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INCREASING THE ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND ASSIMILATORY PIGMENTS CONTENT BY OPTIMIZING THE *IN VITRO* GROWTH CONDITIONS OF *LYCIUM BARBARUM* PLANT

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Abstract
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. The present study was conducted to develop a method of optimizing the content of antioxidants. Therefore, we initiated the in vitro culture of goji plants from meristematic apexes of plants harvested from 30-day-old germinated seeds generated from the substrate septic consisting of peat mixed with perlite. The culture medium used was Murashige & Skoog supplemented with 0.5 mg / l IBA (indolyl butyric acid) and 0.5 mg / l BA (benzyladenine). Plants containers were exposed to light of different colors fluorescent tubes from Osram company and the following lengths wave: white; blue- 473 nm; green- 533 nm; yellow 580 nm; red-680 nm. Chamber growth temperature was $23^0C \pm 2^0C$, between light and $20^0C \pm 2^0C$, during hours of darkness, photoperiod of 16 hours light and 8 hours darkness. At the age of 60 days these vitro seedlings have undergone physiological and biochemical analyzes. Red light, green and yellow stimulates growth length of vitro seedlings and blue light produce small and stocky plants and even growth slowing. Blue light has increased the total content of assimilating pigments and content of antioxidants (phenols, flavonoids) were increased by the yellow and white light. The antioxidant capacity was also higher in vitroplants grown in blue light and lowest in red light.

Key words*: antioxidant capacity, fluorescent tubes, Lycium barbarum L., phenols.*

INTRODUCTION

Since ancient times plants have been acknowledged to be the source of medicines for human healthcare. Antioxidant property is one of the most valuable phytomedicinal values in plant to be used as natural remedies. Through various scientific findings, it has been proven that the consumption of antioxidants is useful in the prevention and treatment of a number of disorders related to oxidative damages. In food industry, the synthetic antioxidants have been frequently used to be incorporated in the food products as a measure to control lipid oxidation reaction. For instance, butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate and tertiary butyl hydroquinone are among the most widely used synthetic antioxidants. Nevertheless, the usage of those synthetic products has raised some questions pertaining to their possible risks of toxicity.

With regard to antioxidant properties, *Lycium barbarum* is one of the herbs that have been recognized to be the source of this phytomedicine. It belongs to the family of *Solanaceae* and found wild around the hills in the Ningxia region of China, and in remote areas of central China near inner Mongolia. Therefore, this has initiated the urge for the searching and discovery of antioxidant substances from natural sources likewise in medicinal plants. *L. barbarum* polysaccharide (LBP) is reported to have efficient immunomodulatory properties and inhibiting tumour growth. It has been recognized to be an

exotic super fruit and very often included in foods especially in preparing Chinese tonic soups. Another interesting finding reported by Li and his contributors (2007) had proven that the polysaccharide of *L. barbarum* is effective in counter-acting oxidative stress damage. In addition to this, Feng and his contributors (2001) indicated that *L. barbarum* is also effective in providing protection for retina from oxidant injury in diabetic subjects.

As a signal and energy source, light is one of the most important environment factors for plant growth and development. Compared with light intensity and photoperiod, light quality shows much more complex effects on plant morphology and physiology. Specific spectrum stimulates different morphological and physiological responses. Red light (R) and blue light (B) absorbed by photosynthetic pigments are more effective than other wave lengths (Pfündel and Baake, 1990). It is well known that red light influences stem elongation, root to shoot ratio, chlorophyll content, photosynthetic apparatus (Appelgren, 1991; Aksenovaetal., 1994; Sæba et al., 1995). Blue light causes physiological responses via phototropins, including phototropism hypocotyl elongation, leaf expansion, stomatal opening, leaf anatomy, enzyme synthesis, chloroplast movements, and genes expression (Christie, 2007; Inoue et al., 2008; Wang et al., 2009). Red light is the primary light source affecting biomass production and elongation through the phytochrome photoreceptor (Sager and McFarlane, 1997). Blue light also affects photomorphogenic responses (e.g., regulation of leaf flattening and compact appearance) through phototropins and cryptochromes acting in an independent and/or synergistic manner with the phytochromes (de Carbonnel et al., 2010; Kozuka et al., 2013).

