

PROTOCOL FOR EFFICIENT *IN VITRO* MULTIPLICATION OF *LYCIUM BARBARUM* L. (GOJI) BY DIRECT ORGANOGENESIS

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Abstract

The plant known scientifically as *Lycium barbarum* L., and commonly in the West as the wolfberry, or simply as "goji", is considered by many authors as the most nutrient rich plant on earth. It has been used for thousands of years by Chinese and Tibetan therapists as a source of health. The plant *Lycium* (Goji) is a shrub of the family Solanaceae is a true national treasure for China used in traditional medicine for over 2000 years. Thus, according to information published in various specialized articles and those taken from traditional beliefs, goji fruit is considered to be an important antioxidant, antidiabetic, and a natural source with excellent effects on the cardiovascular system and in decreasing the level of cholesterol in the human body. Given the properties of this imported super fruit the initiative to develop a propagation protocol to be widely used in Romania too is therefore considered to be one relevant for this plant species. The direct organogenesis protocol used in this study yields explants and microcuttings from meristematic apices consisting of 2-3 node fragments detached from 30 day old goji seedlings germinated in *in vitro* conditions. The average was 89-95% for morphogenetic culture; for the offshoots rooted in a liquid culture supplemented with 1 mg / l IBA, it was 100% .*In vitro* goji rooted plantlets were successfully acclimatized (average survival rate of 90-98%) in a peat substrate mixed with sand. After being placed under *ex vitro* conditions, the vitroplantlets increased rapidly and developed well. Over a period of one month, they formed new branched roots and many axillary shoots with healthy leaves.

Key words: *Lycium barbarum* L., direct organogenesis, goji.

INTRODUCTION

Lycium barbarum L. known as goji, has been recognized as one of the most valuable medicines. Goji berry has a long history of medicinal use, especially among tribes in China. Sorted from various literatures and from traditional beliefs, goji is considered to have an important antioxidant, antidiabetic effect (Nurliyana, et al., 2012; Osman, et al., 2012 Thomson, 2010), repair epidermal damage (Zhao, et al., 2005) and in providing excellent effects on the cardiovascular and cholesterol levels 1-6 (Deli M.A., et al., 2012; Nedelchev, et al., 2012).

Ancient Chinese medical texts celebrated wolfberries for their many uses, including hardening of the body's vital force due to multiple mineral and organic compounds that they contain fruits and seeds vitamins (B1, B6,

A, C and E) 18 amino acids (8 of them essential to life), 21 minerals (including significant amounts of Zn, Fe, Cu, Ca, Se, P and others) essential fatty acids (needed for hormone production and normal operation brain and nervous system) and the current results of researchers confirm these properties. (Peteros, et al., 2012; Potterat O., 2010; Jing and Yin. 2010; Luo, et al., 2004; Feng, et al., 2001). Considered a superfood by specialists, Goji fruit bush has been adopted by Romanian farmers (Tarcza, 2012). A manufacturer in Ciuperceni village (15 kilometers away from Satu Mare, in northern Romania) has set up the first organic goji plantation in Europe, on an area of 2 hectares. He did so after receiving 50 thousand seeds from North America four years ago. Even if at first he did not know much about how to cultivate this plant, he was thinking of future investments.

It was encouraged by the recognized effects of goji fruit, used to treat diabetes or even cancer ([www. https://ro.stiri.yahoo.com](http://www.https://ro.stiri.yahoo.com)).

Micropropagation techniques are used as biotechnological tools that allow the production of a large number of plants in small pieces taken from a mother plant in a relatively short period of time (Cristea, 2010; Stănică, et al., 2002; Roșu, 1999; Cachiță-Cosma D., 1987).

Numerous scientific articles have reported using tissue culture techniques, with many researchers successfully applying these to the goji shrub (Osman, et al., 2013b, 2012; Hu et al., 2001) and by those coming from research centers or universities in Romania (Fira et al., 2012, Fira et al., 2011).

By employing tissue culture techniques, *Lycium barbarum* L. (goji) has been propagated through direct organogenesis (Osman, et al., 2013b; Fira et al., 2011, Hu et al., 2001) and indirect organogenesis (Osman, et al., 2013a; Hu et al., 2008). Different explant sources have been utilized for *in vitro* propagation of goji shrub through direct organogenesis. Researchers Fira A. and Clapa D. (2011) from Fruit Research Station Cluj (Cluj-Napoca town, Romania) reported in their study the use of shoot tip and nodal segments for efficient micro-propagation protocol, whereas Osman, et al., (2013b) and Hu Z., with collaborators (2001) used leaf, stem axillary buds and also root for *in vitro* multiplication of *Lycium barbarum* L. (goji) plants. In most cases, shoot proliferation was achieved by axillary bud growth from nodal explants.

