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Pollen viability and storage life in *Leonurus cardiaca* L.

Aydin Shekari, Vahideh Nazeri*, Majid Shokrpour

Department of Horticultural Sciences, Faculty of Agriculture, University of Tehran, Karaj, Iran

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ABSTRACT

Leonurus cardiaca (Lamiaceae) is an important medicinal plant growing in many regions of Iran. It has been used to cure cardiovascular diseases, stress, anxiety, and nervous irritability. There has been no report on the pollen biology of this species. Therefore, this experiment was designed to investigate viability, *in vitro* germination, optimal storage temperature, and storage life of the pollen grains. Two colorimetric methods using either 2,3,5-triphenyl tetrazolium chloride (TTC) or acetocarmine were employed. In a medium containing 5% sucrose, 100 ppm boric acid (H_3BO_3), and 1% agar, pollen germination rate was examined at five developmental stages including one day before anthesis, and 2 h, 24 h, 48 h, and 72 h after anthesis. Four storage temperatures, 4, 25, −20, and −80 °C, were applied to determine the optimum storage temperature and storage life of the pollens. Pollen germination was tested in a culture medium at 5-day intervals for 4 and 25 °C and every month for −20 and −80 °C. The highest pollen viability was observed by acetocarmine method. The viability of pollens before anthesis was 91.35% and reduced to 2.06% 72 h after anthesis. The highest germination was achieved in pollens gathered 2 h after anthesis (82.84%), which reduced to 0.19% 72 h after anthesis. Pollen germination was stopped after 20, 50, 60, and 60 days of storage at 25, 4, −20, and −80 °C, respectively.

1. Introduction

Leonurus cardiaca (Lamiaceae), commonly known as motherwort, is an important medicinal plant, which grows in many regions of Iran. It has been used to cure cardiovascular diseases, stress, anxiety, and nervous irritability (Russian Pharmacopoeia, 1968; Milkowska-Leyck et al., 2002). It is a perennial herb widespread in Europe, East Asia to the Himalayas, West Asia, Northern Africa, and North America. It is usually found in country areas throughout the hills and plains (Wojtyniak et al., 2013). Chemical compounds such as alkaloids, iridoids, flavonoids, saponins, and cardenolid like glycosides and diterpenoids have been detected and isolated from the leaves and flowers (Milkowska-Leyck et al., 2002). The healing of heart diseases is mainly due to flavonoids (Mockute et al., 2006).

The development of reliable methods for determining the functional quality of pollens helps in monitoring pollen vigor during storage, genetics and pollen–stigma interaction studies, crop improvement and breeding, and incompatibility and fertility studies (Shivanna and Rangaswamy, 1992). Pollen viability can be evaluated by many staining techniques: tetrazolium salts to detect dehydrogenase activity; aniline blue to detect callose in pollen walls and pollen tubes; acetocarmine to detect cytoplasmic content; fluorescein diacetate to determine esterase activity; and the intactness of the plasma membrane, *in vitro* and *in vivo* germination tests or analyzing final seed set (Dafni and Firmage, 2000; Dafni et al., 2005). Staining techniques can be used to assess pollen physiological conditions. As indicators of pollen viability, staining tests are often preferred because they are faster and easier compared to pollen germination, but they tend to overestimate the viability and real germination of pollen grains (Gaaliche et al., 2013). The appropriateness of the viability test depends on the species, since differences have been reported for optimal staining techniques (Khatun and Flowers, 1995; Rodriguez-Riano and Dafni, 2000). *In vitro* pollen germination is a very convenient and effective technique to study many basic and applied aspects of pollen biology (Heslop-Harrison, 1987; Kristen and Kappler, 1990). Therefore, to determine the actual amount of viable pollen, germination tests are necessary. The choice of the method depends on the crop or species (Dafni et al., 2005; Abdelgadir et al., 2012). Storage of pollen is necessary for germplasm conservation, breeding programs, and artificial pollination of dichogamous, self-incompatible, or male-sterile fruit species (Bhat et al., 2012; Lora et al., 2006). Longevity of pollens varies greatly with different plant species and storage conditions such as humidity and temperature (Dafni and Firmage, 2000). Viability and germination capacity of stored pollens have been studied on several plant species including almond

* Corresponding author. Fax: +98 2632248721.
E-mail address: nazeri@ut.ac.ir (V. Nazeri).

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(Martinez-Gomez and Gradziel, 2002), pear (Bhat et al., 2012), strawberry (Aslantus and Pirlak, 2002), and cherimoya (Lora et al., 2006). To investigate pollination potential, pollen quantity and viability and pollen germination capability should be estimated.

Reviewing the literature, we have found no report on the pollen biology of *L. cardiaca*. Therefore, this experiment was designed to (1) investigate the pollen viability; (2) select a reliable staining technique for testing pollen viability, pollen germination, optimal storage temperature, and storage life of *L. cardiaca* pollens.