There is no information available regarding the relationship between the light absorbed by *L. barbarum* plants and the mechanisms underlying the physiology and secondary metabolism under the influence of different light spectra. Secondary metabolites are formed in order for the plant to overcome potential stressful conditions. In plant tissues such as stems and leaves, the secondary metabolite synthesis can change due to environmental, physiological, biochemical and genetic factors (Wink, 2010; Zhao et al., 2005) with light being one of the most influential factor (Kopsell et al., 2004; Kopsell and Sams, 2013). One of the main groups of secondary metabolites is the phenolic group. This group is among the most ubiquitous groups of secondary metabolites in the plant kingdom and represents an example of metabolic plasticity enabling plants to adapt to biotic and abiotic environmental changes (Wink, 2010). Their concentration depends on season and varies at different stages of growth and development (Seigler, 1998; Wink, 2010). Phenolics are pigments exhibiting radical scavenging activity, as well as protective activity against fungi, bacteria, viruses and insects (Lattanzio et al., 2006; Seigler, 1998). Physiological changes are triggered by exposure to varying wave lengths (Samuolien˙e et al., 2013). However, as goji is not locally found in România, thus the acquisition of extracts can only be made through *in vitro* seed germination whereby the seeds were obtained from the imported dried goji berries. Thus, the *in vitro* germination of the species was carried out accordingly beforehand to produce *in vitro* seedlings to serve as the source of plant materials.

The objective of the present study was to determine the efects of diferent wavelengths of fluorescent tubes by investigating the antioxidant capacity, content of assimilating pigments and content of antioxidants (phenols, flavonoids). The results of this study would be used to give guidance on light colour sources design for goji cultivation in a controlled environment.

MATERIALS AND METHODS

1.1. Plant material, growth conditions and light treatments

The culture substrate used in the in vitro cultures experiments consisted of basal medium (MB) Murashige - Skoog (1962) (MS) agar medium, which consisted of macronutrients, Fe EDTA and trace elements, mineral mix according to the original recipe, but with increased addition of vitamins: pyridoxine HCl, nicotinic acid and thiamine HCl (1 ml / l each of the original recipe to where indicated 0.5 mg / l), to which was added m-inositol 100 mg / l, sucrose 30 g / l and agar - agar 10 g / l; this

basic medium (MB) were added as growth regulators 0.5 mg / l IBA (indolyl butyric acid) and 0.5 mg / l BA (benzyladenine). Before autoclaving of the culture medium, the pH value was adjusted to 5.5 with HCl or NaOH, depending on the basicity or acidity of the final medium. For autoclaving, 15 ml of the medium were placed into the clear glass culture containers that were temperature resistant, 8 cm height and 4 cm diameter. After portioning the culture medium, the culture containers were filled with aluminium foil. Sterilization of the containers and culture media was performed by autoclaving at 121 ° C for 21 minutes.

The plant material used for the initiation of vitro cultures was the meristematic apexes of goji plants with a length of about 1 cm and 2-3 leaf primordia, harvested from seedlings regenerated from zygotic embryos that have sprouted from 30 days old seeds that were germinated on a septic substrate consisting of peat mixed with perlite. After cooling of the culture medium, was performed the inoculation of explants on culture medium and then the inoculated containers were transferred into growth chamber that were placed on racks exposed to a temperature ranged from 23°C ± 2°C, in the light regime, 20°C ± 2°C, during darkness and a photoperiod of 16 h light / 24h. Fluorescent tubes emitting coloured light (Osram company) were used, length 590 mm, Ø 26 mm, 120 lux light intensity and the colour varies depending on wavelength: 473 nm blue; green- 533 nm; yellow 580 nm; red-680 nm; white 380-760 nm (used as a control in our experiment).