In this context, the paper presents a new protocol for efficient *in vitro* multiplication of *Lycium barbarum* L. (goji), in order to highlight the importance of explants in inducing the direct organogenesis process.

MATERIALS AND METHODS

The seeds taken from fresh ripe berries of goji (commercial market sources) were surface sterilized in two versions with a product undiluted commercial bleach (ACE) containing 4.85% sodium hypochlorite for 10 minutes (V1) and a dilute bleach product concentration trading 4.50% sodium hypochlorite for 20 minutes (V2), followed by three washes with sterile distilled water.

Aseptic, sterilized seeds were placed individually in glass tubes with a capacity of 19 x 110 mm containing 10 ml of culture medium for germination test. The culture medium was prepared with a composition diluted to the half of the formulation of basal Murashige-Skoog (MS) (Murashige and Skoog, 1962) without hormones, to which were added 20 g /l sucrose, 7 g /l agar and pH 5.8.

In subsequent work steps basal MS (1968) recipe consisting of salts with vitamins normal concentration was used but was supplemented with auxin type phytohormones (IAA, NAA, IBA) cytokinins (BAP and Kin) and gibberellins (GA3) in different concentrații (0.0022 to 2 25 mg /l) for triggering proliferation and morphogenetic processes caulogenesis and rootedness. Cultures were incubated at 25 ± 2°C under fluorescent light 16 hours of light photoperiod.

RESULTS AND DISCUSSIONS

Inoculation and incubation of goji explants

Goji explants were obtained in advance from *in vitro* plantlets developed through the process of seed germination in aseptic culture media without additional plant hormones.

After five weeks, leaves, apexes and nodal parts from the seedlings obtained *in vitro* were detached and used as explants for future culture leaves, apexes and nodal parts from the seedlings obtained *in vitro*. *L. barbarum* explants were excised at about 0.5-1.0 cm length and grown on basal medium Murashige & Schoog (1962) MS in six variants of exogenous plant hormones IAA, NAA, IBA, GA3, BAP Kin and with different concentrations (from 0.0022 to 2.25 mg /l), sucrose 3% and agar 0.8% with pH adjusted to 5.8, according to Table 1.

Inoculation of explants consisted of placing variants of the culture medium chosen for experimentation, distributed in the culture dish sterile operations performed in a laminar flow hood (Stănică, et al., 2002; Roșu, 1999; Cachiță-Cosma, 1987).

After inoculation *in vitro* cultures were transferred into the growth chamber, air conditioner, thus exposing them to an ecophysiological regime required by the nature and type inoculants induced morphogenetic processes.

Table 1. Variants of recipes used to induce experimental organogenesis at goji (*Lycium barbarum* L.)

Medium Variant	Hormonal Balance	Other component	Organogenous processes induced
Var I	MS(1962)+0,5mg/l BAP+0,2mg/l NAA	8 gr./l agar+ 30 gr./l sucrose	initiation of adventitious organogenesis
Var II	MS(1962)+0,5 mg/ l BAP+0,5mg/l NAA	8 gr./l agar+ 30 gr./l sucrose	initiation of adventitious organogenesis
Var III	MS(1962)+ 1mg/l BAP +0,5 mg/l GA3+0,1mg/l IBA	8 gr./l agar+ 30 gr./l sucrose	caulogenesis proliferation
Var IV	MS(1962)+ 2,25 mg/l BAP +0,18mg/l IAA	8 gr./l agar+ 30 gr./l sucrose	caulogenesis proliferation
Var V	MS(1962)+ 1,8mg/l IAA+0,022 mg/l Kin	8 gr./l agar+ 30 gr./l sucrose	rootedness process
Var VI	MS (1962)+ 1mg/l IBA	+ 30 gr./l sucrose without agar	rootedness process
Var 0	MS(1962)- without hormones	7 gr./l agar+ 20 gr./l sucrose	Control seed germination

Legend: MS - Murashige and Skoog Media (Murashige; BAP- 6-benzylaminopurine; NAA –naftyl-acetic acid; IBA - indolyl butyric acid; IAA –indolyl acetic acid; Kin – chinetina; GA3 – giberelic acid

Establishing long-term morphogenetic cultures.

