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A novel approach for breaking seed dormancy and germination in Viola odorata (A medicinal plant)

Tayebeh Barekat[1], Mahmoud Otroshy[2*], Behnaz Samsam-Zadeh[1], Azadeh Sadr-arhami[3] and Arash Mokhtari[2]

1- Msc. Students of Agricultural Biotechnology, Payamnoor University, Isfahan, Iran
2- Department of Tissue Culture, Agricultural Biotechnology Research Institute of Iran (ABRII), P. O. Box 85135-487, Isfahan, Najafabad, Iran
3- Department of Agricultural Biotechnology, Payamnoor University, Isfahan, Iran

***Corresponding author*:** Mahmoud Otroshy

ABSTRACT: *Viola odorata* is a hardy herbaceous flowering perennial Medicinal plant mainly used as an herbal remedy in cases of diabetes and cancer which have a low germination rate under normal laboratory conditions due to hard seed coat and thermal dormancy. Since, creating the optimal conditions for seed germination is essential for development of its cultivation. Hence, the presention investigation was carried out to study the effect of different breaking seed dormancy techniques to optimize and speed up the germination percentage and rate under *in vitro* condition. So the effect of scarification, cold stratification, isolation and culture the endosperm or embryo, hydro-priming, leaching and presence of different kinetin and giberlic acid concentrations in the MS culture medium were compared with the control without any manipulation. The results showed that the highest germination percentage (74.5%) and germination rate (57.5) were observed in endosperm culture followed by, embryo culture and sulfuric acid (96%) for 60 min, respectively.

***Keywords*:** Embryo culture, Plant Growth Regulators, Thermodormancy, Scarification, Sweet violet.
***Abbreviations*:** GA_3- Gibberellicacid, GP- germination percentage, Rs- germination rate.

INTRODUCTION

Viola odorata is a species of the genus viola (MALPIGHIALES: Violaceae) native to Europe and Asia and includes more than 400 species known as sweet violet (Mabberley, 1987). *Sweet violet* is used for the treatment of bronchitis, common digestive disorders, postoperative tumor metastasis, diabetes and cancer. Phytochemically, different groups of compounds have been isolated from various species of this genus like cyclotides, flavonoids, alkaloids and triterpenoids. Some of them already have been scientifically accepted as antifungal, antibacterial, anticancer, antioxidant, antiasthmatic, anti-inflammatory, anti-HIV and antipyretic agents (Ireland et al., 2006; Ebrahimzadeh et al., 2010; Gustafson, 2004).

The plant is conventionally propagated through the divisions of rhizomatous disc, but for large scale cultivation the use of the seeds is preferred. However, the germination rate of this medicinal plant is low due to sever seed dormancy (lord, 1983). Seed dormancy is a physiological occurrence in some medicinal plants caused by external or internal factors such as hard seed coat, immature embryo, rudimentary embryo and inhibitors materials and needs to temperature changes prevent of seed germination, even in optimal conditions (Estaji et al., 2012).

Seed germination has also prevented due to the embryo is constrained by its surrounding structures such as seed coat and endosperm. It is possible to release dormancy by removing the surrounding structures in seed and scarification, embryo culture and endosperm culture techniques are applied to break seed dormancy (Mabundza et al., 2010).

Thermodormancy which is expressed as germination in a narrower temperature range (Corbineau et al., 1993). Low temperatures and/or pre-chilling treatments are considered as the common approach in order to break seed dormancy in Violacea family (Geneve, 2008). It is reported that for some species of pansy (Viola), germination at high temperatures (>30˚C) can be inhibited by thermodormancy. To overcome thermodormancy, cold stratification and growth regulators such as Gibberellic acid alone and/ or in combination with Kinetin and Ethylene also plays an important role to increase the germination rate of the seeds in *Viola odorata* (Cantliffe 1991; Carpenter and Boucher, 1991).

Unfortunately, there is limited information concerning the potential seed dormancy problems for *V. odorata*.We elevated the effect of different chemical and mechanical treatments on breaking dormancy of Sweet violet seeds.

MATERIALS AND METHODS

Seed material and sterilization

This study was conducted in the Plant Tissue Culture Department of Agricultural Biotechnology Research Institute of Iran in 2013. The seeds Of *V. odorata* were prepared from Natural Resources Research Institute, Shahid Fozveh station, Isfahan, Iran.

