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In Vitro Regeneration and Antioxidant Propertiesof *Lycium Barbarum L.* (Goji)

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Graphical abstract

Development al ages (months old)	EC_{50} values (mg/ml) according to plant organs		
	Leaf	Stem	Root
2	0.08	0.08	0.22
3	0.10	0.12	0.10
4	0.11	0.11	0.10
5	0.42	0.12	0.11

Abstract

Ethnobotanical studies has documented the consumption of goji by Chinese tribes since ancient times. Goji or scientifically known as Lycium barbarum L. belongs to Solanaceae family and native to some areas of China. The present study was conducted to determine the best explant and corresponding hormonal compositions for Goji in vitro regeneration. In addition, the age and organ of in vitro seedlings with the optimum level of antioxidant activity were also identified. For in vitro regeneration, leaves and nodes were used as explants and cultured on in vitro regeneration media with varying hormonal concentration, combinations of α-napthaleneacetic acid (NAA) and 6-benzyl amino purine (BAP). The optimum combination for in vitro regeneration was found in leaf explants treated with equal concentration of 0.5 mg/L NAA and BAP each. DPPH assay for antioxidant activity screening done on methanolic extracts of samples from two through five month-old in vitro seedlings.shows the highest antioxidant activity in two-month old leaf and stem samples with the EC50 value of 0.08 mg/ml. Indeed, the results revealed that L. barbarum has the potency to be excellently micropropagated that possesses an outstanding antioxidant activity that is essential in phytomedicines.

Keywords: In vitro regeneration; antioxidant; DPPH assay; Lycium barbarum L.

Abstrak

Kajian etnobotani telah mendokumentasikan pengambilan goji oleh masyarakat Cina sejak zaman dahulu lagi. Goji atau nama saintifiknya Lycium barbarum L. diklasifikasikan dalam famili Solanaceae dan berasal dari sesetengah kawasan di China. Kajian ini dijalankan untuk mengenalpasti eksplan serta kombinasi hormon terbaik untuk regenerasi Goji secara in vitro. Selain itu, aktiviti antioksidan yang paling optimum daripada pelbagai peringkat umur dan bahagian anak-anak pokok turut dapat ditentukan. Bagi regenerasi Goji in vitro, daun dan bahagian nodal digunakan sebagai eksplan dan dikulturkan di atas media dengan gabungan hormon α-napthaleneacetic acid (NAA) dan 6-benzyl amino purine (BAP) yang berbeza.kepekatan Eksplan daun dengan penggunaan 0.5 mg/L NAA dan 0.5 mg/L BAP telah dikenalpasti merekodkan keputusan regenerasi yang optimum. Sementara itu, berdasarkan ujian DPPH yang dijalankan, ekstrak daun dan batang pada umur dua bulan telah menunjukkan aktiviti antioksidan yang paling tinggi dengan rekod nilai EC50 0.08 mg/ml. Keputusan kajian ini telah membuktikan bahawa L. barbarum berpotensi untuk dimikropropagasikan dengan baik di mana ia juga mempunyai aktiviti antioksidan yang tinggi.

Kata kunci: Regenerasi in vitro; antioksidan; ujian DPPH; Lycium barbarum L.

1.0 INTRODUCTION

Lycium barbarum L. or commonly known as goji has been recognized as one of a valuable medicinal. Goji berry has a long history of medicinal use especially among Chinese tribes. Sorted out from various literatures as well as from traditional beliefs, goji is believed to have prominent antioxidant, antidiabetic, as well as providing excellent effects on cardiovascular system and cholesterol level.[1-6] Realizing the properties of this foreign superfruit, the initiative of developing protocol to be used for large-scale plant propagation of this species in Malaysia is therefore found to be relevant.

Micropropagation functions as a tool of biotechnology which allows the production of large numbers of plant from small pieces of a mother plant in a relatively shorter time period. In less than one year, a single explant can be multiplied into thousands plantlets by adopting the micropropagation technique.[7] Whole plant can be regenerated through the application of *in vitro* tissue culture.

Through the *in vitro* regeneration process, the totipotency property of cultured plant with excellent genetic potential can be produce. In this study, the micropropagation process through *in vitro* regeneration of *L. barbarum* is described and the organogenesis potency was determined.

Apart from that, since this species is well recognized as an important source of phytomedicine, therefore determining the optimum antioxidant activity by assessing the free radical scavenging activity of the species was carried out. Antioxidant is one of the most desirable properties in phytomedicines. Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community. This is because; epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer.[8]

In this study, the optimum developmental age and organ of seedlings with the greatest free radical scavenging activity were identified by assessing *L. barbarum* methanolic extracts. The dihenylpicrylhydrazyl (DPPH) assay was carried out through which the EC_{50} value for each sample was determined to serve an indicator of antioxidant activity.

