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ECOLOGICAL EVALUATION AND CONSERVATIONAL
VALUE FOR BULGARIA OF *HIPPOPHAE RHAMNOIDES* L.
TOTAL FLAVONOIDS DETERMINATION AND
EXPERIMENTS ON IN VITRO CULTURE INDUCTION

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(Submitted by Academician V. Golemansky on June 18, 2007)

Abstract

Recent examination of a part of the habitats of *Hippophae rhamnoides* subsp. *caucasica* Rousi, Eleagnaceae in Bulgaria, has shown significant changes, negative as a rule, which imposes that the regional (national) category for the species according to the IUCN Red List should be “Critically endangered” instead of “Endangered”.

In order to assess the pharmacological potential of the plant in the natural environmental conditions of our country, we conducted an evaluation of the total flavonoid content of leaf samples, collected at the site of its natural habitat near Varna and of *Hippophae rhamnoides* L. specimens from the collection of the Botanical Garden, BAS.

As a part of a broader programme for in vitro conservation of medicinal plant species valuable for the Balkan flora, we started the development of a protocol for in vitro propagation of this species. Axillary bud growth induction was achieved on Murashige and Scoog (MS) medium through supplementation with different concentrations of benzylaminopurine (BA).

Key words: *Hippophae rhamnoides* ssp *caucasica*, ecological evaluation, total flavonoids, in vitro culture initiation

Introduction. *Hippophae rhamnoides* L., a member of Eleagnaceae, is thorny nitrogen fixing deciduous shrub or small tree. It is dioecious and propagates through seeds and vegetatively (through root offshoots). The average lifespan of the plants is 30–40 years and fruit bearing begins at the 4th–7th year. The plant occurs naturally throughout a wide area in Europe and Asia [1] mainly at

sandy sea shores and valleys of mountainous rivers at altitudes from the sea level to 2000 m and higher (mainly in Northern Caucasus and Pamir). The southern boundary of its distribution range passes through Bulgaria, where the species is known since the beginning of the 20th century, being distributed in the surroundings of Varna.

In different languages the plant is known as Sea buckthorn, Shaji, Sanddorn, Olivello Spinoso, Oblepicha, Tyrni, Espino Cerval de Mar, Havtorn, etc. The plant has been known for its valuable medicinal properties since ancient times. It is mentioned in the writings of ancient Greek scholars such as Dioscorid and Therophas. Since the ancient Tibetans started using Sea buckthorn more than one thousand years ago, hundreds of Asian traditional recipes have been developed and carried on through generations. Sea buckthorn is a phytoprotective agent to human health. It has antioxidant, anti-ulcerogenic and hepato-protective actions, and its berry oil is reported to suppress platelet aggregation [2–4].

During the past few decades, inspired by the long traditional use and the increasing public recognition of the benefits of natural products, scientists have carried out extensive research on Sea buckthorn. This has resulted in an improved understanding of the health effects and the chemical composition of *Hippophae rhamnoides* L. All parts of the plant are considered to be a good source of a large number of bioactive substances. Research has revealed the presence of carotenoids, tocopherols, sterols, flavonoids, lipids, ascorbic acid and tannins in the different parts of Sea buckthorn.

Catechin, rutin, quercetin, kaempferol and isorhamnetin are the five major flavonoids in the extract of Sea buckthorn leaves [5]. In this work the authors pay attention to the total flavonoid content of the leaves of Sea buckthorn as in a number of studies these bioactive constituents have been reported to suppress platelet aggregation, relieve cardiac disease and have antioxidant, hepatoprotective and immunomodulatory properties [6–10].

The species has an important ecological role as well, as it is effective for soil erosion control and reclamation of marginal land. The plant has an extensive root system and can grow effectively in marginal soils since nitrogen-fixing actinomycetes can form a symbiotic relationship with the roots [11]. Typical contributions of fixed nitrogen by *Hippophae* associations are 27 to 179 kg of N ha⁻¹ year⁻¹ [12].

In Bulgaria the plant is a protected species, included in the Red Book of Bulgaria in the Endangered species category [13]. Detailed information about the habitats and the status of the population of *Hippophae rhamnoides* L. in Bulgaria is available only from 1993 [14], where six natural localities and 3 individual representatives at two places in the Sea Garden of Varna (difficult to assess whether they have natural origin) were observed. Four of the localities were with 2–8 representatives from one sex, and two habitats with a several dozens of both-sex representatives.

The valuable medicinal and ecological properties of the species, as well as the fact that it is native to the Bulgarian flora impose the necessity of its popularization and practical utilization. The Endangered status of the plant species demands serious conservation measures for its preservation. One such measure as recommended by VITKOVA et al. [14] is to create re-introduced populations in appropriate regions, close to the natural habitats.

Micropropagation (in vitro propagation) is a state of the art and efficient method for both ex situ conservation of valuable or endangered species, on one hand, and rapid supply of a great number of planting material, free of pathogens and identical to the parent plant, on the other hand. That is why we have initiated a research work in order to develop a successful in vitro propagation protocol for this valuable medicinal plant.