In order to surface sterilization, the seeds were washed with sterile distilled water 5 times, immersed in 70% ethanol for 1 minute and were washed with sterile distilled water 3-4 times. Then seeds were soaked in 1% sodium hypochlorite (NaOCl) + 1 drop tween-20 for 15 min and finally rinsed with sterile distilled water 4 times under a laminar flow cabinet.

Pretreatments and culture of Seeds

In order to elevate the germination rate and percentage, treatments of hydro-priming with tap water soaking for 48 hours, KNO_3 soaking (0.5%) for 30 min, GA_3 (500, 1000 and 2000 ppm) and Kin (100 and 200 ppm) soaking for 3 days, continues tap water washing (leaching) for 3 days, hot water scarification for 1 hour, sulfuric acid scarification (for 45, 60 and 75 min), seed stratification at 4 °C for 2 months, scratching + stratification at 4 °C for 2 months, Kin (3 and 4 mg/l) + scratching, GA_3 (4 and 6 mg/l) + scratching, endosperm and embryo (Fig.1 a and b) culture were applied. Five pretreated seeds were cultured per bottle containing 30 ml of ¼ MS medium (Murashige and Skoog, 1962). Treatment without any manipulation was considered as a control. All cultures kept at 25º C in the 16/8h (light/dark) photoperiod provided by cool white fluorescent tube with light intensity of 2700 Lux for 7-10 days and allowed to grow.

a b c d

Figure 1. embryo (a), Endosperm and seed (b), Germinated endosperm (c) and seedlings of *viola odorata* grown in 14-day (d)

Data collection and experimental design

To determine the germination rate, number of rooted seeds were counted as the germinated seeds every 24 hours. The following equations were used to calculate the germination rate (Rs) and germination percentage (GP) of the seeds (Equations 1, 2) (shah, 2002).

$$Rs=\sum_{i=1}^{n}\frac{Si}{Di}*100 \quad GP=100*(n/N)$$

Rs: germination rate GP: germination percentage

S_i: number of germinated seeds in each count n: number of germinated seeds

D_i: number of days to (n) counts N: total number of Seeds

n: times counting (Equation 2)

(Equation 1)

The experiment was done in a Completely Randomized Design with 4 replications (cultural battle) and 5 seeds per each battle. Data were analyzed by the SAS statistical computer program (Ver.9). When the ANOVA indicated significant treatment effects (5 or 1%) based on the F-test, the Duncan's Multiple Range Test ($P \leq 0.05$) was used as a method to determine which treatments were significantly different from other treatments.

RESULTS AND DISCUSSION

The results of the ANOVA Procedure (Table 1) indicated that applied treatments affected the germination percentage and the germination rate ($p<0.01$).

Table 1. The ANOVA Procedure for the effect of various breaking seed dormancy Treatment on germination percentage (GP) and germination rate (Rs)

Sources of variation	df	GP*	Rs*
Treatment	20	1937.773**	1073.254**
Error	63	92.811	67.310

**significant at P≤0. 01. *RS: germination rate, GP: germination percentage

According to Table 2, the endosperm culture treatment caused the best responses (74.58%) on germination percentage parameter (Fig.1 c and d). After this, the embryo culture and sulfuric acid for 60 min (49.9%) showed no statistically significant difference and leads to better germination percentage compared with other treatments. The germination rate was highest in endosperm culture (57.5) followed by concentrated sulfuric acid for 60 min.
The use of hydro-priming with tap water, soaking for 48 hours, KNO_3 soaking (0.5%) for 30 min, GA_3 (500, 1000 and 2000 ppm) and Kin (100 and 200 ppm) soaking for 3 days, continues tap water washing (leaching) for 3 days and hot water scarification for 1 hour like control treatment resulted in no germination of seed (Table 2).

