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Botany

Study of Structural Peculiarities of Generative Sphere and *ex-situ* Conservation of Georgian Endemic Plant *Campanula kemulariae* Fomin Included in the Red List of the Caucasus

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ABSTRACT. Structural peculiarities of generative sphere of the local Georgian endemic *Campanula kemulariae* Fomin (VU), included in the Red List of the Caucasus, were studied at different stages of development. Capacity for seed production was evaluated, germination capacity and seed viability determined. Course of formation of generative sphere of *C. kemulariae* proceeds mainly within the norm. Anther develops by centrifugal (efferent) type. The wall of anther consists of epidermis, endothecium, 2 intermediate layers and tapetum. Insignificant deviations (1-2%) occurring at early stage of meiosis do not affect its normal course. Tetrads are formed by simultaneous mode. Pollen grain is triporous. Fertility of pollen attains 90%. Conditions and terms optimum for seed germination were established under controlled temperature and illumination. Early stages of ontogenesis – latent and pregenerative were studied. *Ex-situ* conservation works were carried out at the Department of Plant Conservation of the National Botanical Garden of Georgia. *© 2015 Bull. Georg. Natl. Acad. Sci.*

Key words: *endocytosis, microsporocyte, meiosis, germination capacity, ontogenesis.*

Campanula kemulariae Fomin (Campanulaceae, sect. Symphyandriformes) [1, 2], species included in the Red List of the Caucasus [3] is a local Georgian endemic, characterized with narrow distribution. Its populations occur in west Georgia, Imereti, on Nakerala range, in the gorge of river Kvirila, on limestone rocks situated between Chiatura and village Darkveti. The species is lythophilic, grows in lower and upper mountain belts. Its occurrence is sporadic, plants are scattered on rocks. Populations occur on limestones and marls. Coenotic environment of *Campanula kemulariae* is presented by rock xeric groupings and hornbeam stands developed on slope depressions [1].

Campanula kemulariae is characterized by a creeping rhizome, the stem is 30-35 high; leaf is bare or hairy, with tomentum scattered on veins. Lower leaves are with long stalks, cordate, serrate on margins; middle leaves are short-petiolate; upper leaves are sessile. Flowers are arranged in panicles. Calyx is wide, triangular, pointed, ciliate. Corolla bell-shaped, with long hair on the margin, twice as long as calyx, dark blue. Stamens -5, ovary three-locular, stigma three lobed. Style is heavily protruded from the corolla. Fruit is a capsule with many seeds. Seeds are small, oval [1].

The aim of the present investigation was to study structural peculiarities of the generative sphere of *Campanula kemulariae* at different stages of development and to observe the processes of seed formation, as the study of reproduction biology is of primary importance for the survival of species of the primary conservation concern.

The presented study is important from the viewpoint of the Global Biodiversity Strategy. The species is also valuable in terms of study of the history of Flora. The species, being distinguished by ornamental properties, seems to be prospective for the use for decoration of rocky slopes.

Materials and Methods

In the course of investigation the consecutive phases of microgametogenesis were studied; germination capacity and viability of seeds was determined; *ex-situ* conservation works were carried out via establishing reserve of seeds and seedlings of *Campanula kemulariae*.

Material used in our studies (plants and seeds) was collected from the wild population of *Campanula kemulariae* located in its natural distribution range, gorge of the river Jrucha and cultivated population, growing on the collection plot of the Department of Plant Conservation of the National Botanical Garden of Georgia.

Phenological observations on the object were carried out at the collection plot of the Department of Plant Conservation of the National Botanical Garden of Georgia.

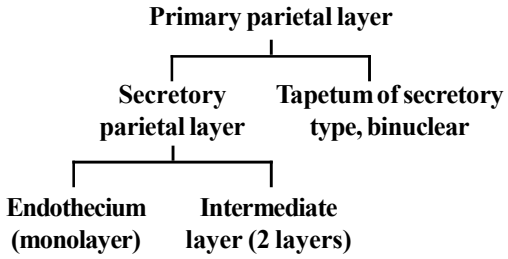
Laboratory trials were conducted using the methods accepted in structural and experimental embryology [4].

