Reproductive capacity and *in vitro* cultivation of the glacial relict *Papaver degenii* (*Papaveraceae*)

Rossen N. Gorgorov, Elina P. Yankova, Georgi A. Baldjiev, Iva I. Apostolova, Petka D. Yurukova-Grancharova, Marina I. Stanilova

Department of Plant and Fungal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Acad. Georgi Bonchev St., bl. 23, 1113 Sofia, Bulgaria, e-mail: maris@bio.bas.bg (corresponding author)

Received: June 06, 2011 ▹ Accepted: October 31, 2011

Abstract. In Bulgaria, *Papaver degenii* is found only in the alpine area of the Pirin Mts and is considered a local endemic. The species' vulnerability and limited occurrence raise questions about its reproductive capacity and conservation, both *in situ* and *ex situ*. An embryological study has revealed that *P. degenii* can reproduce sexually, but has a low pollen viability (20,4 %) and a high rate of unviable embryos (68,9 %), which suggests a prevalence of vegetative reproduction. To improve propagation, the plant was cultivated *in vitro*, with seeds as initial material. Several MS-based nutrient media were tested, including in a temporary immersion system. Adaptation of plantlets to ambient conditions was started.

Key words: germination, *in vitro* micropropagation, pollen and seed viability, poppy, reproductive biology

Introduction

Bulgarian flora includes nine *Papaver* L. species, distributed mostly at lower altitudes, and only *Papaver degenii* (Urum. & Jav.) Kuzmanov is an alpine species (Fig. 1). It is localized in the Pirin Mts and is considered a local endemic (Kuzmanov 1970). The diploid chromosome number established for the studied population is 2n=14 (Andreev 1981). The taxonomical status of this species has changed during the years (Urumoff 1920; Markgraf 1958; Kuzmanov 1970; Schönswetter & al. 2009) as a reflection of the polymorphic structure of *P. alpinum* s.l. The latest molecular and morphological studies of *P. alpinum* do not support the recognition of *P. degenii* as a separate species but as a part of *P. alpinum* subsp. *alpinum* (Schönswetter & al. 2009). Irrespective of the taxonomical interpretation, *P. degenii* is considered a vulnerable species (VU D1+2) at national level (Stoeva 2009).

Fig. 1. *Papaver degenii* in its native Bulgarian population near peak Vihren, July 2010.

The species originates from the Pleistocene (Kuzmanov 1970, Schönswetter & al. 2009). This is regarded as a reason for its adaptation to the cooler climate of the high mountains. *P. degenii* grows exclusively on marble screes or rock fissures. It has a branched rhizome and semi-rosette leaves, which support its persistence in a moving substrate with very shallow soil.

P. degenii takes part in distinct communities confined to the *Papaveri degenii-Armerietum alpinae* association, considered a pioneer community for crystalline limestone screes (Mucina & al. 1990). This vegetation type is endemic for the Pirin Mts and belongs to *Veronico kellererii-Papaverion degenii* alliance of the class *Thlaspietea rotundifolii*. The total projection cover within the sample plots of 16 m^2 registered in 2010 did not exceed 35 % and the abundance of *P. degenii* was on the average 5 % there.

Restricted occurrence of this species requires special attention for its further preservation. The current threats are mostly linked to an enhanced tourist flow. The plant is beautiful and attracts the hikers, or suffers from being trampled on near the trails. The size and persistence of *P. degenii* population also depends on the pollen and seed viability, as well as on its resistance to climate warming as a glacial relict. The present studies are aimed at elucidation of the reproductive capacity of the species and at application of *in vitro* techniques for rapid plant multiplication and *ex situ* conservation.

Material and methods

Plant material

Flower buds, open flowers, stems, and mature seeds of *P. degenii* were collected in July and August 2010 from its Bulgarian native population near peak Vihren (Pirin Mts), at an average altitude of 2800 m. Mature seeds were kept in paper bags at room temperature and stems were stored in wet paper. The quantity of the collected plant organs was in conformity with the vulnerability status of the species.

In vivo study of the reproductive capacity

To estimate the *in vivo* reproductive capacity, tree major parameters were studied: pollen viability; seed viability and features of the reproductive sphere. Flower buds, open flowers, and mature seeds were fixed in FAA mixture (formaldehyde: glacial acetic acid:70 % ethanol, in proportion of 5:5:90). The material was subsequently treated according to the classical paraffin methods: embedded in paraffin, cut with rotary microtome into 8–20 µm slices, stained with Heidenhain's haematoxylin and embedded in Entelan.

