The estimation of pollen viability of snowdrop (*Galanthus nivalis* L.)

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Summary: In the present experiment pollen viability of snowdrop were tested using four vital dyes (TTC, Baker' procedure, MTT and FDA) to determine their potential to differentiate fresh living pollen from pollen heated for 12 hours at 80 °C (killed pollen). We found that two of the four dyes previously employed to determine viability also stained killed pollen in the case of snowdrop, while the FDA and MTT did not. We suggest that the two latter are probably the best method to test snowdrop pollen viability, since they do not normally stain either killed or aborted pollen.

Introduction

The need for assessing pollen viability of natural populations is very important in the understanding and monitoring of the effects of environmental stresses during the flowering period (*Thomson,* 1994). Pollen viability and performance highly depends on the environment. In this regard, environmental factors such as temperature and soil fertility affect *in vivo* pollen performance (*Zamir & Gaddish,* 1987). Adverse climatic and environmental conditions are deleterious to pollen viability and can result in decrease of population size caused by complete pollen abortion. The consequent study of pollen viability and performance of natural populations can help to monitor the negative changes in the environmental conditions such as air and soil pollution (*Bellani* et al., 1988), accumulation of toxic compounds, different temperature and water stresses, etc. Unfortunately, there is only a limited number of studies available on the pollen viability of the early flowering bulbous horticultural crops, which are generally frost tolerant.

Snowdrop – the "Fair Maid of February" – is the first flower of the year, generally it flowers as early as February. It is a native of Switzerland, Austria and of Central- and Southern Europe generally, but where naturalized elsewhere spreads into considerable masses, and is abundant wherever it occurs. There is probably no bulbous plant, however, which possesses all its extreme hardiness in resisting cold, shows such a marked preference or distaste for certain localities, even though there may be little variation in soil or altitude. In some districts snowdrops will grow and spread in woods; in others, with apparently identical conditions, it is difficult to get them to grow and they will refuse to spread.

Nowadays, natural snowdrop populations are in a critical situation in many places around Europe, as their original growing territories are partially occupied by the human setlements. On the other hand, this nice flower is frequently collected in early spring and snowdrop is also a major source of some important medical drugs (*Gilljam,* 1993, *Hammar* et al., 1995). Beside of the human activity the changing environmental conditions (e.g. pollution) are also dangerous for the natural populations. Although the measurement of pollen viability of snowdrop are not defending the natural populations, the establishment of such monitoring systems can help in the maintenance of some, still untouched populations in their original form. Unfortunately, according to our knowledge, no detailed studies have been carried out in examining pollen viability of snowdrop.

A large variety of dyes have been used to test pollen viability of several species, but only a few studies have tested the potential risk of these dyes to stain killed pollen (*Rodrigez-Riano & Dafni,* 2000). The most common nuclear and vital dyes, which indicate the presence of cytoplasm or enzymes, respectively, used thus far (Alexander's procedure, acetocarmine, aniline blue in lactophenol, TTC, MTT and X-Gal) have recently been strongly criticized, as they also stain killed pollen (*Kapyla,* 1991, *Parfitt & Ganeshan,* 1989, *Khatum & Flowers,* 1995, *Sedgley & Harbard,* 1993). Therefore the aim of the present study was to compare different vital dyes to determine their potential efficacy as indicators of snowdrop pollen viability.

Materials and methods

Plant material

Plants of a native population of snowdrop (*Galanthus nivalis* L.) was studied in our present experiment *(Figure 1).* Flowers were collected from the park of our institute at the

time of anthesis and brought into the laboratory. The flowers were kept in a germination chamber at 14 °C under 150 mmol/s/m^2 light intensity until the anthers opened. Depending on the amount of pollen per anther one or two anthers per flower were used in the different viability tests. Pollen was extracted and mixed on a microscope slide and then divided into two samples. The first sample was immediately stained with one of the vital stains, while the other part of the pollen was heated to 80 °C for 12 hours (overnight). The second sample was designated as dead pollen control.

Pollen performance and viability tests

The morphology of the mature snowdrop pollen was studied using the Alexander's procedure (*Alexander,* 1969) in comparison of an unstained pollen population. In the case of unstained pollen studies the pollen grains were suspended in an appropriate concentration of sucrose. The number of normally developed, young and aborted pollen grains were differentiated.