Materials and methods. The changes of the population were visually observed, which is the only practical possibility, because of the prevailing type of the habitat of the species in Bulgaria (steep, almost vertically inclining friable sea banks). The conservational evaluation is made according to the methodology of IUCN (2001), applied on a regional level [15].

Plant material from naturally growing plants in Bulgaria was collected at the site of the habitat of *Hippophae rhamnoides* ssp. *caucasica* – the area between Chernomorets rest-house and Pasha Dere near Varna; and of the introduced species – from the collection of the Botanical Garden, Bulgarian Academy of Sciences, Sofia. The group of individuals, which form the collection in the Garden, was received from the Botanical Garden, Varna and Evksinograd Park in 1991. Now, because of the sprouting of the plants, it is difficult to distinguish between them. Herbarium specimens were deposited at the Herbarium of the Faculty of Biology, Sofia University – SO 104 538 for the Pasha Dere specimen; and SO 104 539 for the Botanical Garden, Sofia specimen.

As an assessment of the pharmacological potential of Sea buckthorn growing in Bulgaria, total flavonoids concentration of leaf samples of the plant was determined. It was measured using a colorimetric assay in accordance to a modification of the method of ZHISHEN et al. [16] and expressed in milligrams of (+)catechin equivalent per 1.0 g air dry weight of the sample. For this purpose, whole branches were air dried in shade and leaf samples were ground and kept in a dessicator until constant weight at room temperature before the analysis. 100 mg of the samples were extracted with 80% methanol, 0.4 ml of the extract was placed in a volumetric flask and 0.6 ml distilled water, 0.06 ml 5% NaNO₂ and 0.06 ml 10% AlCl₃ were added. After the addition of 0.4 ml 1 N NaOH and 0.45 ml distilled water, the absorption at 510 nm was measured and the concentration was calculated by means of a calibration curve of (+)catechin.

For in vitro culture initiation, plant material was collected at the Botanical Garden and was immediately manipulated. The top most parts of young offsets of the freshly collected branches were cut into segments approximately 1.5 cm in

length, containing one to four nodules; the adjoining petioles and laminae basis were kept intact in order to preserve the axillary buds. Explants were kept in sterile distilled water prior to the sterilization procedure to prevent drying. For each experiment a number of 120 explants was manipulated and placed in nutrition media in separate micropropagation glass test-tubes. Our preliminary investigations had shown that pre-sterilization of the explants with 70% v/v ethanol led to a fast decay of the explants up to seven days after they were placed in the nutrition media. We attributed this to the dissolving of the explants' cuticle, so that is why we excluded pre-sterilization from our procedure. Explants were sterilized in 0.1% HgCl_2 , triple washed in sterile distilled water and placed into the nutrition media. They were kept in a cultivation room at a temperature of 25°C , photoperiod of 16 h light and 8 h dark regime and light intensity $60\ \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (Fig. 1). In our initial experiments explants were placed in conventional Murashige and Scoog nutritional media (MS-nutrition media) [17].



Fig. 1. Surface sterilized explants of the intact *Hippophae rhamnoides* L. placed in the nutrition media



Fig. 2. Shoot initiation of *Hippophae rhamnoides* L. in modified MS nutrition media

Evaluation of the present status of the species in Bulgaria. In the biggest known in Bulgaria locality of the plant, that of Pasha Dere, which is the only place in Bulgaria with the characteristic for the species monodominant community, some changes were observed. Ten years after the former report [14], in 2003, a visual evaluation reveals an almost unchanged number of plants. Shoot observation showed a positive tendency in their growth rates for the period 2000–2003. This might be explained by the reduction of the number of pine forestation which was developed in the time of the first report. Drying and cutting have restricted this grove now. However, there still are no young individuals of Sea buckthorn observed, i.e. there is no seed regeneration and the number of the population remains critically low. The locality second in number of specimens is situated to the northwest of the Galata cape. In 1992 it consisted of several dozens of male and female plants. In 2004 their number was similar, but the specimens were smaller in size. The fruit bearing has also been very poor, with few dispersed fruits observed. There is a strong potential danger of extinction of the whole locality, as it is close next to a big soil drag-down, which developed at the end of 1990s. Up to this moment the drag-down restricts human activities, but there still is the danger of new soil dragging process, as well as direct or indirect extinction because of changes of water regime during fortification activities. The habitat in the outskirts of the Galata neighbourhood, to the right of the old road Varna – Galata is extinct due to building activities. In the 1990s there were 7–8 specimens from both sexes there. From the rest of the habitats, reported by Vitkova et. al (1993), the single male representative in the Sea Garden on the slope of the seabank is preserved and in good vegetative condition in 2005. The 3 other groups reported in 1993 (at the sea bank between Galata cape and Pasha Dere and two more in the region of Studentska bus stop) have not been recently observed. However, the extensive building activities in the last decade in these regions most probably imply the extinction of the species at one of them, situated at the sandy beach, very close to the road.

