Research Article

Turk J Bot
36 (2012): 55-62
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doi:10.3906/bot-1008-42

Reproductive biology studies towards the conservation of two rare species of Colchic flora, *Arbutus andrachne* and *Osmanthus decorus*

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Received: 31.08.2010
Accepted: 05.04.2011

Abstract: Our study examines the structural peculiarities of the generative sphere at different phases of development, and the processes of pollination, fertilisation, and seed formation in 2 species of wild flora: *Arbutus andrachne* L. (Ericaceae), the eastern strawberry tree, and *Osmanthus decorus* (Boiss. & Balansa) Kasapligil (Oleaceae), Caucasian osmanthus. These Arcto-Tertiary plants are included in the *Red Data Book of the Georgian SSR*. As germinable seeds are the main factor determining species distribution and the complete transfer of genetic information, the aim of our research was to establish the self-regeneration capacity of the species under study and choose optimum conditions for seed germination, the further development of seedlings, and their ex situ conservation. It has been stated that abnormalities taking place at different stages of sexual reproduction are responsible for the low capacity for seed formation found in the studied species. In particular, these factors are as follows: a limited capacity for allogamy; a low percentage of pollen tube development, at 20%-30% for *Arbutus andrachne* and 15%-20% for *Osmanthus decorus*, despite the high fertility of pollen grain of both species (approximately 70%-80%); and the frequent occurrence of embryo degeneration. The germination of *Arbutus andrachne* seeds on petri dishes on agar reached 80%. The results of the present study allow for the elaboration of protocol for the propagation of these relic species and for their further ex situ conservation on the collection plot of the Department of Plant Conservation.

Key words: Conservation, fertility, pollen grain, embryo, *Arbutus, Osmanthus,* Georgia

Introduction

Nowadays many rare and unique plant species are threatened by extinction due to interference from human economic activities Studying the seed formation processes of these species and evaluating the capacity for production of viable seeds is of great significance, as germinable seeds are often the main factor determining species distribution and the complete transfer of genetic information.

In this sense, embryological investigations play a key role, as they allow for the detection of weak points in the chain of reproduction processes and further elaborate methods of overcoming them. The results of investigations of this kind can serve as a basis for the further ex situ conservation of rare species. *Arbutus andrachne* L. (Ericaceae), the eastern strawberry tree, and *Osmanthus decorus* (Boiss. & Balansa) Kasapligil (Oleaceae), Caucasian osmanthus, are 2 such rare

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species and are representatives of the flora of the Arcto-Tertiary period included in the *Red Data Book of the Georgian SSR* (Kacharava et al., 1982).

The aim of the present investigation was to establish the self-regeneration capacity of the species under study and to determine the optimum conditions for seed germination, the further development of seedlings, and their ex situ conservation.

Materials and methods

The present article incorporates experimental work performed between 2006 and 2010, which was targeted at the conservation of the above-mentioned rare species of Colchis flora.

Arbutus andrachne is distributed in the gorge of the river Bzyb (Abkhazia), in the state-run Miussera Nature Reserve, located in the hills of the coastal zone in the lower mountain belt. The area has an altitude of 100-110 m and an incline of 30-40° on the slopes with southern exposure (Kacharava et al., 1982; Borodin et al., 1984). In the republic of Adjara, these strawberry trees are distributed in the gorge of the river Ajaristskali, near the village of Gorkhanauli (Memiadze, 1973; Gagnidze, 1985; Kuthatheladze, 1985; Diasamidze, 2006). Additionally, several samples of the strawberry tree grow in territory belonging to the Department of Plant Conservation of the Tbilisi Botanical Garden and Institute of Botany.

Arbutus andrachne is a very ornamental multistemmed tree of low height (from 3 to 8 m), with a wide and rounded crown, coral-red bark, and leathery evergreen leaves of an oval shape. The flowers are white, bisexual, assembled in terminal panicles, and entomophilous (Figure 1). Its fruit is a spherical berry, many-seeded, 5-locular, and of a roundish shape not larger than 1 cm in diameter.