1.2. Quantification of phenols, flavonoids, assimilating pigments content, antioxidant capacity and fluorescence

Chlorophyll fluorescence was evaluated by measuring ϕPSII on 5 leaves per treatment. ϕPSII was measured at normal light regime.

Assimilatory pigments were measured with a non-destructive portable chlorophyll content meter (CCM 200 Plus, Opti-Sciences Ltd.) that measures optical absorbance, the readings being expressed as CCI units.

Extracts were prepared by macerating 5 g of ground fresh plant in 95 ml of distilled water or 30% w/v ethanol for 24 hours. Total phenolic content was assessed using the Folin Ciocalteu reagent method, by spectrophotometric readings at 760 nm of the colour of incubated extracts (Makri, 2008).

Total flavonoid content was determined according to the method described by Makri (2008), by evaluating the absorbance at 510 nm of extracts reacted with 5% $NaNO_2$ and 10% $AlCl_3$.

In this study, the method of DPPH assay was used and the comparative antioxidant assessments were conducted mainly to compare the antioxidant capacity of this species by exposing the vitro plantlets to light coloured fluorescent tubes of different wavelengths. Free radical scavenging activity was determined in the DPPH method. In such assay, DPPH is best corresponds to a model radical which will be reduced by antioxidant properties derived from the extracts. DPPH is a relatively stable free radical and can be reduced by electron-rich radical scavengers from medicinal plant extracts. Free radical scavenging activity was determined in the DPPH method (Herald, 2012), measuring the decolouration of DPPH solution reacted with extracts at 515 nm for 3 hours.

1.3. Statistical analysis

The statistical analyses conducted were represented by analyses of variance among treatments and the Tukey test at $p<0.05$, the results being expressed as means and standard errors.

RESULTS AND DISCUSSIONS

The research made by us on the goji in vitro plants, cultivated on Murashige-Skoog medium culture exposed 60 days in light colored fluorescent tubes have allowed revealing that the lighting regime favored synthesizing antioxidants. As can be seen in figure 3 and 4 yellow light treatment determines the highest amount of polyphenols, followed by the quantity produced in vitro plants grown under white light where flavonoid synthesis is the biggest. Comparing the results of the extract obtained in the control variant of vitroseedlings illuminated with fluorescent white tubes with those obtained from the vitroseedlings illuminated with fluorecent colored tubes we found that in terms of content of assimilating pigments, vitroseedlings grew in blue light

contain the highest amount of assimilating pigments and remains the same in red and yellow light and decreases in green light (Figure 1).

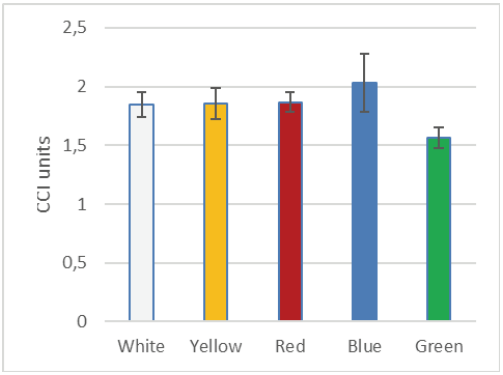

Figure 1. Assimilatory pigments in goji plants (*-significant differences from control plants at $p < 0.05$).

A high productivity with red light treatment is known (Darko et al., 2014), and is described in other species such as *Lycopersicum esculentum* (Suyanto et al., 2012). The amount of carotenoids is related to green and blue light treatments (0.4 to 0.48 mg / g). These pigments absorbing especially at these wavelengths (Govindjee et al., 1960).