Actually the two biggest habitats at Pasha Dere and northwest to the Galata cape are at the number boundary of 50 individuals, at a visible evaluation. Having in mind that a part of the visible plants are connected through their roots, genotypically the number of these subpopulations is below 50 individual plants. Therefore the general population of the species is below 250 individuals, with a number below 50 individuals per subpopulation.

Determination of total flavonoid content. *Hippophae rhamnoides* subsp. *caucasica* Rousi, Eleagnaceae, samples, collected at the site of its natural habitat near Varna contained 12.75 mg/g dw (1.28%), and samples of the Botanical Gargen, Sofia collection – 7.48 mg/g dw (0.75%) total flavonoids, expressed as (+)catechin equivalent. For comparison other authors report quantities of total flavonoids 0.83–2.0% [18], the highest value being at elite selections; and others –

0.87 mg/g dw – 38.88 mg/g dw [19]. Cited literature references are for air-dried leaves total flavonoid concentration.

In vitro culture initiation. The experiments on in vitro culture initiation were conducted at the Laboratory of Plant Biotechnology, Department of Plant Physiology, Faculty of Biology, Sofia University. After approximately 10 days cultivation in the basal MS nutrition media, the total number of explants placed in it showed visible signs of drying and decay. Darkening of the nutrition media, most probably due to anthocyanins leaking, was also observed. In a second repetition of the procedure we obtained the same result. In order to achieve faster break of lateral bud dormancy, we added benzyladenine – BA (6-benzylaminopurine) to the nutrition media. We also simultaneously decreased the standard MS-macrosalts concentration in order to decrease the osmotic potential of nutrition media and prevent explant drying. Benzyladenine was added in concentrations 0.2, 0.3, 0.5 and 0.7 mg/l, and standard MS macrosalts formula was reduced to $\frac{1}{2}$ and $\frac{1}{4}$ of the conventional content. Formulas with only reduced macrosalts content, but without addition of BA, did not give satisfactory results, therefore the combination of both osmotic potential reduction and cytokinin addition was further implemented. In all explants axillary bud growth induction was observed, as 0.5 and 0.7 mg/l BA concentrations led to faster bud break and branching, but slower shoot growth, and $\frac{1}{4}$ macrosalts concentration gave better vitality to the shoots. That is why our media of choice for axillary bud induction were 0.2 and 0.3 mg/l BA with macrosalts concentration $\frac{1}{4}$ of the conventional Murashige and Scoog nutrition medium (Fig. 2). Axillary bud dormancy of *Hippophae rhamnoides* L. ex situ explants was successfully broken by the addition of benzyladenine to the MS nutrition media. However, no successful in vitro multiplication could be achieved due to the drying of the in vitro induced shoots during long term cultivation. Further experiments are needed in order to optimize the components of the nutrition media for establishing the multiplication step of the micropropagation protocol of *Hippophae rhamnoides* L. There also are other reports in literature for micropropagation of the species. Some of them are in accordance with our results in the addition of 1 μ M BA (0.22 mg/l) to the conventional MS nutrition medium and differ in macrosalts concentration of the media of choice for in vitro propagation, as the authors achieve results without altering the conventional formula [20].

Conclusions. Due to the extinction of one of the localities of *Hippophae rhamnoides*, the probable extinction of the locality at the Studentska bus stop, as well as the total area of occurrence and occupancy of the population, we consider that the regional (national) category of the species according to the IUCN (IUCN 2001) criteria must be “Critically endangered” instead of “Endangered”. The argument for this is that the area of occurrence in Bulgaria is below 100 square km and the area of occupancy is below 10 square km. There also is an ongoing decrease of the total area of occupancy, the number of mature individuals, the number of subpopulations – i.e. the species complies to criterion B for Critically

endangered. As the number of the total population for Bulgaria is below 250 adult individuals, below 50 individuals per population, the species complies with criterion C as well. Our opinion is that the evaluation of the species in Bulgaria is to be CR [B1ab(ii,iv,v)+2ab(ii,iv,v); C2a].

The rich flavonoid content of the leaves of Sea buckthorn shows the potentially high pharmacological value of the plant cultivated in our country. Moreover, the considerably higher values for the specimens collected at the natural habitat of *Hippophae rhamnoides* ssp. *caucasica*, Eleagnaceae near Varna, in comparison to the Botanical Garden collection sample, is a strong argument for the necessity of taking measures for re-cultivation and recovery of the natural resources of this valuable medicinal plant for our country, not only in ecological aspect, but also as a source of valuable health products.

Further research is in progress for development of an efficient in vitro propagation protocol for this species, being at the present stage only at the first phase – a successful in vitro culture induction. We consider that at the current stage of the experiments a determination of the total flavonoids content of in vitro cultured *Hippophae rhamnoides* L. would not be relevant. The accumulation of a sample of sufficient number of successfully in vitro cultured individual plants is necessary for obtaining of a statistically valid result of the secondary metabolites biosynthesized by the plant in vitro in laboratory conditions.

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