Osmanthus decorus is distributed in Adjara in thin mixed forests or light chestnut forests and on rocks and rocky mounds reaching 1800 m in altitude. Caucasian osmanthus is an evergreen bush or small tree of up to 5 m height. Its branches are bare and leaves are 8-10 cm long, lanceolate, and smooth-edged with short stalks. The plant's bisexual flowers are arranged in bunches in the leaf axils (Figure 1). The calyx is short, divided into 4 parts, and petals are pale. The fruit is a greyish-black drupe, elliptic in shape, which contains a single seed.

Seed processing and banking was performed according to the methods of Baskin and Baskin (2001) and Iriondo and Perez (1999). Work aimed at ex situ conservation of the target species was carried out in 2006, prior to the banking of seeds. In particular, the germination capacity of each species was tested.

In terms of storage behaviour, the seeds of *Arbutus andrachne* are classified as orthodox. Some species belonging to the genus *Osmanthus*, in particular *Osmanthus ilicifolius* Mouill., also show orthodox storage behaviour (Hong et al., 1998). This implies that the seeds can be dried without damage to the low moisture content (Hay & Smith, 2003; Leprince, 2003).

Before banking, the seeds of our target species, collected from the wild, were cleaned, placed into paper bags, and put into a barrel with silica gel in order to remove any extra moisture. After this, the relative humidity was measured using a hygroscope (Rotronic, Austria). Seeds with a relative humidity of 15%-20% were sealed in foil bags and placed in long-term storage at the Caucasus Regional Seed Bank (CRSB) at a temperature of –20 °C.

Ex situ conservation activities are undertaken for these species in the Department of Plant Conservation of the Tbilisi Botanical Garden and Institute of Botany. Seeds of *Arbutus andrachne* and *Osmanthus decorus* (500 seeds for each species) were deposited in the CRSB, which is managed by this department, and the accompanying herbarium vouchers can be found at the National Herbarium of Georgia (TBI). *Arbutus andrachne*: Georgia, Adjara, Shuakhevi district, Gorkhanauli village, 29.11.2006, 610 m, 41°37.537′N, 42°10.261′E, rocky slopes, collection #286. *Osmanthus decorus*: Georgia, Adjara, Batumi Botanical Garden, 01.09.2006, 638 m, 41°41.906′N, 41°43.156′E, collection #295. Duplicates of the seed collections (500 seeds for each species) and the accompanying herbarium vouchers were sent to the Millennium Seed Bank, Royal Botanical Gardens, in Kew, UK, for storage.

The flower buds for the embryological control were collected at different developmental stages. All specimens came from plants grown in their natural