2. Material and methods

2.1. Study site and plant materials

Seeds of one population of *L. cardiaca* were collected from Khansar, Isfahan, Iran. Plants were grown during April to September, 2014, in the greenhouse of Horticulture Department, University of Tehran, Karaj, Iran. Flowers were collected at anthesis stage, during 8:00–10:00 am, and transferred to the laboratory for further measurements.

2.2. Viability and in vitro germination of pollen

To evaluate pollen viability and germination at different stages of flower development, pollens were collected at five developmental stages including one day before anthesis, and 2 h, 24 h, 48 h, and 72 h after anthesis. To determine the viability with two colorimetric tests, pollens were distributed uniformly in a drop of 1 percent tetrazolium solution (2,3,5-triphenyl tetrazolium chloride) (Norton, 1966) and one or two drops of 2 percent acetocarmine (Marks, 1954) on microscopic slides. Five replicates were used for each test. The observations were made by light microscope (KF2, Zeiss, Germany). A pollen grain was considered viable if it turned red. The percentage of pollen viability was determined as the ratio of the number of viable grains to the total number of grains.

To determine pollen germination, pollen grains were sowed in Petri dishes containing culture medium (15% sucrose, 100 ppm boric acid [H_3BO_3] and 1% agar) with a clean brush, according to the method of Dane et al. (2004). The Petri dishes were incubated for 24 h at a constant temperature of 25 °C under normal light conditions. A pollen grain was considered germinated when its tube length was greater than its diameter. A minimum of 100–150 pollens were counted per petri dishes with 3 replicates. Germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollens per field of view. A light microscope was used to determine pollen germination.

2.3. Storage life

Pollens were collected from flowers at anthesis and stored in sealed 1.5 ml Eppendorf tubes. Before storing, the collected pollens were desiccated by incubating at 25 °C for 4 h. Four storage temperatures, 4 °C, 25 °C, −20 and −80 °C, were applied to determine the optimum storage temperature. A refrigerator and an incubator were used for 4 and 25 °C temperatures, respectively, and a freezer for sub-zero temperatures. Pollen germination was tested in petri dishes containing 10 ml of culture medium containing 15% sucrose, 100 ppm boric acid (H_3BO_3), and 1% agar by 5-day intervals for temperatures of 4 and 25 °C, and 30-day intervals for −20 and −80 °C.

2.4. Statistical analysis

Pearson correlation coefficients were calculated to investigate relationship between Pollen viability with TTC and pollen germination. Data were analyzed according to a one way ANOVA model (SPSS version 22), and the means were compared following Duncan's Multiple Range Test ($P < 0.05$).

Table 1
Pollen viability and *in vitro* pollen germination at different flower developmental stages.

Flower developmental	Pollen viability		*In vitro* germination (%)
	TTC (%)	Acetocarmine (%)	
One day before anthesis	86.93[a]	91.35[a]	73.63[b]
2 h after anthesis	83.40[b]	85.57[b]	82.84[a]
24 h after anthesis	33.06[c]	36.48[c]	24.37[c]
48 h after anthesis	18.76[d]	20.31[d]	11.04[d]
72 h after anthesis	1.33[e]	2.06[e]	0.19[e]

Means followed by the same letter in a column are not significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 2
Correlation coefficients among pollen viability with TTC and pollen germination.

Pollen germination	Pollen viability	
	1	Pollen viability
1	0.990[a]	Pollen germination

[a] Correlation is significant at the 0.01 level.

3. Results

3.1. Effect of flower development stage on viability and in vitro germination

The pollen viability at different stages of flower development are shown in Table 1. Determination of viability by TTC and acetocarmine showed maximum viability at one day before anthesis, while in the following days, these values reduced to 1.33 and 2.06 at 72 h after anthesis, respectively. The maximum pollen viability was observed in acetocarmine (Fig. 1A and B). Significant differences were observed among TTC and acetocarmine ($P < 0.05$) and five flower developmental stages ($P < 0.01$). The values of *in vitro* pollen germination at different time intervals after anthesis are shown in Table 1. The highest germination was found at 2 h after anthesis (82.84%), which reduced afterwards and reached the lowest germination at 72 h after anthesis (0.19%) (Fig. 1C). Pearson correlation coefficient showed that Pollen viability with TTC had positive significant correlation with pollen germination (Table 2).

3.2. Storage life

Pollen germination was assessed at 5-day intervals for temperatures of 4 and 25 °C and in monthly intervals for −20 and −80 °C and expressed as percentages of germinated pollen grains (Table 3). The viability of pollens stored at 4, 25, −20, and −80 °C reduced gradually to 2.34 (after 50 days), 5.62 (after 20 day), 7.63, and 9.58% (after 60 days), respectively. Differences in germinations among the temperatures tested were significant during the entire period of storage ($P < 0.01$). However, the results showed no significant differences among pollens stored at sub-zero temperatures. The pollen germination showed the same decreasing linear trends with increase in storage period in sub-zero degrees (Fig. 2). Additionally, decreasing linear trends with different slopes were observed for both temperatures of 4 and 25 °C, as pollen germination decreased more rapidly at 25 °C than did at the other temperatures (Fig. 3). Thus, there was an inverse relationship between pollen germination and the duration of storage.