Pollen viability was estimated by counting the pollen grains in thirty mature anthers. For estimation of seed viability, the tetrazolium test was used (Peters 2000). Mature seeds were incubated in water at 30–35 °C for 24 hours, then embryos were isolated from them and placed into 1 % solution of 2,3,5-triphenyltetrazolium chloride for 24 h. The colourless tetrazolium solution turns red when it comes into contact with the hydrogen derived by enzymes of the seed respiration process. Seed viability was evaluated by the colour pattern of embryos: those showing active respiration turned red and were considered viable (the darker the colour, the stronger the respiratory activity in the seed); pink colour indicated less viable embryos, while colourless embryos were counted as unviable.

Data obtained for pollen and seed viability were analyzed statistically and presented in tables and figures. The observations and microphotographs were carried out with Olympus CX21 light microscope and Infinity lite light camera (1.4 Mpx).

Sterilization and *in vitro* cultivation conditions

Stems and mature seeds were surface-sterilized in 70 % ethanol for 30 s or 1 min, respectively, followed by 10 min in a solution of NaClO (chlorine < 5 %), and afterwards were washed three times with sterile distilled water. Due to the extremely small size of seeds, the sterilization technique was modified, so that they were continuously rinsed with sterilizing solutions and water on filter paper in a funnel, rather than being soaked in such solutions. Stems were cut into 1 cm long segments and inserted in MS-based nutrient medium (Murashige & Skoog, 1962) containing 30 g/l sucrose and solidified with 6,5 g/l agar; seeds were placed in the medium by pressing filter paper against its surface.

Additionally, two sets of seeds, with 250 seeds each, divided into five lots of 50 seeds were exposed to 23 °C (in the light) and to 15 °C (in the dark), in order to check the temperature effect on the germination of this relict species. In this case, the seeds were placed on the medium in Duchefa® plastic containers with grids. The photoperiod was 16/8 h, with light intensity of 2000 lux. Seeds were also placed on water agar without any nutrients (270 seeds in six lots of 45 seeds), and on moistened filter paper (270 seeds in two Petri dishes).

Seedlings were multiplied by subcultivation on fresh medium. Propagation coefficient (PC) was calculated as a number of newly-formed plantlets per initial plantlet.

Media with different composition and consistency, as well as different cultivation vessels were tested to enhance the *in vitro* propagation rate and to stimulate the rooting of plantlets. Medium without plant growth regulators (MS), and media supplemented with benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) were used in two concentrations: 3 mg/l BAP and 1 mg/l NAA (BN1), and 2 mg/l BAP and 0,2 mg/l NAA (BN2), or only 1 mg/l NAA (N); as well as plastic containers (Duchefa®) with solid and liquid media, and RITA® temporary immersion system (TIS). The plants in TIS were immersed four times daily for five minutes.

Adaptation of the rooted plants was attempted in a tray with moistened mix of soil, sand and marble powder (1:1:1), covered with transparent plastic foil, progressively habituated to room temperature and humidity.

Results and discussion

Pollen viability

The counting of mature pollen grains showed that few of them were viable. The estimated average pollen viability in the thirty anthers observed was only 20.44 % (Table 1). These results corresponded to the degeneration processes observed during the anther ontogenesis, microsporogenesis and development of the male gametophyte that led to formation of a different amount of sterile (empty) and darkly stained (degenerating) inefficient pollen grains.

Seed viability and germination

The performance of a seed can be determined by studying its germination and viability potential. Many seeds are neither completely dead, nor completely alive. One of the most significant advances in seed testing technology in recent years has been the topographical tetrazolium method. The tetrazolium test is a quick chemical test that can be conducted in a short time period with minimal equipment. The staining pattern after the use of tetrazolium test reveals the live and dead areas of the embryo and enables the analyst to determine if the seeds have the capacity to produce normal seedlings (Copeland & Mc Donald 2001).

Table 1. Pollen grain viability of *Papaver degenii* – statistical data.