Four different methods for staining were used to test pollen viability.

a] Tetrazolium test

The tetrazolium test (*Aslam* et al., 1964) is based on the reduction of a colorless soluble tetrazolium salt to a reddish insoluble substance called formazan, in the presence of dehydrogenases. Nitroblue tetrazolium and 2,3,5-triphenyl tetrazolium (TTC) are the most commonly used tetrazolium salts. The test solution consisted of 0.2–0.5% TTC in 0.1 M phosphate buffer (pH = 7.2) and in sucrose solution of suitable concentration to prevent bursting of pollen grains. The pollen grain was considered viable if it turned red.

b) Baker's procedure

Baker's procedure (*Dafni,* 1992) detects the presence of alcohol dehydrogenase. The test solution consisted of 7 mg phosphate buffer/10 ml water (pH 7.3); nitroblue-tetrazolium just give a slight yellow color; 6 mg nicotinamid adenine dinucleotid and 0.5 ml of ethanol (35%). The pollen grain was considered viable if turned violet or pink.

c) MTT test

MTT test (*Khatum* & *Flowers,* 1995, *Norton,* 1966) detects the presence of dehydrogenase. The test solution consisted of a 1% concentration of the substrate 2,5-diphenil tetrazolium bromide (MTT) or thiazol blue in 5% sucrose. The pollen grain was considered viable if it turned deep pink or if it presented no color but showed irregular black lines over its surface (according to *Rodriguez-Riano* & *Dafni,* 2000).

d) Fluorochromatic reaction (FCR) test

When pollen grains are mounted in fluorescein diacetate (FDA) solution, the nonpolar, nonfluorescent FDA readily enters the pollen cytoplasm. Cytoplasmic esterases hydrolyze FDA and release fluorescein, which is polar and fluorescent (*Heslop-Harrison* et al., 1984). Unlike FDA, fluorescein passes sparingly through an intact membrane and therefor accumulates in the cytoplasm of *viable* pollen grains and gives a bright green or yellowish green fluorescence under the fluorescence microscope. For testing pollen viability fluorescein diacetate was dissolved in acetone (5 mg/ml) and used at 10^{-6} M in 60% (w/v) sucrose according to *Heslop-Harrison* (1970). The pollen was considered viable if strong bright fluorescence was observed under fluorescent microscope (490–510 nm).

All non fluorescent pollen viability tests were conducted by incubating the pollen in the medium for at least 30 min at 35 °C. Five replications per each samples and five random groups of at minimum 300 pollen grains per replica was counted in the present experiment. In the case of FDA test similar amount of pollen grains was studies 2–5 minutes after staining.

Statistical analysis was carried out using SPSS for Windows 8.0 statistical software package.

Results and discussion

Snowdrop pollen grains are relatively small and the majority of the freshly shed pollen has a very good performance *(Figure 1).* According to the pollen morphological studies *(Table 1)* about 99% of the pollen grains show normal phenotype, while only 1% of the population was still vacuolated immature microspore. Interestingly, no aborted or sterile pollen grains were observed under light microscopy without staining. The cytoplasm ripeness test (Alexander test) is very sensitive to osmotic potential of the dye and therefore several pollen grains burst, which made the study a little bit complicated. Although the majority of the pollen grains showed normal pollen morphology, the ratio of the aborted or sterile pollen grains become higher probably as a result of staining.

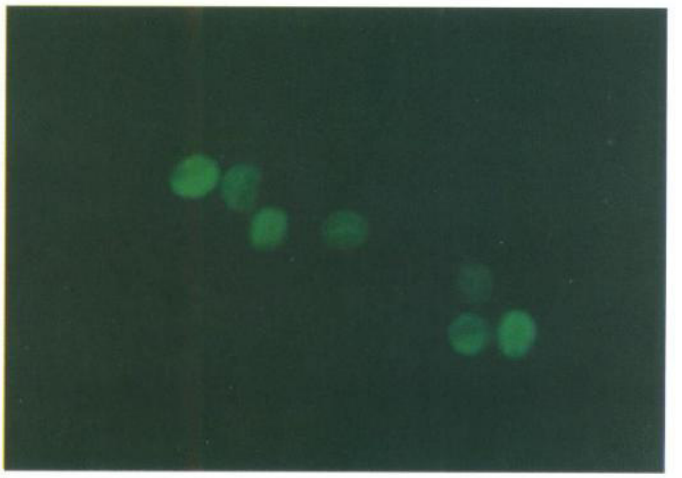

Figure 2 Living pollen grains, stained with FDA

Table 1 Morphological features of the freshly shed snowdrop pollen grains

Treatment	% of mature pollen	% of immature pollen	% of sterile abnormal pollen
Without staining	99.0	1.0	0.0
Alexander test	97.2	0.8	2.0*

* significantly differ from the unstained control at p= 0.05 probability level, respectively

The cytoplasm ripeness test stained completely the killed pollen grains also, and therefore it cannot be of use as a viability dye. The high temperature treatment to kill the pollen grains did not modify the pollen performance, significantly (data not shown).

According to the results obtained in our experiment snowdrop pollen viability rates fluctuate depending on the test used. The maximal values were obtained with TTC test, the values given by Baker's procedure and MTT were next to each other but of a lower level, and the minimum were given by the FCR test *(Table 2)*.