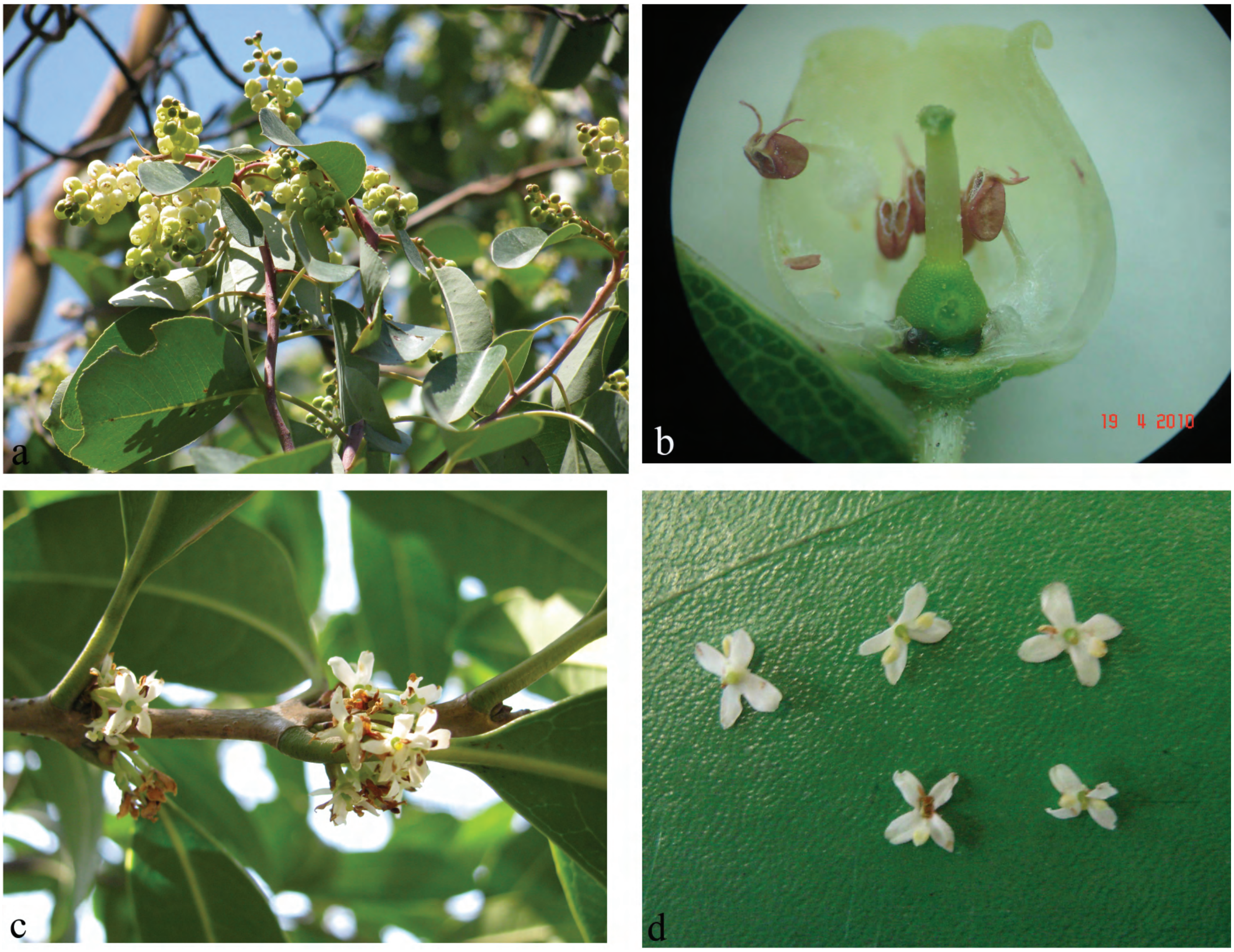

Figure 1. Inflorescences and flower details of *Arbutus andrachne* and *Osmanthus decorus*. *A. andrachne*: a) inflorescences, b) flower details. *O. Decorus*: c) inflorescences, d) flower details.

habitat and were gathered in spring between the years of 2006 and 2008.

Embryological control was performed using accepted cytoembryological methods (Herr, 1971; Pausheva, 1974). Materials were fixed using FAA, Carnua, and FPA (formalin-glacial acetic acid-ethyl alcohol, 5:5:90). The chromosome number was determined in the mother cell of the pollen grain in prophase, M1.

Microscopic study was performed using a light microscope (Carl Zeiss, Germany). Images were taken using a Sony digital camera featuring 8.1 megapixels, a high-sensitivity ISO of 800, and a 3-inch touch panel.

Seeds used for germination experiments were collected from individuals of both species grown in their natural habitat. Specimen collection was performed in autumn, 2008-2010. Because only single individuals or small groups of the mentioned species occur in the places of their distribution in Adjara, fruit was collected randomly from 4-6 individuals of each species. Where possible, up to 100 seeds were gathered from each individual.

