$ | 15 $^{e}$ |
| G3 | | 32 $^{ab}$ | 30 $^{abc}$ | – | 17 $^{de}$ |
| G4 | | 24 $^{bcde}$ | 22 $^{cde}$ | 19 $^{de}$ | – |

Values with different letter are significantly (P<0.01) different. ♂ = male parents, ♀ = female parents.

**Pollen germination in different genotypes**

Pollen germination percentage depended on the genotype. As shown in Figs. 5 and 6, there were significant differences among the genotypes. Variation in pollen germination percentage of different cyclamen genotypes has already been reported by Ewald and Schwenkel (1997) and Nan (2008).

Some genotypes showed low germination rate (Fig. 7). Rapid pollen tube growth might be beneficial during the fertilization process. Stigma receptivity in cyclamen is 16 days approximately (Schwartz-Tzachor *et al.*, 2006). However, pollens with low germination capability may be followed by low seed yields, especially if the pollination is done late.

**Cross-pollination success**

Averagely, 24% of crosses led to fruit set (Table 1). Naturally, a few ovaries are able to produce seeds. The main reason for the low fruit yield in cyclamen is the high rate of inflorescences abortion (Ewald and Schwenkel, 1997); furthermore, most (approximately 80%) ovules fail to be fertilized. Callose inclusion is also essential for fertilization in cyclamen (Reinhardt *et al.*, 2008).

According to Ewald and Schwenkel (1997), the number of pollen tubes in styles varied between 0 and 100, and a few of them were capable to reach the ovules. In our study, pollen germination percentage was 44–84% (Fig. 5); it was reported to be 15–51% in other experiments (Ewald and Schwenkel, 1997; Nan, 2008). *In vitro* condition seems to be more favorable for cyclamen pollen germination than *in vivo* condition. Cross success was higher when male parent showed higher pollen germination. Furthermore, female parents had different fruit set when crossed with a specific male parent (Table 1), which shows ovules quality the quality depends on genotype (Reinhardt *et al.*, 2008) to be important for fruit set. These studies suggest that the pollens germinability and ovules quality determine final seed yield in cyclamen.

With respect to our result, genotypes with high germination percentage are useful for use

as male parents in cross breeding. Two genotypes with higher pollen germination led to 10% higher cross-pollination success. Considering the fact that fruit set in cyclamen is low, the 10% higher cross-pollination success would be beneficial in large scale forimproving F1 seed production.

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