Materials were fixed using Carnoy's fixing agent (3:1). Material was embryologically studied using the binocular light microscope Carl Zeiss (Germany). Phases of ontogenesis were established using methods by Ponomarev [5]. Productivity of seed formation, germination capacity and viability were determined according to the method by Rabotnov [6] and methods accepted in the Millennium Seed Bank [7, 8].

Results and Discussion

In the process of experiment male and female stages of flowering process were studied consecutively.

Microscopic studies of male generative sphere revealed that the pollen grain is tetralocular, introrse. Lower, villous part of the filament, widened in the form of triangle, covers oval nectary, which opens with pores near the style, on the surface of stigma (Fig. 1). Anthers develop by (centrifugal) efferent type.



The cells of the primary parietal layer divide periclinally to form the secondary parietal layer and tapetum. As a result of division of the secondary parietal layer endothecium and intermediate layers are formed. The wall of mature anther consists of epidermis, intermediate layer and tapetum (Fig. 2). The outer layer of epidermal cells is thick and is not covered with cuticle, as specified for *Campanula sibirica* [9]. In postmeiotic period cells of endothecium significantly increase in size and fibrous thickenings of different configuration are formed.

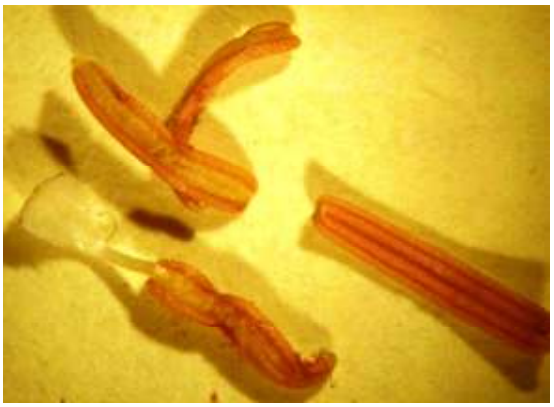


Fig. 1. The stamen of *Campanula kemulariae*.

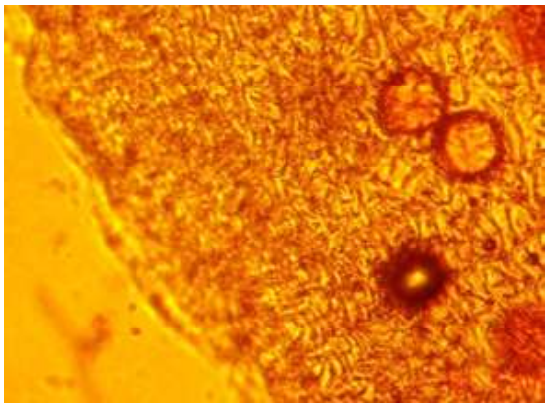


Fig. 2. The wall of anther of *Campanula kemulariae*.

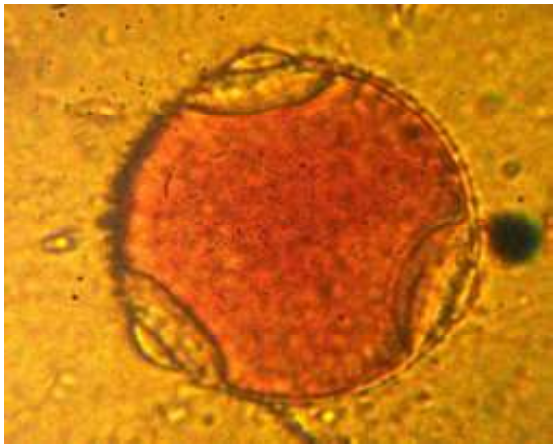


Fig. 3. The triporous pollen grain of *Campanula kemulariae*.

Fig. 4. The anther of *Campanula kemulariae* with fertile pollen.

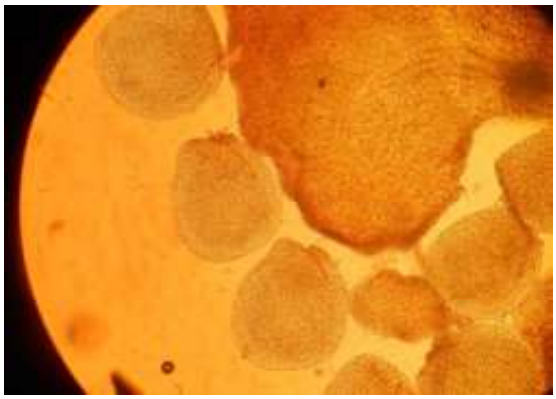


Fig. 5. Ovary of *Campanula kemulariae* with numerous ovules.