Table 2. Effects of various breaking seed dormancy treatments on germination percentage and germination rates of Viola odorata

Treatments	GP	Rs
Control	0.00 ^{f}	0.00 ^{f}
Embryo culture	54.31 ^{b}	22.75 ^{cd}
Endosperm culture	74.58 ^{a}	57.50 ^{a}
Cold stratification	28.33^{cd}	24.75 ^{cd}
GA_3 – 4 mg/l	15.62 ^{fde}	13.75 ^{def}
GA_3 – 6 mg/l	29.99^{cd}	24.50 ^{cd}
Kin – 3 mg/l	21.66 ^{cde}	16.75 ^{de}
Kin – 4 mg/l	18.33 ^{de}	18.00 ^{cde}
Concentrated sulfuric acid 45 min	5.00 ^{fe}	6.75 ^{fe}
Concentrated sulfuric acid 60 min	49.99 ^{b}	42.50 ^{ab}
Concentrated sulfuric acid 75 min	39.10^{bc}	32.50 ^{bc}
Scarification and Cold stratification	10.00 ^{fe}	11.75 ^{def}
Hydro-priming	0.00 ^{f}	0.00 ^{f}
KNO_3-0.5%	0.00 ^{f}	0.00 ^{f}
GA_3 soaking -500 ppm	0.00 ^{f}	0.00 ^{f}
GA_3 soaking -1000 ppm	0.00 ^{f}	0.00 ^{f}
GA_3 soaking -2000 ppm	0.00 ^{f}	0.00 ^{f}
Kin soaking -100 ppm	0.00 ^{f}	0.00 ^{f}
Kin soaking -200 ppm	0.00 ^{f}	0.00 ^{f}
Leaching for 1 hour	0.00 ^{f}	0.00 ^{f}
Hot water scarification	0.00 ^{f}	0.00 ^{f}

Values followed by the same letter(s) within a column are not significantly differentp≤0.05 (LSD test)

As shown in Table 2, unscarified seeds did not show good germination, so it indicates that *V. odorata* seeds have typical hard and impermeable seed coats (physical dormancy). Generally, seed germination status depends on embryo growth potential and chemical or physical inhibitors (Koorneef et al., 2002).

This potential depends on the seed structure especially mechanical resistance present in the structures covering the embryo and affective factors on embryo growth such as plant growth regulators and environmental factors. Previous studies have been suggested that the seeds of Violaceae (violet) have a seed coat with a

mucilaginous inner layer that contains inhibitors. Therefore, it was hypothesized that such inhibitors present in the seed coat and endosperm interfered with seed germination (Benech et al., 1998; Mares, 2005).

Foley, (2004) and Lopez, (2005) reported that unscarified seeds of closely related species *Euphorbia esula* and Euphorbia heterophylla did not exhibit any imbibition or germination as a result of their hard seed coats and germination of them was enhanced by manual removing of the seed coat from the endosperm surrounding the embryonic axis and scarification by sulfuric acid pre-treatments, respectively.

Hence, seed coat and endosperm removal by various scarification methods or using the endosperm and embryo culture techniques possibly can eliminate such inhibitors and subsequently could be enhanced the potential of seed germination in V. odorata*,* which is in agreement with the results obtained from this study (table 2).

Further physical dormancy, it has also been reported that in some species of viola, germination at high temperatures (> 30˚C) can induce thermal dormancy (Cantliffe, 1991; Carpenter and Boucher, 1991).

As respects, cold stratification and adding plant growth regulators to culture medium have been able to promote the GP and Rs, it seems possible that these results are due to the presence of thermal dormancy in this plant. This finding is in agreement with Cantliffe (1991) and Carpenter (1991) findings which have stated that thermal dormancy can be relieved by using cold stratification and exogenous combinations of plant growth regulators.

In conclusion, the results of various treatments in our study confirmed that seed of *V. odorata* exhibit dormancy due to its hard seed coat. Various seed scarification methods break down *V. odorata* seed coat impermeability which is resulting in a considerable increase in germination percentage and rates. Also, cold stratification and exogenous plant growth regulators relatively enhanced the seed germination. This finding further strengthens the possibility of thermal dormancy existence in the seeds of this plant. As regards, the seeds of *V. odorata* are dormant due to hard seed coat so it is propagated conventionally by the rhizomes, nevertheless for the large-scale production to facilitate the seedling propagation and to accelerate the generation turnover in breeding programs, development of a rapid seed germination method is desirable. Therefore, it is possible to increase a significant germination percentage and save the time required for platelet production of *v. Odorata* using endosperm or embryo culture techniques described in the present study.

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