Anther number	Nonviable pollen grains	Viable pollen grains	Sum total	Pollen viability, %
1	360	140	500	28.00
2	286	91	377	24.14
3	320	59	379	15.57
4	282	67	349	19.20
5	265	41	306	13.40
6	236	50	286	17.48
7	258	37	295	12.54
8	328	103	431	23.90
9	335	82	417	19.66
10	288	82	370	22.16
11	339	117	456	25.66
12	403	62	465	13.33
13	254	52	306	16.99
14	339	43	382	11.26
15	388	31	419	7.40
16	368	35	403	8.68
17	281	22	303	7.26
18	328	27	355	7.61
19	219	115	334	34.43
20	362	91	453	20.09
21	313	71	384	18.49
22	281	80	361	22.16
23	319	69	388	17.78
24	293	50	343	14.58
25	351	59	410	14.39
26	174	135	309	43.69
27	289	136	425	32.00
28	348	205	553	37.07
29	254	120	374	32.09
30	288	79	367	21.53
total	**9149**	**2351**	**11500**	**20.44**

Mean	20.08
Standard Error	**1.67**
Median	18.84
Standard Deviation	**9.13**
Sample Variance	83.36
Kurtosis	0.28
Skewness	0.75
Range	36.43
Minimum	**7.26**
Maximum	**43.69**
Sum	602.53
Count	30.00
Confidence Level (95,0 %)	3.41

Depending on staining, the embryos were estimated as viable and unviable, and classified in two classes: Class I – viable embryos (light- and dark-red coloured – Fig. 2 A, B, C); Class II – unviable embryos (colourless – Fig. 2 D). Most embryos fell into Class II (68.93 %) and only 31.07 % into Class I.

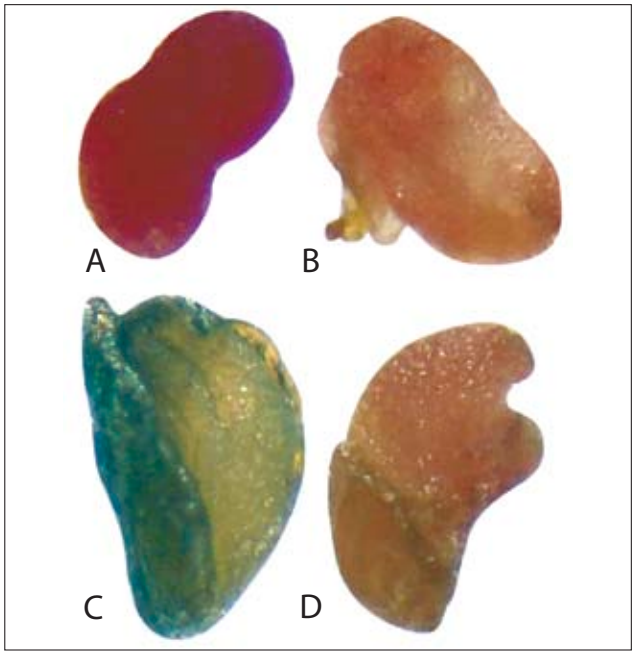

Fig. 2. Seed (embryo) viability after tetrazolium test: **A**. Red-stained – viable embryo; **B**, **C**. Light-red stained – viable embryo; **D**. Colourless embryo – nonviable (×40).

Peculiarities of the reproductive sphere

A preliminary study carried out into the reproductive biology of *P. degenii* has revealed that the species reproduces sexually, which is in correlation with its diploid chromosome number.

Sexual reproduction in many angiosperm plants involves self-incompatibility, which is one of the most important mechanisms to prevent self-pollination, as well as inbreeding (East 1940; Lewis & Crowe 1958; Franklin-Tong & Franklin 2003a; Thomas & al. 2003). It has been proved that in the family *Papaveraceae,* genus *Papaver*, and especially in *P. nudicaule* (Kurup & al. 1998), *P. alpinum* and *P. rhoeas* (Hiscock & McInnis 2003; Franklin-Tong & Franklin 2003b; Thomas & al. 2003; Thomas & Franklin-Tong 2004; Li & Franklin-Tong 2008), gametophytic self-incompatibility exists. Our results suggest that the high percentage of unviable pollen grains (79.56 %) and embryos (68.93 %) in *P. degenii* could be related to this mating system. Thomas & al. (2003) reported that in the poppy self-incompatibility manifests itself as a highly specific cell-cell recognition interaction between the receptive style surface and the own pollen, which inhibits the pollen tube growth and leads to pollen death. On the basis of a molecular study supporting this opinion, Franklin-Tong (2007) underline that self-incompatibility prevents self-fertilization by using a highly discriminating cellular recognition and a rejection "antigen-antibody" mechanism between the own pollen and the style.