From a physiological standpoint, the values of the chlorophyll fluorescence PSII and Fv/Fmϕ were not significantly altered suggesting a minimal impact on plant physiological condition as a result of the use of colored light sources. The difference in content of chlorophyll expressed through light absorption by leaves wasn't statistically significant in vitroseedlings illuminated to all variants of light (Figure 5).

In this study, the antioxidative proprieties of *L.barbarum* were measured spectrophotometrically by DPPH assay. DPPH (diphenyl picrylhydrazyl) assay is the most widlely reported method for screening of antioxidant activity of many plant drugs. The basis of DPPH assay activity relies on the concept of delocalisation of spare electron over the molecule of DPPH. Upon addition of substance with hydrogen donor property, DPPH will undergo reduction process and the colour of solution changes from deep violet to yellow. These disappearance of DPPH radical chromogens reflects the presence of antioxidant in the tested extracts (Molyneux, 2004). The colour changes after reduction can be quantified by its decrease of absorbance at wave length 517 nm. The quantified reduction of absorbance reflected the reduction capability of DPPH radical by antioxidative agents, namely the tested extracts.

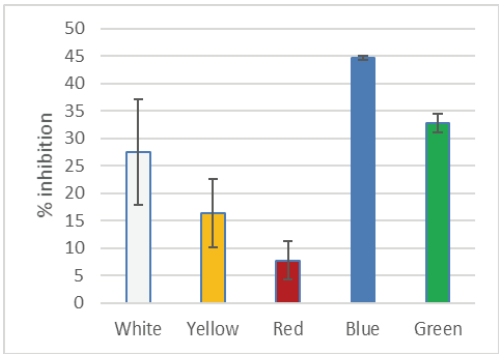

Figure 2. Free radical scavenging activity in goji plants (*significant differences from control plants at $p < 0.05$).

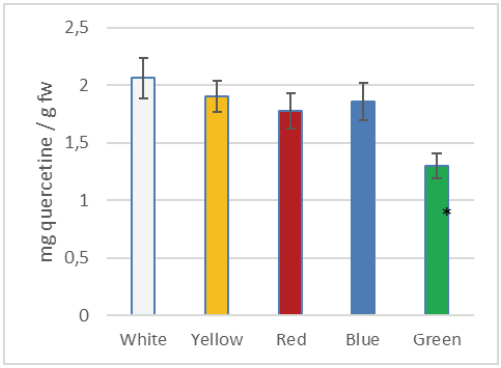

Figure 3. Total flavonoid content in goji plants (*significant differences from control plants at $p < 0.05$).

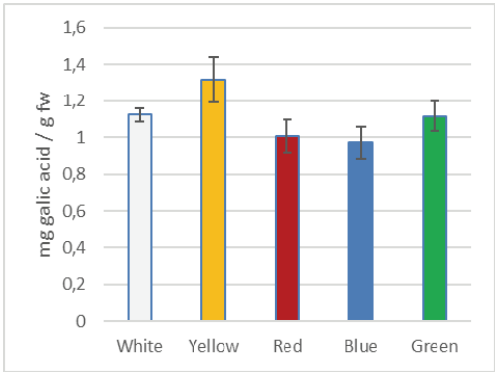

Figure 4. Total phenolic content in goji plants (*significant differences from control plants at $p < 0.05$).

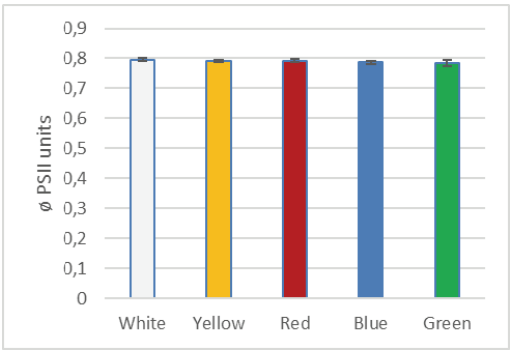

Figure 5. Chlorophyll fluorescence in goji plants (*significant differences from control plants at $p < 0.05$)

DPPH assay method is preferred due to its simplicity, convenience and time-saving properties. The concept involved in this method is particularly focusing on the ability of the tested extract to scavenge a stable DPPH free radical. The DPPH assay method is preferred due to its convenience which can evaluate the activities in a relatively short duration of time (Molyneux, 2004).