In order to test viability and evaluate germination capacity, seeds of both species were sown on petri dishes. Tests were completed using both filter paper wetted with distilled water, according to the method described by Rabotnov (1960), on 1% agar (Baskin

& Baskin, 2001) at room temperature. Seeds were later transferred to the incubator and refrigerator. Petri dishes containing seeds were then placed in different conditions. The following experimental variants were developed: sowing on filter paper at room temperature (20-22 °C), on agar in refrigerated conditions (at 5 °C), and on agar in the chamber of the incubator with a 12:12 photoperiod at alternating temperatures (14 °C during the dark period and 18 °C during the light period). The process of germination was monitored every week.

Embryology

***Arbutus andrachne*:** Anthers are 4-locular. Wall of anther consists of epidermis, endothecium, intermediate layer, and the secretory tapetum. Its walls develop from the periphery to the centre (Figure 2). Primary parietal layer divides to form endothecium, and the secondary parietal layer develops into the middle layer and tapetum. In the late stage of pollen grain development, epidermal cells thicken unevenly, become bigger, sometimes become serrate.

Cells of endothecium also significantly enlarged. Endothecium is single-layered. Unlike the middle layer, no fibrous thickenings are seen in its structure. Middle layer is 3-layered. It degenerates when meiosis starts. Tapetum is multinuclear, cellular, secretory. It retains cellular structure until the tetrad stage. In the mother cell, which gives rise to the pollen grain, the course of meiosis is within the norm. In the prophase of the first meiosis (pachynema), mainly 10 bivalents of closed type and 3 bivalents of open type are formed (2n = 26). Univalents were not observed. Along with this, slight abnormalities were noted: disorientation of bivalents, presence of chromatinous globules in the area of spindle apparatus. These deviations do not affect normal course of meiosis. Very rarely, triads and pentads are formed, as well. Formation of tetrads is simultaneous, tetrahedral.

Young microspores are mononuclear. Nucleus is of large size and round shape, sometimes elliptic, and mainly holds a central position.

Mononuclear pollen grains differ according to the shape of nucleus and its position and size, though difference is not significant. In binuclear pollen grains, vegetative nucleus is spindle-shaped. Generative nucleus is situated opposite the pore of pollen grain. Mature pollen grain is 2-membranous, with smooth outer layer**.**

Exine is thin, transparent, slightly defined. Normal, mature pollen grains are homogenous. Besides fertile pollen, a certain number of sterile grains are observed. Sterile pollen grains lack cytoplasm; sometimes the nucleus is absent. Its size is much smaller when compared with fertile grains.

The formation of structures of female generative sphere proceeds without deviations in early stages of development. Ovary is superior, 5-locular. Ovule is anatropous, tenuinucellate. Embryo sac is monosporic, of *Polygonum* type. Mature embryo sac