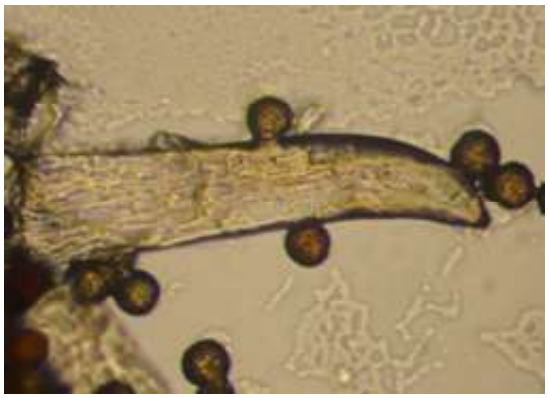


Fig. 6. Pollen-collecting hair of *Campanula kemulariae* with pollen grains attached to it.

Fibrous thickenings occur in the intermediate layer as well. In contrast to *Campanula sibirica* intermediate layer is not single-layered. It is represented by two layers. In the beginning of meiosis intermediate layer undergoes degeneration, rarely one layer, adjacent to the endothecium remains. Tapetum is of secretory type, binuclear, undergoes lysis at the stage of mononuclear pollen grain. Meiosis in microsopocytes proceeds within the norm. Mainly 17 closed bivalents are formed, open bivalents are rarely observed (1-2). Univalents are not registered. Slight deviations – deterioration of orientation of bivalents, asynchronism in metaphase and anaphase of the second meiotic division, rarely formation of

triads or pentads do not affect the normal course of meiosis. Finally formation of pollen tetrad proceeds normally. Tetrads are formed by simultaneous type. Arrangement of microspores in tetrads is tetrahedral. Anthers contain great amount of inulin. Complex carbohydrate of plant origin – inulin occurs as storage substance in other representatives of Campanualceae family as well [9]. Pollen grains in small-size (7mm), buds of light colour are mononuclear. Binuclear pollen grain occurs in coloured approximately 10-15mm long buds. In binuclear pollen grain generative nucleus, which is oval by shape and small in size is located near the pore. Mature pollen grain is binuclear, triporous, monosiphonous, though some representatives of order Campanula are characterized by polysiphonous pollen grain [10, 11] (Fig. 3). Number of pores is different as well. Together with fertile pollen small portion of sterile pollen is found as well. Pollen fertility is high (90%), which is a prerequisite for successfull pollination(Fig. 4). As the staining with acetcarmine does not always show physiological activity of pollen, we tested pollen germination ability on 15% solution of saccharose. Pollen tube intensively grows at 20-21°C. In conditions of low temperature (4°C), growth of pollen grain stops. Viability of pollen grain, tested by Tetrazolium [12,13] lasts for 12-15 days.

Development of the female sphere of *Campanula kemulariae* is normal as well. Ovary is three locular, with numerous ovules (Fig. 5). Length of the style in open flower is at average 21-24 mm. Middle and upper parts of a style are covered with thin-envelope villi of epidermal origin. Stigma is three-lobed. Like other species of Campanulaceae family, embry sac of *Campanula kemulariae* is monosporic, of Polygonum type. [9, 14] Campanulaceae are characterized by peculiar pollination, investigation of which has a long history. Despite the great interest of researchers, there is discrepancy in opinions between researchers on issues, connected with pollinataion process.Some authors think that after donation of pollen, collecting hairs shed from the style; others assume that the pollen collecting hair do not shed, but there takes place their retraction, invagination into the superficial tissue of the style (A. Broniar, 1839 cited according to [15]). Mechanism of invagination of collecting hair is also debatable. According to Kirchner (Kirchner, 1897 cited according to [15]), visitor insects can adopt pollen only in case when the hair is half invaginated into superficial tissue of the style.

