MICROPROPAGATION AND ACCLIMATIZATION OF *LYCIUM BARBARUM* L. MICROPLANTS TO *EX VITRO* CONDITIONS

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Abstract. With a high content of antioxidants, vitamins, amino acids, essential minerals and essential fatty acids, goji (*Lycium barbarum* L.) contributes to general well-being, supports the immune system, being considered a superfood. Goji berries have a long history of medicinal use, they are considered to have an important antidiabetic, anti-aging and anticancer effect, repair epidermal lesions, provide benefits to the cardiovascular system and contribute to effective cholesterol control. Micropropagation techniques are used as biotechnological tools that allow the production of a large number of plants from small fragments taken from a mother plant in a relatively short period of time. An applicable and efficient protocol for sterilization, initiation, multiplication, rooting and acclimatization in goji was developed. The results obtained in this study highlight the usefulness of tissue cultures in obtaining a high-quality planting material and the use of healthy biological material produced *in vitro* for large-scale propagation in *ex vitro* conditions.

Keywords: goji, plant tissue culture, variety, micropropagation, acclimatization

INTRODUCTION

Since ancient times, plants have been used as a source of health in human life. *Lycium barbarum* L. (goji) is a shrub of the family *Solanaceae* used in traditional medicine for thousands of years (Dănăilă-Guidea et al., 2015). With a high content of antioxidants, vitamins and essential minerals, goji (*Lycium barbarum* L.) contributes to general well-being, supports the immune system and eye health, being recognized as one of the most valuable medicines. Goji berries have a long history of medicinal use, they are considered to have an important antidiabetic and even anticancer effect, repair epidermal lesions, provide benefits to the cardiovascular system and contribute to effective cholesterol control (Nurliyana et al., 2010; Osman et al., 2012; Thomson, 2010; Zhao et al.,2005; Deli et al., 2012; Nedelcheva, 2012; Luo et al., 2004; Jing and Yin, 2010).

The properties of *L. barbarum* as potent antioxidant had been proven in many different scientific studies (Wu et al., 2004; Li et al., 2007; Bucheli et al., 2011; Osman et al., 2012 cited by Osman et al., 2013). Cells in humans and other organisms are constantly exposed to a variety of oxidants that cause many diseases, including heart disease, cancer, and even aging. Consumption of antioxidants has been proven by various scientific findings to be helpful in preventing and treating a number of disorders related to oxidative damage. In the food industry, synthetic antioxidants have been frequently used to be incorporated into food products as a measure to control the lipid oxidation reaction. Therefore, this initiated the desire for the search and discovery

of antioxidant substances from natural sources, also in medicinal plants (Osman et al., 2012).

Current scientific results (Peteros et al., 2012; Potterat, 2010; Jing and Yin, 2010; Luo et al., 2004; Feng et al., 2001 cited by Dănăilă-Guidea et al., 2015) confirm that this plant is valued for its many uses: strengthening the body's vital force thanks to multiple mineral and organic compounds containing vitamins from fruits and seeds (B1, B6, A, C and E) 18 amino acids (8 of them essential amino acids), 21 minerals (including significant amounts of Zn, Fe, Cu, Ca, Se, P and others) essential fatty acids (required both hormonally and for the normal functioning of the brain and nervous system).

Since goji berries are considered a superfood by specialists, this crop has also been adopted by Romanian farmers. The first certified organic plantation in Romania was established in Augustin village, Brasov county in 2014. The plantation was established with goji certified planting material from the Goji Bio Brasov nursery, the only certified Bio nursery in Europe. The surface of the plantation was 2.5 hectares with 6000 goji fruit trees. The variety is patented by the same company under the name "Kronstadt", a sweet and productive variety. The tests carried out on these fruits highlighted the rich content of vitamins and antioxidants. Goji culture is not pretentious, it can be cultivated in all regions of the country, with exceptional results and minimal coordination. For optimal development results, a pH test is is required (the pH of the soil must be higher 5.5-6).

Since the beginning of the activity, the Goji Bio Brasov nursery has helped to establish hundreds of crops of different sizes, as well as to the creation of processing solutions for these fruits.

Thanks to the numerous properties of this plant species, the initiative of developing a protocol for plant micropropagation through tissue culture procedures and acclimatization of *in vitro* produced microplants for large-scale plant propagation in *ex vitro* conditions is found to be appropriate. Micropropagation techniques are used as biotechnological tools that allow the production of a large number of plants from small fragments taken from a mother plant in a relatively short period of time. Numerous scientific articles are published on the use of biotechnologies in the cultivation of this fruit tree. By using tissue culture techniques, *Lycium barbarum* L. was propagated by direct and indirect organogenesis (Osman et al., 2012, 2013; Hu et al., 2008; Dănăilă-Guidea et al., 2015; Fira and Clapa, 2011; Cao et al., 1999; Sidhu, 2010; Tian et al., 1993). Different sources of explants have been used for direct *in vitro* organogenesis propagation of the goji (growing tips, nodal segments, axillary buds, stems and also root). In most cases, proliferation was achieved by growing axillary buds from nodal explants. The paper presents a protocol for efficient *in vitro* micropropagation of *Lycium barbarum* L., Romanian variety Kronstadt, and also the acclimatization in protected area of *in vitro* obtained microplants for cultivation in *ex vitro* conditions. The study took one year to be accomplished and had started in July 2022.