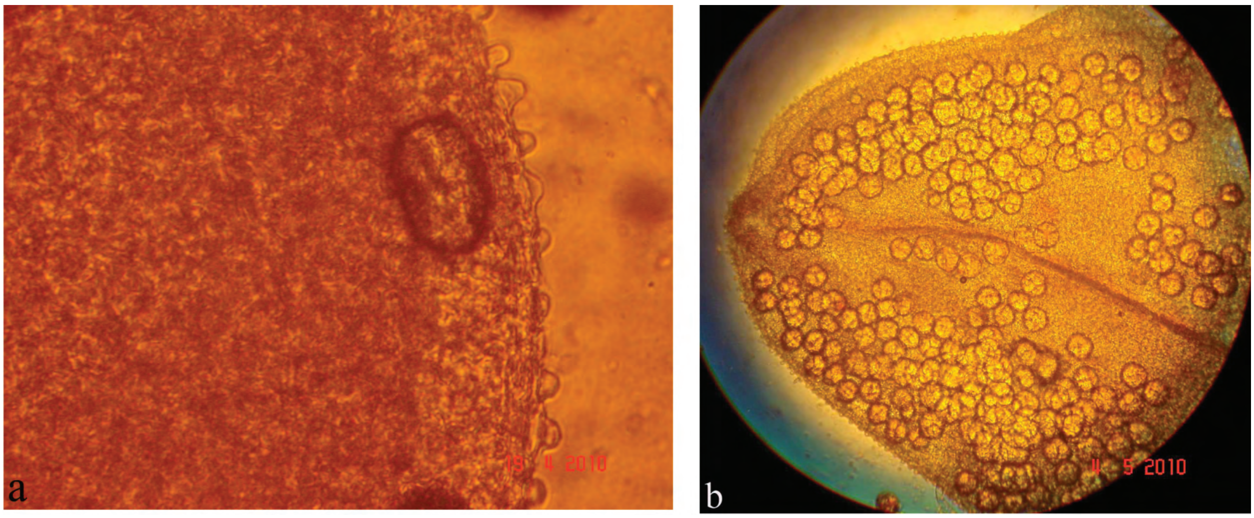

Figure 2. The structure of the anther of *Arbutus andrachne*: a) wall of the anther, b) pollen grains.

is 8-nuclear, 7-celled, of prolonged shape, broadened at micropile. Embryo sac is surrounded by 2 layers of tapetum cells. At chalazal end the haustorium is developed. Nucellar cap is formed at micropylar part, as in some angiosperms (Coşkun & Ünal, 2010). Fertilisation is of premitotic type. The zygote, formed as a result of normal process of fertilisation, then notably enlarges in size and starts to divide.

Embryogenesis proceeds as the Solanad type and a 4-celled tetrad is formed. The apical cell of the 2-celled embryo gives rise to the development of the embryo. In mature seed, embryo is of straight form (Figure 3). Endosperm is cellular, and at the final stage of development it surrounds the embryo from all sides.

Figure 3. A longitudinal section of an *Arbutus andrachne* seed with embryo.

Very often series of deviations were observed, causing the process of fertilisation to be hampered. In particular, penetration of the pollen tube into tissues of the style was impeded, or, in case of successful penetration, depression of the zygote or elimination of the already-formed embryo took place. Some abnormalities were also noted in the process of division of the primary nucleus of endosperm into 2 daughter cells, which caused ceasing of the process of endosperm formation at this stage.

***Osmanthus decorus*:** Anther is 4-locular. In endothecium, fibrous thickenings are marked (Figure 4). Tapetum is of secretory type. Meiosis proceeds normally and 18-19 closed and 4-3 open bivalents are formed (2n = 44). Sometimes univalents were observed, as well. Often anomalies took place, in particular: delay of chromosomes in anaphase of the first meiotic division; and cases of asynchronous division marked in the metaphase of the second meiotic division, resulting in a certain number of sterile pollen. Pollen grain is bicellular (Figure 4). Sperms are of prolonged type with large nucleus.

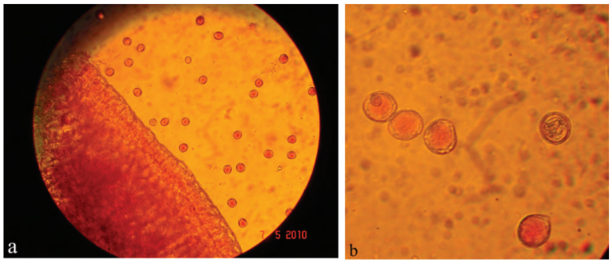

Figure 4. The structure of the anther of *Osmanthus decorus*: a) wall of the anther, b) pollen grains.

Ovule is anatropous, tenuinucellate, with a single integument; 2 stamens, ovary bilocular, with 2 ovules. Embryo sac is of *Polygonum* type. Fertilisation is postmitotic. Sperm fuses with secondary nucleus. Endosperm is cellular. Embryo differentiates with 2 cotyledons. Embryo is of Onagrad type. Despite normal development, very often formation of egg-cell apparatus was hampered.

Results and discussion

Seed germination experiments

As seen from the results of this investigation, the seeds of *Arbutus andrachne* started germination at room temperature (20-22 °C) after a month and the germination percentage in this group attained 5%-10% on the 49th day, after which the number of germinated seeds did not increase (Figure 5).