Concerning the generative sphere, some deviations were observed in *P. degenii*. Very likely, they might also be considered a consequence of self-incompatibility: degeneration of the own pollen in contact with the style that consequently inhibits the pollen tube growth (Fig. 3 A); formation of a high amount of degenerating and sterile pollen in the anthers (Fig. 3 B); early degeneration of some ovules at the stage of archesporium (Fig. 3 C); positioning of the central cell nucleus near to the antipodals but not to the egg cell that prevents, or at least impedes its fertilization (Fig. 3 D). Nevertheless, our observations have shown that in some ovules double fertilization takes place (Fig. 3 E, F).

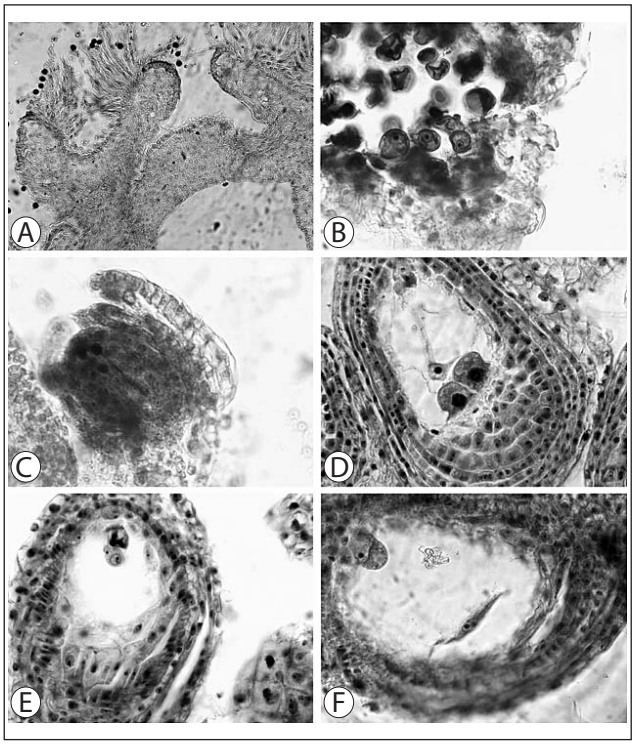

Fig. 3. Peculiarities of the reproductive sphere in *Papaver degenii*. **A**. Stigma with degenerating mature pollen grains on its surface (×100) ;**B**. Viable and degenerating pollen in an anther locule; **C**. Degeneration ovule at the stage of archesporium; **D**. Embryo sac with egg apparatus and central cell near the antipodals; **E**. Egg apparatus in the embryo sac and pollen tube destroying one of the synergids; **F**. Two-celled embryo and nuclear endosperm. **B**–**F** (×400).

In vitro germination and culture initiation

Sterilization of seeds was successful (no contaminated seeds), unlike that of the stems, none of which survived. The first seeds to germinate were observed a week after inoculation, at both temperatures, on MS medium, as well as on water agar and on wet filter paper without nutrients. A good half of the seeds germinated due to the presence of water alone: 56.7±3.3 % on water agar, and 55.8±2.6 % on filter paper. The germination rate was higher on MS medium containing macro salts, microelements and vitamins. A temperature of 23 °C was more favourable and 84.4 % of the seeds on MS medium germinated. Dependence of growth on temperature has been also demonstrated with other *Papaver* species (Tisserat & Berhow 2009). The number of germinated seeds was lower at 15 °C, so they were transferred to the same conditions as the first group of seeds (23 °C, in the light) two weeks after inoculation, and finally reached 75.2 % (Fig. 4). Germination energy was the highest during the second week in both variants, fast germination being normal for species with a short vegetation period. At the end of the third week, 78.3 % of the seedlings developed their first leaves, while the rest withered and died at the cotyledon stage. One-month old plantlets had several leaves and good roots (Fig. 5 A). They were subcultured on fresh medium, with removal of the roots. Root cutting stimulated formation of new shoots, and more or less thick rosettes formed (Fig. 2 B). Usually, the shoots at the periphery withered, while one or several new shoots appeared from the inner part of the rosette. The withering of rosettes required frequent transfer on fresh medium, every three or four weeks. Similarly, Savona & al. (2001) subcultured the seedling shoots of *Papaver nudicaule* every 15–20 days on media containing BAP or 2iP.