MATERIALS AND METHODS

Collection of plant materials

The study was commenced with the procurement of biological material, consisting of actively growing stem fragments detached from young and healthy plants,

typical of the variety. The explants necessary for the initiation of *in vitro* cultures were obtained from goji mother plants cultivated in open field in Codlea, Brasov county. Young shoots used as a source of explants were detached from the mother plants in early July and brought to the laboratory.

Kronstadt Romanian goji variety is a sweet and very productive variety of goji, with results of over 4 kg of fruit/plant per year, with a high content of vitamin C and other antioxidants. It requires relatively little care, focusing on irrigation and occasional foliar treatments. It can grow up to 3 meters, and the goji fruits are found at the ends of the branches, easy to pick. It starts producing fruits with the year following planting and into full production starting with the 3rd year. Goji fruits can be used in various mixtures, for human consumption or treatments. It can be processed by dehydration, transformation into powder or cream as well as by lyophilization.

Sterilization of explants

Microbes are common cause of contamination in tissue culture. Sterilization is one of the reliable means to control the pathogenic effect of microbes. The use of nodal explants for *in vitro* propagation promotes direct regeneration of cultures, but may cause high levels of microbial contamination as well, due to the size of explants.

The protocol for sterilizing the stem fragments involved the completion of specific steps that must be strictly followed. Goji young shoots were rinsed under running tap water for 3 minutes. After removing the leaves (keeping a petiole portion to protect the bud during sterilization), the shoots were cut into 2 cm segments containing one axillary bud. Explants were then transferred to clean containers, covered and sent to flow hood. All surfaces were wiped with sterile tissue paper soaked in 70% ethanol. Culture vessels, work tools, tissue paper and all other materials required for inoculation have been pre-sterilized and then placed in the hood.

For surface sterilization, goji explants were subjected to different sterilant conditions (variants from V1 to V4). The uninodal segments were dipped in 70% ethanol for 1, 2, 3, and 4 minutes respectively, after which, without rinsing, they were immersed in 1% sodium hypochlorite (NaClO) solution for 5, 8, 10, and 15 minutes, respectively (Table 1). Four drops of Tween-20 were added to the NaClO solution. After applying the sterilization treatments, to remove the sterilization agents, the explants were rinsed 3 times successively in sterile distilled water for 10 minutes and then draying on filter paper. All operations were carried out in the sterile room, in the hood with laminar air flow (sterilized with the UV lamp).

Table 1

Sterilant conditions for *in vitro* initiation of goji cultures

Sterilant conditions	
V1	70% ethanol for 4 minutes 1% NaClO for 15 minutes
V2	70% ethanol for 3 minutes 1% NaClO for 10 minutes
V3	70% ethanol for 2 minutes 1% NaClO for 8 minutes
V4	70% ethanol for 1 minute 1% NaClO for 5 minutes

Inoculation of explants on the culture medium. For initiation of *in vitro* cultures, WPM (Woody Plant Medium - Lloyd şi McCown, 1981) supplemented with 30 g/l sucrose, 0.1 g/l myo-inositol and 9 g/l agar was used as nutritive medium for shoot growth. For control of microbial contamination, 3 ml/l of a broad-spectrum product PPM (Plant Preservation Mixture) that inhibits the growth of pathogens in plant tissue cultures was added to the medium. The pH of the medium was adjusted to 5.7. Test tubes containing 5 ml of culture medium were covered with aluminum foil and autoclaved at 121 °C for 20 minutes (Table 2). When the shoots had approx. 7-8 cm high (2-3 nodes) were fragmented (cuttings with 1 node and related leaves) and transferred from the growth medium to the rooting medium (Fig. 1).