The extract which functions as antioxidant reacted with DPPH, chemically named after 1-diphenyl-2-picrylhydrazyl thus resulted in the formation of 1-diphenyl-2-picrylhydrazine. This conversion is visible by the change of colour from deep violet to pale yellow or almost colourless.

Osman and his collaborators have shown in a study done in 2012 that the leaves and stems of the two months old *L. barbarum* were the most optimum extract with the greatest antioxidant activities. After assessing the antioxidant properties of *L. barbarum* by DPPH assay, we have found that almost all variants of vitroseedlings possessing considerable activity of antioxidant with the two months old leaf and stem. Nevertheless, among all the extracts assessed, the most optimum extract which possesses optimum antioxidant activity was seen to be in the leaf and stems *L. barbarum* illuminated with blue light which was found to be higher (44.6%) than that of vitroplants illuminated with others colours of light (Figure 2), especialy with red light (7.73%).

After 60 days of vitro culture, if comparing vitro seedlings in terms of growth, we can see that the red light, green and yellow stimulates increase in length of vitro seedlings and blue light produce small and stocky and even slowing growth of plants (Figure 6).

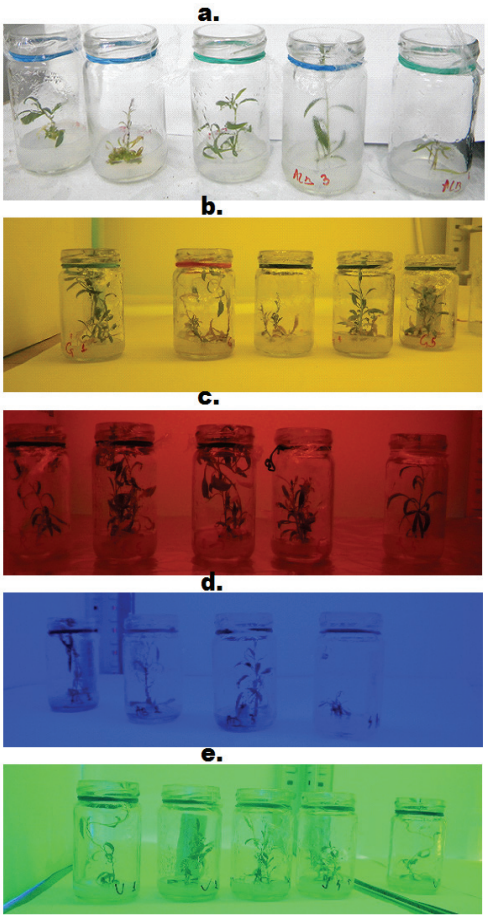

Figure 6. Aspects of *Lycium barbarum* vitro seedlings in the 60th day after initiation of vitro cultures exposed to fluorescent tubes of different colors: a- white; b-yellow; c-red; d- blue; e- green.

CONCLUSIONS

The results from the present study clearly suggested that *Lycium barbarum* plants may serve as an excellent source of antioxidants and bearing health promoting factors not only by the consumption of the berries, but also by optimally consuming leafs and stems of the plants.

These findings are also beneficial in providing a useful benchmark in determining the optimum colour of light for this species at its best, most promising antioxidative effects.

The results of this study would be used to give guidance on light colour sources design for goji cultivation in a controlled environment.
In addition, it may serve as a basis for even more extensive researches to be done on this species with the focus of interest directed towards its phytomedicinal values, hence would be incorporated into health-promoting supplementary foods and pharmaceutical preparations.

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