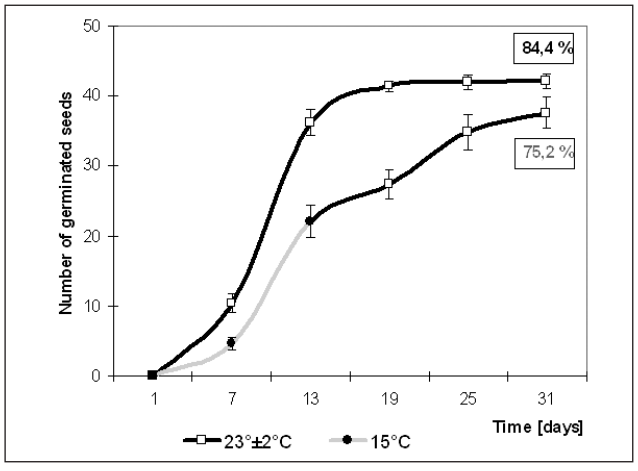

Fig. 4. Dynamics of seed germination at different temperature and light conditions: 23±2°C under 16/8 h photoperiod, and 15°C in the dark (after the second week all seeds were transferred to 23±2°C, 16/8 h photoperiod).

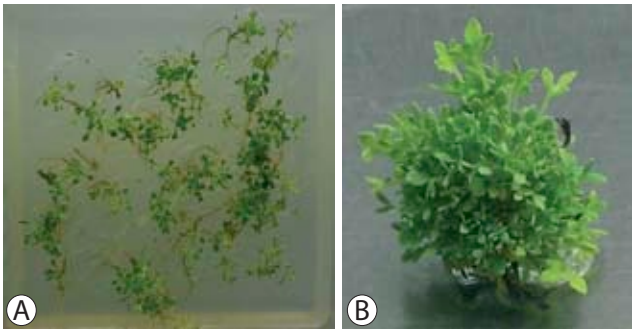

Fig. 5. **A**. One-month old seedlings, germinated *in vitro*; **B**. Formation of rosettes after subcultivation on agar medium.

In vitro cultivation

The MS medium free of plant-growth regulators proved better than those supplemented with BAP and NAA. The plants cultivated on MS solid medium had a 90 % survival rate and a propagation coefficient of 2.4 for four weeks of cultivation (120 plants derived from 49). Survival rate decreased with the increase of BAP concentration: 85 % in the BN1 medium, and only 56 % in the BN2 medium, due to plant vitrification. Savona & al. (2001) also reported hyperhydricity in media with 0.5 mg/l BAP and decreased its concentration twice to avoid it.

Plantlets transferred to a liquid static MS medium had larger leaves than the ones on the solid medium with the same composition; however, only 9 % of 110 plantlets survived.

To combine the advantages of solid and liquid media, a temporary immersion system (TIS) was used for cultivation of *P. degenii* (Fig. 6 A). As in the static liquid medium, the plants were generally larger than those on solid media. No vitrification was observed; however, many of the plantlets withered and died. The survival rate and propagation coefficient were highest on the agar medium. On the other hand, cultivation in TIS stimulated spontaneous rooting of the plantlets (Table 2). *In vitro* rooting is of great importance for the success of further *ex vitro* adaptation of the regenerated plantlets. The parameters of the system need to be improved and withering to be avoided; however, the initial trials with TIS were very promising. Up to 52.9 % of the plantlets formed long roots after five weeks in RITA® containers (Fig. 6 B, C). Furthermore,

