ANALYSYS ON POLLEN AND SEED PRODUCTIVITY AND EFFECTIVENES IN GENTIANA LUTEA L.

E. Yankova¹, G. Baldzhiev¹, M. Petrova², E. Zayova² and P. Yurukova¹
¹Bulgarian Academy of Sciences, Institute of Botany, Sofia, Bulgaria
²Bulgarian Academy of Sciences, Institute of Genetics “Acad. D. Kostoff”, Sofia, Bulgaria
Correspondence to: Elina Yankova
E-mail: e_jankova@abv.bg

ABSTRACT
A study on the viability of pollen grains and embryos in Gentiana lutea distributed in Bulgarian flora has been carried out. Degenerative processes predominantly of the mature pollen were observed. As a result of that, in the studied populations, the effective mature pollen grains in the anthers reduce in different amount. The used tetrazolium test shows that in the mature seeds the viability of embryos reduced progressively during the three consecutive years of the present study.

Keywords: Gentiana lutea, pollen and embryos viability, tetrazolium test

Introduction
Gentiana lutea L. is an important medicinal plant species whose roots are used as a remedy for augmentation of the gastric secretion and digestion from ancient times. The native populations of this species in Bulgaria are under high anthropogenic influence. Despite that they are distributed in higher parts of the mountains and some of them are included in protected areas, the active collection of the plants as a source for the pharmaceutical industry leads to extinction of G. lutea.

The species is protected by the Law of biodiversity (6) and included in the Red Data Book of PR Bulgaria (11) and in the Red list of Bulgarian vascular plants (8) in the category “endangered species”. G. lutea is also presented in the List of endangered medicinal plants in the Anex to the Council Regulation (EC) No. 338/97 of EU (9) which purpose is the protection of the plant species by control of the trade with them. In addition, G. lutea is included in the Directive 92/43/EEC of 21 May 1992 (2) on the conservation of natural habitats and of wild fauna and flora.

It is established that the state of the populations of G. lutea is under influence of different factors, such as: conditions in the natural habitats (soil composition, climatic conditions); slow development of the species and the fact that the mature individuals blossom and produce fruits at the tenth year of its development; the difficult germination of the seeds as well as a limitation on seed regeneration and domination of the vegetative one (4).

The aim of this study is to analyze the viability of the pollen grains and embryos of G. lutea in order to estimate its role on the realization of reproductive capacity of the species in natural conditions.

Materials and Methods
Sites and populations
As plant material, flowers buds, flowers and seeds originated from three Bulgarian native populations of G. lutea were used:

1. Vitosha Mt (Western Bulgaria). The population is situated on the nord slope in the foot of the peak Golyam Rezen. The altitude is of 1900 m and inclination 10%. The projective amount of G. lutea is 10%.
2. Pirin Mt. The population is situated on the rout from chalet Vihren to Kazanite and under the way connecting the chalets Vihren and Banderitsa, on the east slope, at altitude in the interval of 2150 to 2250 m. The projective amount of G. lutea is 2%.
3. Rodopi Mts (Eastern). The population is situated on the ridge part of peak Shabanitsa, above the village Kesten, at altitude of 1800 m. The projective amount of G. lutea is 30%.

Pollen viability test
For an estimation of the pollen viability, flower buds and flowers were collected and fixed in a mixture of FAA (formalin:glacial acetic acide:70% ethanol in correlation 5:5:90 parts). A continuation the material was embedded in paraffin, cut into 12-25 μm sections and treated according to classical paraffin methos. The sections were stained with Heidenhain’s hematoxilyn. The permanent slides were mounted in Canada balsam. The observations were carried out with “Olimpus CX 21” light microscope.
Embryo viability test

To estimate embryo viability, a quick test completed within 24 h was applied – the known as a tetrazolium test. The isolated embryos were incubated in a diluted (1 %) solution of 2, 3, 5-triphenyltetrazolium chloride, according to Peters (7). Initially, the tetrazolium solution is colorless, but changes to red when it comes into contact with hydrogen (reduction) derived from enzymes in the respiration process. Embryos showing active respiration turn red and are considered viable. The darker the color, the greater the respiratory activity in the seed (Fig. 1f). Light pink indicates an embryo with less viability than an embryo staining dark red (Fig. 1f). Depending on the staining, the embryos were classified into three classes and deemed viable and nonviable: Class I – embryos stained 100 % (red colored embryos) (Fig. 1d.), Class II – embryos stained 10 % (only root stained in red – Fig. 1e), Class III – embryos stainless Viable embryos represented the color patterns of class III (Fig. 1g). The microphotographs were made with “Infinity lite” Camera, 1,4 Mpx.