Pollination is a crucial step in the reproduction cycle of *Campanula kemulariae*. It consists of several stages: 1) liberation of pollen from the male structure; 2) transfer of pollen on the female structure; 3) growth of pollen grain. Liberation of pollen from the anther takes place in the male phase. The male phase embraces the stage of bud. In the stage of bud stamen and stigma are of equal height. Stamen is attached to the style, dehisces in introrse direction and pollen grains liberate from it with the aid of radial appendixes of medial septum – placentoids. Pollen attaches to the pollen collecting hairs (Fig. 6). At this stage the pistil is not functionally ready to accept pollen. This is confirmed by the peroxidase test [5, 11-13]. Enzymes, which oxidize polyphenols and aromatic amines-peroxidases catalyze reactions of oxidation. When functional activity of an organ is high, peroxidises contained in its cells actively participate in oxidation reactions. When adding H_2O_2 to the pistil (stigma), its enzymatic decomposition takes place, which is manifested by intensive isolation of bubbles (Fig. 7). Perixodase test in the closed bud has revealed low functional activity of stigma. In the stage of bud the pollen grain is functionally active. Transfer of pollen grain to the female reproductive organ (sphere) proceeds in the female phase, which includes early, middle and late flowering phases.

In early flowering phase filament deflects and the anther retracts from the style by 45° angle and curls up like a spiral. Flower starts discharging the nectar. Style grows in size going out of margins of the corolla. According to Y. Nyman,1993, secondary presentation of pollen from the pollen-collecting hairs of the style takes place, which sweep up the pollen after dehiscence of the anther [16]. The style brings the

Fig. 7. Testing of functional activity of stigma of *Campanula kemulariae* by peroxidase test.

Fig. 8. Early, middle and late phases of flowering of *Campanula kemulariae*.

Fig. 9. Germination of *Campanula kemulariae* seed on agar medium.

Fig. 10. Seedling of *Campanula kemulariae*.

pollen out of flowers and this mechanism makes pollen available for visitor insects.

In the middle phase of flowering pollen dries up, only its lower part of triangular shape retains turgor. Stigma opens (splits) and becomes functionally active, which is proved by the peroxidise test – active elaboraton of bubbles on the surface of stigma after dropping the peroxidise on it. Pollen grain is transferred to the female sphere by insects. Pollinator insects belong to Hymenoptera.

Self-pollination in *Campanula kemulariae* is avoided by hercogamy, dichogamy – clearly expressed protandry. Entomophilic pollination is the ecological problem. It depends not only on the abiotic factor (humidity), but also the presence/number of pollination insects.

Efficiency of pollination in the period, studied by us in nonuniform, being reflected in number of formed seeds.

Number of ovules in ovary fluctuates within the range of 95-128. In case of successful pollination (2013) fruit contains 70-112 seeds. Under unfavourable conditions, at frequent preceiptation, only 16-18 seeds develop in a capsule. In a late flowering period stigma becomes more inclined to outer direction, touching the remaining pollen. At this stage autogamy is not excluded and this is proved by limited number of seeds, obtained from isolated flowers (6-12 seeds). The cases of autogamy are described in representatives of this order *Campanula rotundifolia*, *C. latifolia* and *C. rapunculoides* [17]; facultative autogamy was reported in *Campanula kachetica* Kantsch. by isolation of buds in the wild population [18].

Autogamy points to the plasticity of reproductive sphere and promotes survival of a species. In case of successful pollination a three-locular capsule with numerous seeds develop. Seeds fall out of

the capsule via three pores, situated on the lower surface of a capsule. Growing of pollen tube into the style proceeds by the endotropic mode.

In the process of experiment with the aim of checking seed viability the seeds were sown at different terms (immediately after harvesting, in autumn and in spring). Different conditions were tested (medium (1% agar or filter paper): temperature regime – in conditions of controlled temperature and illumination, and under naturally changing temperature and illumination)

I variant: fridge – 1 week at +4°C, in darkness with the following transfer to the incubator on 1% agar medium, alternating temperature 21/14ºC, day/night 12/12 hours;

II variant: in the incubator from the beginning - on 1% agar medium, alternating temperature 21/14ºC, day/night 12/12 hours.

To determine germination capacity germinated seeds, seeds that remained fresh after termination of germination tests, mouldy seeds and seeds, infected with insects were counted separately. After termination of germination test the remainder seeds were cut-tested to determine presence of empty and infected seeds.