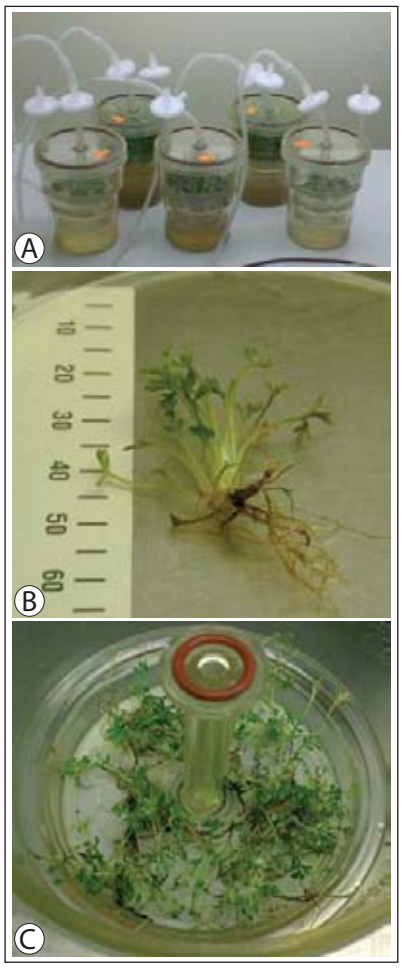

Fig. 6. **A**. TIS; **B**. *In vitro* plantlet rooted in TIS; **C**. Newly-formed small plantlets during cultivation in TIS.

Table 2. Subcultivation of *P. degenii* on agar-solidified MS medium and in MS liquid medium in a temporary immersion system (TIS), (PC – propagation coefficient).

Medium, cultivation vessel, and time	No. of initial plantlets	Survived plantlets		Obtained plantlets		Rooted plantlets	
		Number	%	Number	%	Number	%
MS agar medium, 4 wk	49	44	89.8	120	2.4	0	0
MS agar medium after TIS, 4 wk	46	38	82.2	156	3.4	5	3.2
MS in TIS (first time), 5 wk	75	51	68.0	51	0.7	27	52.9
MS in TIS (second time), 5 wk	23	4	17.4	48	20.1	35	72.9

during consecutive cultivation in TIS, numerous small rosettes appeared from the inner basal part of the dying initial explants, with short white roots, and occasionally forming small callus pieces. Usually one plantlet formed 2–8 new plantlets. After five weeks, the non-rooted plantlets were transferred again to solid MS medium, in order to reduce the relatively high necrosis rate in TIS. The propagation coefficient increased to 3.4 new plantlets formed for four weeks, and several plantlets rooted, which could be related to the preceding cultivation in TIS. Cultivation on N medium containing only auxin did not stimulate root formation. Other species were successfully propagated in TIS (Wawrosch & al. 2005). *Ex vitro* adaptation of the first rooted plantlets is in progress (Fig. 7). Transfer of the *in vitro* plants to ambient conditions with lower humidity is one of the main bottlenecks in the *in vitro* micropropagation technique. The choice of suitable substrate is also important, which was the reason to mix the soil with sand and marble powder, in order to approximate the soil of the plant's natural habitat.

Conclusions

The results of the present study show that sexual reproduction of the glacial relict *Papaver degenii* has probably a less significant role in sustaining its population *in situ* than the vegetative one.

Probable reason for the low degree of expression of the reproductive capacity in *P. degenii* is a combination of unfavourable environmental conditions (short period of vegetation, short visitation of flowers by pollinators, low temperatures and other biotic and abiotic factors) with some species' peculiarities, such as vegetative to sexual reproduction ratio, self-incompatibility, low amount of pollen and low seed viability. All these factors influence the character and size of *P. degenii* population in time and space and, respectively, its preservation under natural conditions. Besides the particular species characteristics, rarity of *P. degenii* is most probably due to its low competition ability, as it is confined to unproductive habitats. In this respect it can be considered a stress-tolerant species.

The higher rate of the observed seed germination *in vitro* suggests that some seeds have germinated which would not survive under natural conditions. The applied *in vitro* techniques are suitable for enhancement of the species' multiplication. They are very appropriate for an *ex situ* conservation of the species vulnerable to competition, as *P. degenii,* as well as for strengthening of their populations *in situ.*

Acknowledgements. This study was supported by the EEA Grants/Norway Grants within the framework of project "Conservation of biodiversity in hot-spots of glacial relict plants in Bulgaria" (BG 0034), including the purchase of the RITA® Temporary Immersion System.

References

Andreev, N. 1981. Reports. – In **Löve, À.** (ed.), IOPB Chromosome numbers reports, 70. – Taxon, **30**(1): 74-75.

Copeland, L.O. & Mc Donald, M.B. 2001. Principles of seed science and technology. Fourth edition. Kluwer Academic Publishers, Massachusetts.

East, E.M. 1940. The distribution of self-sterility in the flowering plants. – Proc. Amer. Phylos. Soc., **82**(1434): 449-518.

Franklin-Tong, V.E. & Franklin, F.C.H. 2003a. Gametophytic self-incompatibility inhibits pollen tube growth using different mechanisms. – Trends Pl. Sci., **8**(12): 598-605.