Seeds of strawberry tree, sown on petri dishes and placed in the refrigerator at the beginning of the test, germinated in a shorter period, with a germination percentage of 50% determined after 20 days. In order to continue the experiment, seeds germinated in the refrigerator were then transferred to the incubator. At this time, an essential increase was observed in the number of seedlings, and, after 3 months, the germination rate had reached 80% (Figure 5). Seedlings were finally transplanted to pots and open ground.

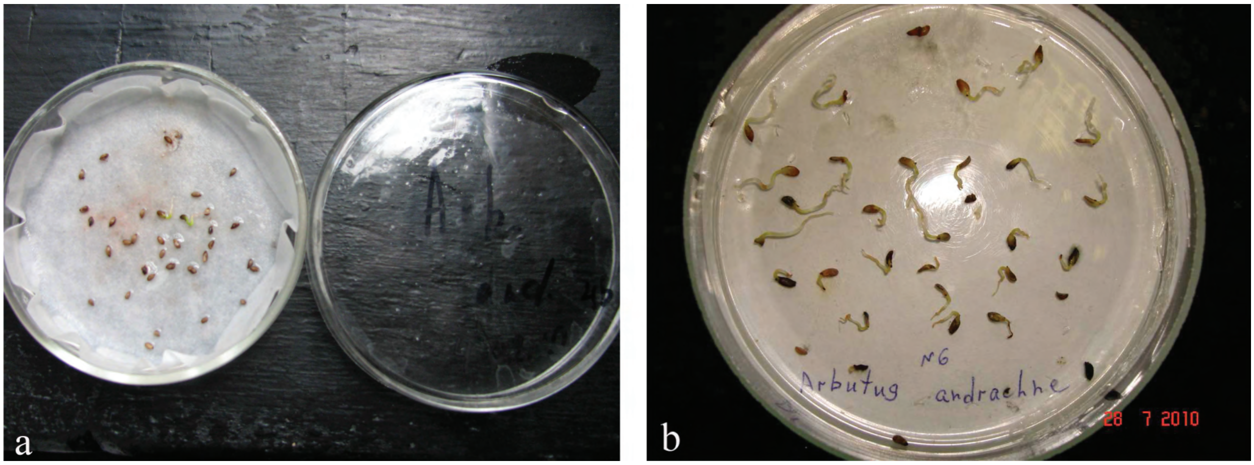

Figure 5. Germinated seeds and seedlings of *Arbutus andrachne*: a) germination on filter paper, b) germination on agar.

Those *Arbutus andrachne* seeds on petri dishes that were placed into the incubator at the beginning of the test did not germinate.

The seeds of Caucasian osmanthus did not germinate in any of the variant groups of the experiment. After 6 months without apparent germination, seeds from each variant were dissected and examined under the microscope. When the various experimental groups were compared, the embryo was in a better state in seeds that had been transferred from the refrigerator to the incubator. Embryos found in these seeds were large in size, occupied the central portion of the seed, and had well-developed endosperm (Figure 6), though the embryo was alive in all of the examined seeds. The state of the embryo proves that the seed remained potentially germinable in all cases. Observations on this process are still in progress.

Summarising the results of our embryologic studies, it is evident that the formation of female and male generative spheres and the processes of fertilisation proceed mainly in a normal fashion despite a series of violations and, as a result, a certain number of fertile seeds is formed.

It has been stated that abnormalities occurring at different stages of sexual reproduction of both species may be responsible for the low seed formation capacity seen in the studied species. More specifically, these abnormalities are a low rate of pollen tube development (20%-30% for *Arbutus andrachne* and 15%-20% for *Osmanthus decorus*) despite a high rate of pollen grain fertility in both species (approximately 70%-80%), and the frequent occurrence of embryo degeneration. Because of these factors, the normal seed set in these plants is somewhat restricted. Due to the small size of the distribution range of these species, the possibility of allogamy in this case is significantly impaired and artificial conditions were found to be unfavourable for fertilisation. It has also been noted that the biological plasticity of the system of reproduction is conditioned by the system of incompatibility and structural peculiarities of flowers (Shevchenko, 1983).