In regeneration systems "*in vitro*" caulogenesis express themselves by developing unipolar structure represented shoots and directly shoots structure from explant or via callus, cytokinins having an essential role in inducing this kind of morfogenesis; by posting strains develop "*in vitro*" and rooting their on special rhizogene media can be regenerated independent plants, a process that is sitting at the base of the nonconventional technology vegetativ way .

During the initial phase of establishing the cultures morphogenetic included in the first 6-8 weeks of culture, the leaf explants developed callus nodules and small roots while the nodal explants were formed by direct organogenesis leaf primordia and shoots.

After 8 weeks of culture goji plantlets regenerated *in vitro* on induction medium (VarI and VarII) variable subcultivations were performed on fresh MS basal culture medium of variants VarIII VarIV for induced proliferation and morphogenetic crops processes.

After 14 weeks of culture, a thorough assessment at explants (Goji) of *Lycium barbarum* on *in vitro* regeneration was performed and recorded (Table 2). Referring to the data obtained, it showed us that treatment with 0.5 mg /l NAA and 0.5 mg /l BAP (variable) used in the cultivation of leafy explants page is the best combination to induce

initiation of *in vitro* adventitious organogenesis initiation to *L. barbarum* species.

Table 2. Influence variants used to induce experimental recipes from goji organogenesis, after 14 weeks of culture; (average values)

Medium Variant	Inoculated explant type	Elongated adventitious shoots/explant	
		No.	Length Sprouts (cm)
Var III	leaf	5	5,80
	apexes	3	2,2
	nodal fragments	15	7,1
Var IV	leaf	5	4,7
	apexes	5	2,5
	nodal fragments	10	5,5

The advantage of the culture "*in vitro*" apical or axillary buds situated at the explants used as inocula (apexes and parts nodal) is due miniaturized indicated at the tip of the shoot growth, such as regenerating an independent plants require only induce elongation and rootedness process of the new obtained vitroplants. In the case of leaf explant inoculated on to the VarIII the hormone balance was made up of 1 mg /l BAP 0.5 mg /l GA3 + 0.1 mg /l IBA morphogenetic cultures was produced concurrently with the proliferation of multiple shoots and the formation of adventitious roots *in vitro* conditions (Figure 1).



Figure 1. Morphogenetic crops from goji (*Lycium barbarum*) initiated from nodal explants *in vitro* conditions.

Induction rootedness process

Subcultivation were made at intervals of 3 to 4 weeks in culture medium variant used in initiation, because it has proven so effective in stimulating the development of multiple shoots and in terms of their elongation. On the occasion of each elongated subcultivations shoots over 2 cm in size were detached and transferred on two (VarV and VarVI) rizogenetic media (Figure 2).



Figure 2. *Lycium barbarum* elongated shoots (Goji), transfer on the rizogenetic culture media variants: VarV- (with agar) and VarVI- (without agar)

The first root primordia were formed after 3 weeks and efficient root system to develop after 6 weeks of incubation under these conditions (Figure 3).



Figure 3. Goji *vitro*-plantlets with well developed roots system, suitable for acclimatization after 3-4 maintenance in liquid rooting medium

CONCLUSIONS

This study was conducted to determine the best protocol subculturing and hormonal compositions suitable for *in vitro* regeneration of *Lycium* plant. They were also identified age and body seedlings *in vitro* with an optimal level of proliferation. For the *in vitro* regeneration of leaves and nodes were used as transferable items on regeneration medium and cultivation in presence of various concentrations of hormone combinations of- α naphthalenacetic acid (NAA) and 6-benzyl amino purine (BAP).

Nodal explants were identified as being more receptive than leaf explants, since the latter took them two weeks to produce adventitious buds.

The yield of morphogenetic crops from goji meristematic apexes explants detached from seedlings germinated *in vitro* conditions was 89-95% and the rooting of shoots in the liquid culture medium supplemented with 1 mg /l IBA it was 100%. Goji rooted plantlets in *in vitro* conditions were successfully acclimatized (median survival rate of 90-98%) in a peat substrate mixed with sand. *Lycium* plantlets placed *in vivo* have grown rapidly and have developed well branched adventitious roots and the numerous side shoots with leaves healthy over a period of one month (Figure 4).



Figure 4. Goji vitropplants transferred to pots after 6 to 8 weeks of acclimation period

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