Franklin-Tong, V.E. & Franklin F.C.H. 2003b. The different mechanisms of gametophytic self-incompatibility. – Philos. Trans. Roy. Soc. London. B, **358**: 1025-1032.

Franklin-Tong, V.E. 2007. Inhibiting self-pollen: self-incompatibility in *Papaver* involves integration of several signaling events. – J. Integr. Plant. Biol., **49** (8): 1219-1226.

Hiscock, S.J. & McInnis, S.M. 2003. The diversity of self-incompatibility systems in flowering plants. – Pl. Biol., **5**(1): 23-32.

Kuzmanov, B. 1970. *Papaver* L. – In: **Jordanov, D.** (ed.), Fl. Republ. Popularis Bulgaricae. Vol. **4,** pp. 282-283. In Aedibus Acad. Sci. Bulgaricae, Serdicae (in Bulgarian).

Lewis, D. & Crowe, L.K. 1958. Unilateral interspecific incompatibility in flowering plants. – Heredity, **12**(2): 233–256.

Kurup, S., Ride, J.P., Jordan, N., Fletcher, G., Franklin-Tong, V.E. & Franklin, F.C.H. 1998. Identification and cloning of related self-incompatibility S-genes in *Papaver rhoeas* and *Papaver nudicaule*. – Sexual. Pl. Reprod., **11**(4): 192-198.

Li, Sh. & Franklin-Tong, V.E. 2008. Self-incompatibility in *Papaver*. A MAP kinase signals to trigger programmed cell death. – Pl. Signal. Behav., **3**(4): 243-245.

Markgraf, F. 1958. Eine neue Gliederung der Alpenmohne. – Phyton (Horn), **7**(4): 302-314.

Mucina, L., Valachovič, M., Jarolímek, I., Šeffer, J., Kubinská, A. & Pišut, I. 1990. The vegetation of rock fissures, screes, and snow-beds in the Pirin Planina Mountains (Bulgaria). – Stud. Geobot., **10**: 15-58.

Murashige, T. & Skoog, F. 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. – Physiol. Plant., **15**(3): 473-497.

Peters, J. 2000. Tetrazolium Testing Handbook. Contribution No. 29 to the Handbook on Seed Testing revised, AOSA

Savona, M., Semeria, L., Allavena, A., Carli, S., Profumo, P. & Ruffoni, B. 2001. In vitro culture of *Papaver nudicaule* L. – In: **Sorvari, S. & al**. (eds) Proc. IV Int. Symp. In Vitro Cult. & Hort. Breed. – Acta Hort., **560**: 469-472.

Schönswetter, P., Solstad, H., García, P. & Elven, R. 2009 A combined molecular and morphological approach to the taxonomically intricate European mountain plant *Papaver alpinum* s.l. *(Papaveraceae)* – taxa or informal phylogeographical groups? – Taxon, **58**(4): 1326-1343.

Stoeva, M. 2009. *Papaver degenii* (Urum. & Jav.) Kuzmanov. – In: Petrova, A. & Vladimirov , V. (eds), Red List of Bulgarian Vascular Plants. – Phytol. Balcan., **15**(1): 86.

Thomas, S.G. & Franklin-Tong, V.E. 2004. Self-incompatibility triggers programmed cell death in *Papaver* pollen. – Nature, **429**(6989): 305-309.

Thomas, S., Osman, K., de Graf, B.H.J., Shevchenko, G., Wheeler, M., Franklin, F.C.H. & Franklin-Tong, V.E. 2003. Investigating mechanisms involved in the self–incompatibility response in *Papaver rhoeas*. – Philos. Trans. Roy. Soc. London. B, **358** (1434): 1033-1036.

Tisserat, B. & Berhow, M. 2009. Production of pharmaceuticals from *Papaver* cultivars *in Vitro.* – Engineering in Life Sciences, **9**(3): 190-196.

Urumoff, I. 1920. Neue und seltene Pflanzen Bulgariens. – Magyar Bot. Lapok, **19**: 33-40.

Wawrosch, Ch., Kongbangkerd, A., Köpf, A. & Kopp, B. 2005. Shoot regeneration from nodules of *Charybdis* sp.: a comparison of semisolid, liquid and temporary immersion culture systems. – Pl. Cell Tissue Organ Cult., **81**(3): 319-322.