Germination capacity was calculated using the formula:

$$G = \frac{q \cdot 100}{q + F + M}$$

where: G is germination percent, q – a number of germinated seeds, F – a number of full seeds, remained fresh by the end of the test, M – a number of mouldy seeds.

Seed viability was determined according to the formula:

$$V = \frac{q + F \cdot 100}{q + F + M}$$

Results of germination tests, calculated using the above given formula are presented on charts 11-15 below.

Biochemically seed viability was determined using TZ test (staining with tetrazolium chloride [19].

Percentage of good seeds was determined using the formula:

$$j = \frac{h \cdot k}{m}$$

Where j is percentage of good seeds, h – total number of seeds, k – number of cut tested good seeds and m – total number of cut-tested seeds.

As seen from Fig. 11-15, variant 2 (placing Petri dishes with seeds in incubator from the beginning - on 1% agar medium and alternating temperature 21/14ºC, day/night 12/12 hours), yielded the higher germination percent, than conditions of the variant 1 (Fridge+Incubator). In different years germination percent in these conditions varied from 49 (Fig. 12) to 66.7% (Fig. 13). The highest germination percent 66.7% was achieved in autumn sowing of 2012-2013 period (Fig. 13). Seeds started to germinate from the second week from sowing. Seeds sown upon harvesting – freshly harvested seeds are characterized with lower germination percent.

As is known [20, 21] different species united in the family Campanulaceae are characterized with 3 ontogenetic periods: 1. period of dormancy or latent period; 2. pre-generative period, which includes 4 age groups of individuals (seedlings, juvenile plants, immature plants and virginal plants); 3. generative period.

We studied initial period of ontogenesis: latent period-seeds and pre-generative period-seedling.

Seeds of *Campanula kemulariae* can be classified as Orthodox, that means that they are tolerant to drying and freezing in the process of *ex-situ* conservation. By shape the seed is oval, light or dark brown. Outer surface of seed is covered with small dark dotted lines with indefinite twists, its length – 8-10 mkm, width – 3.5-4 mkm.

Embryo is straight, linear, located in the centre and it is surrounded by the endosperm.

Indicative sign of the seedling is the presence of cotyledons and the primary leaf. Seedlings develop simultaneously in 15-20 days from sowing. Emergence

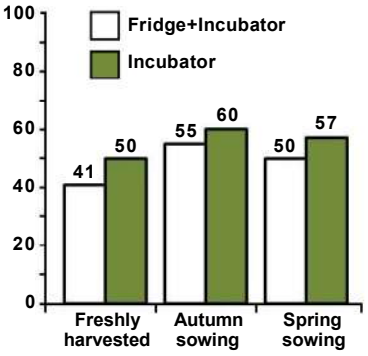


Fig. 11. 2010-2011, Germination %.

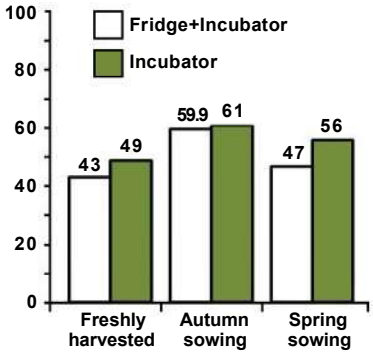


Fig. 12. 2011-2012, Germination %.

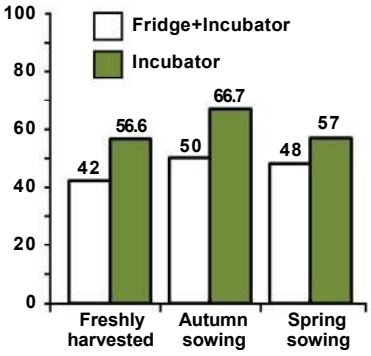


Fig. 13. 2012-2013, Germination %.

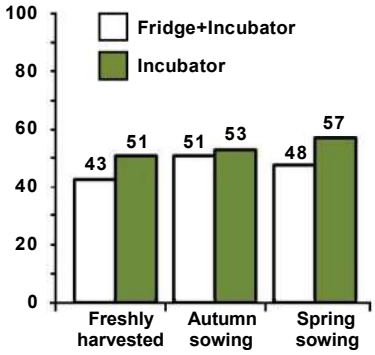


Fig. 14. 2013-2014, Germination %.