Table 2

Nutritive medium for *in vitro* growth and rooting of goji shoots

Components	***Growth medium*** *WPM (Lloyd and McCown, 1981)*	***Rooting medium*** *MS (Murashige and Skoog, 1962)*
Sucrose	30 g/l	30 g/l
Myo-inositol	0.1 g/l	-
Agar	9 g/l	9 g/l
PPM	3 ml/l	3 ml/l
IAA	-	0.5 mg/l

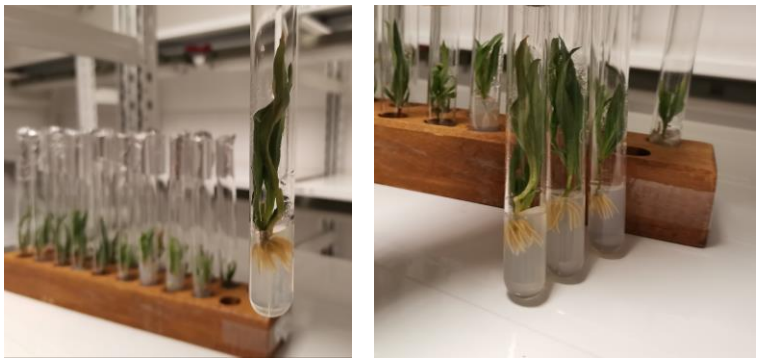

Fig. 1. Root appearance of goji microplants on rooting medium supplemented with IAA

After explants inoculation, the cultures were transferred to a growth chamber and exposed daily to 16 hours light. The growth chamber temperature was maintained at 25±2 ºC. At an interval of approximately 30 days, the cultures were pasted on a fresh medium (Fig. 2).

Fig. 2. Goji microplants incubated in growth chamber

In order to transfer the *in vitro* obtained microplants in a protected area, for acclimatization, in January 2023 we started to inoculate shoots also on plastic green filter boxes (140 mm long, 80 mm wide and 80 mm high). In each box, 100 ml of nutritive medium were distributed and 3 shoots were inoculated. Culture medium MS supplemented with 30 g/l sucrose, 0.5 mg/l NAA, 9 g/l agar and 3 ml/l PPM was used. Well-developed plantlets with vigorous shoots and a developed root system were obtained in this type of culture pot. These microplants were then transferred in protected area, for acclimatization, but some of them can be used as a source of explants for new *in vitro* cultures (Fig. 3).

Fig. 3. Well-developed rooted goji plantlets before being transferred in protected space

Acclimatization of in vitro obtained microplants. At the end of May the goji plantlets were transplanted into pots, in greenhouse (plantlets grown in green filter boxes, well developed, with vigorous root system were used; those that were transplanted from test tubes did not survive - they were smaller and with less developed roots). Pots with a volume of 2 l were used (Fig. 4). As a substrate, a mixture of peat + perlite (in a ratio of 1:1) was used. Treatments with CROPMAX (foliar fertilizer) have been performed in 2 applications: June 6 and June 13. At the beginning of July the goji plants, acclimatized in the greenhouse, were transferred to open-field conditions for *ex vitro* cultivation.

Fig. 4. Acclimatizated goji plants in greenhouse

RESULTS AND DISCUSSION

Observations were made every day for culture contamination and general growth of plantlets. The first microbial contamination occurred 11 days after cultures initiation, on the explants to which the V4 sterilization treatment was applied. Regarding the sterilant conditions used, the best results were obtained in the case of variants V2 and V3. In this case, the number of microbial infections was reduced, and the explants grew normally and generated well-developed microplants. In the case of the V1 sterilization variant, the treatment was too harsh (sterilizing agent concentration and exposure time were higher) and the explants did not survive. Also, even on the V4 sterilization variant, the results were not the desired ones. Although the explants survived, the presence of microbial infections was very high.

After obtaining a stock of microbial contamination free cultures, the explants that survived performed well both on the shoot growth medium and the rooting medium. The composition of the culture medium and the conditions in the growth room ensured a favorable climate for the growth and development of goji plantlets. Thus, very vigorous goji microplants with a well-developed root system were obtained. Furthermore, the use of green filter boxes as culture vessels was a very good choice in order to transfer microplants from *in vitro* to *ex vitro* conditions, compared to using plantlets grown in test tubes. In green filter boxes the goji plantlets were better developed (both in terms of shoots and roots aspect), they were more resistant and passed the acclimatization stage more easily.

About 1 month after transfer to the greenhouse of the *in vitro* obtained microplants, the acclimatized goji plants were cultivated to open field-conditions for large-scale propagation. Goji Kronstadt plants transferred in *ex vitro* conditions are characterized by uniformity, production entry being faster than other forms of propagation. It needs a period of acclimatization after transfer from the protected area. Periodic treatments make the Goji shrub remain healthy and productive.

CONCLUSIONS

The results obtained in this study provide valuable information regarding the *in vitro* cultivation of *Lycium barbarum* L. An applicable and efficient protocol for sterilization, initiation, multiplication, rooting and acclimatization in goji was developed. The obtained results highlight the usefulness of tissue cultures in obtaining a high-quality planting material and the use of healthy biological material produced *in vitro* for large-scale propagation in *ex vitro* conditions.

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