Table 2 Viability percentage of fresh and killed snowdrop pollen as determined by four vital dyes

Test	Fresh	Killed
TTC	85.6	75.4*
Baker's	83.6	81.7ns
MTT	85.1	1.2***
FDA	80.1	0.0***

ns – not significant, *,*** – are significant at p= 0.05 and 0.001 probability level, respectively

According to the results of the experiment, three groups of dyes are apparent; those that always or almost always stained killed pollen in the case of snowdrop, those that stained killed pollen in some replications but not consistently across the experiment and those that never stained killed pollen. Dyes in the first group (TTC and Baker's) always stained some killed pollen. The difference between this two dyes was that Baker's stained killed pollen faster than fresh pollen and TTC stained killed pollen at the same rate as fresh pollen. With the TTC staining solution, where 100% of pollen stained red color even after killing by heating, presumably the reduction is brought about by some heat stable component of the pollen. Whatever is the mechanism of the reduction, the use of TTC is obviously not appropriate for testing viability of snowdrop pollen. Similar results were obtained by *Rodriguez-Riano & Dafni* (2000) in the case of Baker's procedure. Therefore, we recommend that TTC and Baker's test should not be used to test snowdrop pollen viability.

The second group (MTT) showed many different color tonalities and sometimes the very dark pink pollen was difficult to distinguish from the black. In addition, MTT seldom stained killed pollen, although when it did the stain was always lighter than with fresh pollen. Therefore MTT should be used with caution, taking into consideration the sensitivity of species being tested. *Parfitt & Ganeshan* (1989) found that, in the case of some Prunus species, heat-killed pollen was intensely stained, and *Riano-Rodriguez & Dafni* (2000) also found that the two hours heat treated pollen could be stained in the case of several species.

The use of fluorescein diacetate test was the most reliable method to distinguish between fresh and killed pollen, since dead pollen were completely black under UV light and did not show any fluorescence at all. Within 2–3 minutes incubating fresh pollen in fluorescein diacetate living pollen grains appeared bright green. Unfortunately during counting some of the pollen grains showed decrease in its fluorescence and after 5–10 minutes the pollen population became highly variable. Some grains still fluoresced very bright while others less brightly, some were half bright and others were non-fluorescent. This result suggests that snowdrop pollen is highly sensitive to UV light, which gradually kills the pollen grains. According to this we have to be very careful using this method in monitoring snowdrop pollen viability *(Figure 2)*.

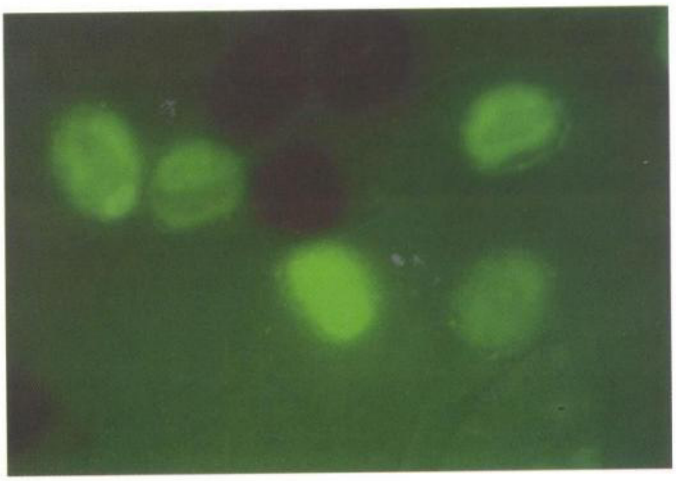

Figure 3 Mixed pollen population of snowdrop after 5 minutes illumination with UV light (bright fluorescent pollen grains are living ones, while the dark black ones are killed by the UV light.

Out of the four dyes tested, only the MTT and FDA test showed a high correlation with pollen viability as they did not stain either killed or aborted pollen. The variability of color tonalities and fluorescence may sometimes make it difficult to use this dyes also to differentiate between fresh and killed pollen. The prompt use of FDA probably is the most promising method to solve this problem.

According to the results of the present study we recommend using some type of control (such as killed pollen) to check the potential of the method to test pollen viability before using it. If it stains killed pollen, then it must be avoided in agreement with the suggestion of *Rodriguez-Riano & Dafni* (2000). The next step should be to test the potential capability of the dye to stain non-germinated pollen at different ages, because sometimes the use of vital

dyes is unsatisfactory (*Sedgley* & *Harbard* 1993). Based on our results we suggest to use the MTT or FDA test to monitor pollen viability in snowdrop, and the use of this vital dyes could help to follow environmentally caused changes in natural snowdrop populations.

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