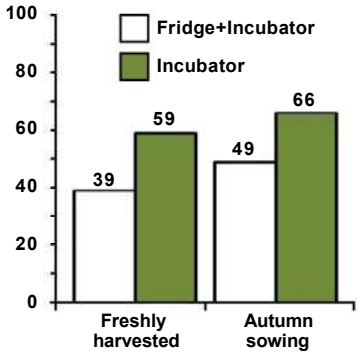


Fig. 15. 2014, Germination %.

of single seedlings continues until the 30th day. Seedling length – 20 mm. Cotyledons are ovoid. Cotyledons of Campanula are uniform by shape, glossy green with bare surface.

Sizes are variable, at average 0.3-0.5 mm, epycotyl is not developed. Primary leaf emerges immediately above the cotyledons. Leaf lamina is ovoid, with short, rounded petiole at a base. Leaf margins and lamina ciliate. Hypocotyl is 5-10 mm long.

Indicative sign of juvenile period is development of 2-4 leaves and growth of the secondary root, formation of lateral roots. As leaf lamina grows, serration becomes more expressed. Leaf venation is reticulate, leaf pubescent. Leaf petiole and abaxial surface of lamina are of reddish colour. In juvenile period formation of rosette-like basal leaves takes place.

In virginal period vegetation lasts until November. Plant overwinters with green leaves. By the end of February leaves develop from shoot apex. Rhizome develops additional roots, root system becomes stronger and number of rosette-like leves increases.

The first generative phase commences on the second year.

Generative shoot is orthotropous, monocarpic. Internode is shortened on upper and lower terminals. Arrangement of leaves is alternate.

Thus results of investigation show that the process of formation of the generative sphere of *Campanula kemulariae* proceeds within norm and the species is characterized with high reproductive performance.

ბოტანიკა

კავკასიის წითელ ნუსხაში შეტანილი საქართველოს ენდემური სახეობის *Campanula kemulariae* Fomin გენერაციული სფეროს სტრუქტურული თავისებურებების შესწავლა და *ex-situ* კონსერვაცია

ლ. გაბედავა

საქართველოს ეროვნული ბოტანიკური ბაღი, თბილისი

(წარმოდგენილია აკადემიის წევრის გ. ნახუცრიშვილის მიერ)

პირველად არის შესწავლილი კავკასიის წითელ ნუსხაში შეტანილი საქართველოს ლოკალური ენდემური სახეობის ***Campanula kemulariae*** **Fomin** გენერაციული სფეროს სტრუქტურული თავისებურებები განვითარების სხვადასხვა ეტაპზე. განისაზღვრა თესლის წარმოქმნის შესაძლებლობა, თესლის სიცოცხლისუნარიანობა და აღმოცენების ხარისხი. ჩატარდა ***ex-situ*** საკონსერვაციო სამუშაოები. მიღებული შედეგები საფუძველს გვაძლევს დავასკვნათ, რომ ***C. kemulariae***-ს გენერაციული სფეროს ფორმირება ნორმის ფარგლებში მიმდინარეობს; სამტვრე პარკის განვითარება ხდება ცენტრიდანული ტიპით. სამტვრე პარკის კედელი შედგება ეპიდერმისის, ენდოტეციუმის, 2 შუალედური შრისა და ტაპეტუმისაგან. მეიოზის ადრეულ ეტაპზე გამოვლენილი გადახრები (1-2%) არ არღვევს მის ნორმალურ მსვლელობას; ტეტრადის წარმოქმნა სიმულტანური ტიპით ხორციელდება. მტვრის მარცვალი სამფორიანია. მტვრის ფერტილობა 90%-ს შეადგენს. სვეტში სამტვრე მილის ჩაზრდა ენდოტროპულად ხდება. დადგინდა თესლის გაღივებისათის ოპტიმალური პირობები და ვადები კონტროლირებადი ტემპერატურისა და განათების პირობებში. შესწავლილია ონტოგენეზის საწყისი ეტაპები: ლატენტური და პრეგენერაციული.

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