**TABLE 1**

<table>
<thead>
<tr>
<th>Locality</th>
<th>No. of flowers</th>
<th>No. of pollen grains analyzed</th>
<th>% of fertile pollen grains</th>
<th>Standart deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitosha mountain</td>
<td>30</td>
<td>7149</td>
<td>11,69</td>
<td>14,46</td>
</tr>
<tr>
<td>Rodopi mountains</td>
<td>30</td>
<td>5993</td>
<td>54,76</td>
<td>32,06</td>
</tr>
<tr>
<td>Pirin mountain</td>
<td>30</td>
<td>13635</td>
<td>8,24</td>
<td>21,31</td>
</tr>
<tr>
<td>Total:</td>
<td>90</td>
<td>26777</td>
<td>19,58</td>
<td>31,08</td>
</tr>
</tbody>
</table>

These results show that the size and state of the population are affected from the amount and productivity of the seeds. The highest amount of viable pollen is produced in the flowers of the population from the Rodopi Mt, which is characterized with highest projective cover of the species (30%), optimal development, flowering, regular fruit production and a significant degree of seed formation. The characteristics observed are due to the fact that this population is located at the frontier with Greece and the access to it is limited, which in its turn helps to keep the natural character of the habitat. In the other two populations located in areas that form a part of active tourist routes, the projective cover of the species is lower then the above mentioned - respectively 20% in the population from Pirin Mt and 10% in the population of Vitosha Mt. The low percent of effective pollen in these populations might be explicated with the conclusions of some authors (1, 3, 10) that the unfavorable conditions in the alpine belt (low temperatures and short growing season) limit the plant pollination and fertilization.

In *G. lutea*, a high seed productivity is established [94 % - (5), 95,52 % - (4)] and a high viability of embryos [89 % - (4)] that indirectly show for a high viability of the pollen needed for a high seed production. However, this is not in correlation with a low efficiency of the mature pollen observed in populations of Pirin Mt and Vitosha Mt. In an embryological study, an aposporous embryo sacs initiation in *G. lutea* is found (12) that like meiotic legitimate ones can also lead to a seed formation and in its turn probably is a reason to increase the reproductive capacity of this species.

The high viability of embryos of *G. lutea* in the moment of the maturation of seeds established by fuchsin test (89% - (4) and by cultivation in KNOP solution [81% - (4)] decrease quickly from 60% in the first 5 months up to 35% during a year (Table 2). Later on, the viability is maintained only in the root apex in about 1/3 of the embryos (33 %) that according the used test for viability of the embryos are classified as unviable embryos.

Results and Discussion

The previous embryological study on *G. lutea* (12) shows that the micro-, megasporogenesis and development of the male and female gametophyte in this species run normally and only insignificant deviations are registered during the meiosis in the microspore mother cells (MMCs). It is important to notice that degenerative processes affect predominantly the mature pollen despite that they begin during the homeotypic division of the meiosis in MMCs and cover only some microspore tetrads in the anthers. One-nucleate pollen grains formed after the disintegration of the tetrads are viable (Fig. 1c). Later on, the pollen begins to degenerate and as a result in the studied populations the amount of the effective mature pollen decreases (Table 1). The lowest percent (8,24 %) fertile pollen was found in Pirin Mt population. Relatively higher amount of viable pollen grains (11,69 %) was established in the population from Vitosha Mt, while in Rhodopi Mt population the pollen fertility rate is significantly higher (54,76 %).
Embryo viability assessed in three consecutive years by tetrazolium test

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of embryos analyzed</th>
<th>Viable embryos %</th>
<th>Empty seeds % (without embryo and/or endosperm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>60</td>
<td>0 %</td>
<td>26.67 %</td>
</tr>
<tr>
<td>2008</td>
<td>60</td>
<td>35 %</td>
<td>12 %</td>
</tr>
<tr>
<td>2009</td>
<td>60</td>
<td>60 %</td>
<td>5 %</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>31.25 %</td>
<td>6.67 %</td>
</tr>
</tbody>
</table>

Fig. 1. Pollen and embryo viability in Crepis lineata L. a, b - degenerated mature pollen grains (a x100, b x400); c - one-nucleate viable pollen grain (c x100); d - viable mature embryos (x20); e - nonviable embryos (x20); f, g - viable seed after tetrazolium testing (x20).
Conclusions
The results of the present study give the reason to conclude that:

The germination of the seeds of *G. lutea*, besides announced in the literature physiological dormancy is also influenced from a rapidly decreasing of the embryo viability.

Analysis on the pollen and embryos viability in this species carried out provides an explanation of the connection between them. The reflection of the viability established during the study on the reproductive capacity of the populations as an important factor that impact the size of the populations of *G. lutea* was revealed.

Because of the limited distribution of *G. lutea* in Bulgaria, its status as a threatened species, and its importance for medical practice is necessary to cultivate the species. In this direction the embryo cultures are more appropriate to overcome the seed dormancy as well as the rapid reduction of seed viability in the course of time.

Acknowledgments
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REFERENCES