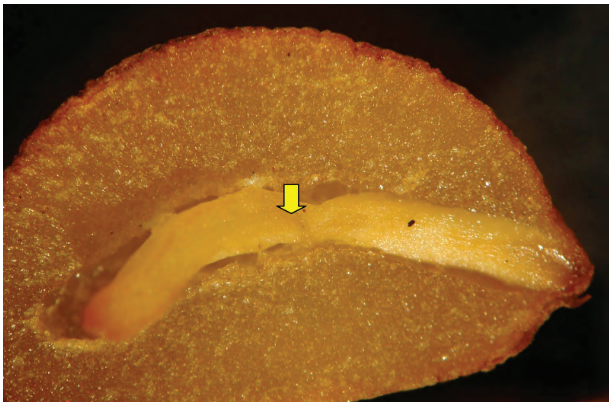

Figure 6. A longitudinal section of an *Osmanthus decorus* seed with embryo.

The results of our germination tests show that no special treatment is required to trigger the germination process of strawberry tree seeds, which start to germinate shortly after harvesting. The seed germination rate was better when the seeds were kept in a cold environment from the beginning of the experiment, though a higher germination percentage was attained when the seeds were transferred to the warmer conditions created in the incubator. Thus, the possibility of obtaining seedlings from strawberry tree seeds, together with the established seed bank of this species, created according to international standards, establishes the basis for the successful conservation of this rare species.

With regard to *Osmanthus decorus*, seeds of this species collected in 2006 were tested for germination before banking, though they failed to germinate within the standard 49-day period (Figure 7). Inspection of the embryo via microscopic examination of dissected seeds revealed that the embryo was alive, and thus the seeds were banked. Further experiments were carried out using the material collected over the next years and different conditions were tested. These later experiments have shown that the seeds are dormant and may require a longer period to germinate. It seems that transferring seeds from a cold environment to one with a higher temperature positively affects the process of germination of Caucasian osmanthus seeds. Presumably, the germination of these seeds is suppressed by inhibitors contained within the seed. The effect of inhibitors on seed germination may be alleviated by application of different growth regulators, namely gibberellic acid and kinetin (Terzi & Kocaçalişkan, 2010). The effect of gibberellic acid and kinetin on the germination process of Caucasian osmanthus seeds is being tested and experiments will be continued further.

Acknowledgements

The authors would like to express their gratitude to Dr. Zurab Manvelidze (Batumi Sh. Rustaveli State University, Batumi Botanical Garden) for assistance and advice during the field work carried out in the Adjara region.

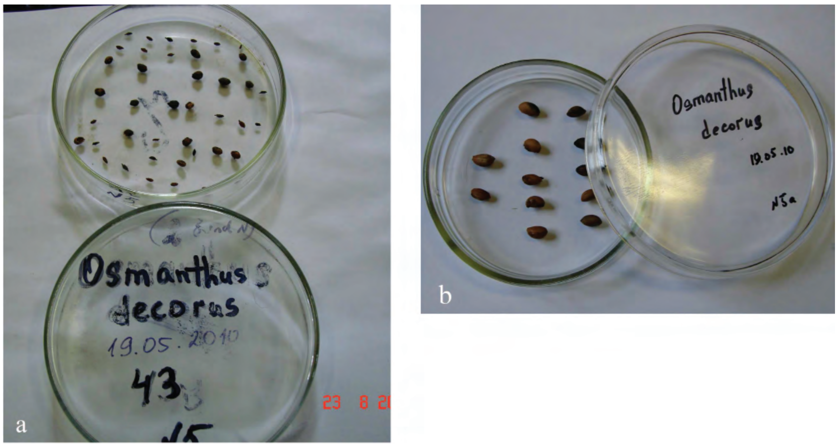

Figure 7. Germinating seeds of *Osmanthus decorus*: a) germination test on filter paper, b) germination test on agar.

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