Table 1 *In vitro* regeneration of *L. barbarum* from leaf and nodal explants on different combination of NAA and BAP after 14 weeks[a]

Explant	Concentration of NAA and BAP (mg/L)		No. of leaves per plantlet	Height of plantlets (cm)	Degree of rooting
	NAA	BAP			
Leaf	0.0	0.0	0	0	NA
	0.1	0.1	0	0	NA
	0.1	0.5	0	0	NA
	0.1	1.0	0	0	NA
	0.5	0.1	3.80±1.33	0.90±0.36	Profuse
	0.5	0.5	7.60±2.80	1.69±0.75	Profuse
	0.5	1.0	0	0	NA
	1.0	0.1	0	0	NA
	1.0	0.5	0	0	NA
	1.0	1.0	0	0	NA
Node	0.0	0.0	0	0	NA
	0.1	0.1	0	0	NA
	0.1	0.5	0	0	NA
	0.1	1.0	32.90±7.27	2.60±0.99	Very few and almost absent
	0.5	0.1	0	0	NA
	0.5	0.5	0	0	NA
	0.5	1.0	0	0	NA
	1.0	0.1	0	0	NA
	1.0	0.5	0	0	NA
	1.0	1.0	0	0	NA

[a]Data are expressed as Mean ± Standard Error

2.0 EXPERIMENTAL

2.1 In Vitro Regeneration of *Lycium Barbarum* (Goji)

The explants were obtained from the seedlings grown by aseptic *in vitro* seed germination process beforehand. The goji seeds were surface-sterilized and cultured on sterilized germination media consisted of 4.4 g/L MS (Murashige and Skoog's, 1962) salts with vitamins, 3% sucrose and 0.8% agar with the pH of 5.8. The cultures were incubated at 25±2ºC under continuous daylight fluorescent illumination with 16-hour light photoperiod. After eight weeks in culture, the leaf and nodal parts of the *in vitro* seedlings were used as explants. The explants of *L. barbarum* were excised to approximately 0.5–1.0 cm and cultured on MS media with varying hormonal combinations (0.1, 0.5 and 1.0 mg/L) of NAA and BAP and they were combined in every possible combination to produce 9 different treatments. The cultures were incubated at 25±2ºC under continuous daylight fluorescent illumination with 16-hour light photoperiod and monitored on weekly basis. After 8 weeks in culture, the *in vitro* regenerated plantlets were subcultured onto fresh MS basal media and the subculturing procedures were carried out every two-week intervals. *In vitro* regeneration of shoots and/or roots were recorded. The height, number of leaves and degree of rooting of the plantlets were all observed and recorded.

2.2 Antioxidant Properties of *Lycium Barbarum* (Goji)

Samples of *L. barbarum* were taken and divided into different organs namely leaf, stem and root for each developmental age of two, three, four and five months old *in vitro* seedlings. The samples were dried at 50°C for 48 hours in the oven before being weighed and crushed into coarse powder. Extraction procedure was carried out by referring to Azizah *et al.* [9]with some modifications. An amount of 100 mg of sample was extracted with 50 ml of 70% aqueous methanol for 120 minutes in orbital shaker and filtered using Whatman filter paper. The filtrates were used for the assay. The antioxidant assay was conducted according to Nurliyana *et al*. [10] with some modifications. Sample amounting to 1.0 ml was added to 2.0 ml of 0.15 mM DPPH. They were allowed to stand for 20 minutes before the absorbance readings were taken at 517 nm by using UV/Vis spectrophotometer. Ascorbic acid was used as positive standard. The tests were run in duplicate and the readings were averaged. Percentage of Inhibitions (% In) of DPPH radical by test compounds were determined by the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance}_{(control)} - \text{Absorbance}_{(sample)}}{\text{Absorbance}_{(control)}} \times 100\%$$

The Percentage of Inhibitions (%In) values were used to produce graphical plots of dose-response curve. The graphs plotted were constructed between the scavenging activities (%In) versus the samples' concentrations. The EC_{50} values were determined from the curves constructed for each month by graphical interpolation of the concentration of sample at which the %In is 50%. Theoretically, the activity is expressed as effective concentration, EC_{50} whereby it signifies the effective concentration of sample required to scavenge the DPPH radical by 50%. [11-12] The lower the EC_{50} value, the greater the free radical scavenging activity that the sample possesses.

3.0 RESULTS AND DISCUSSION

3.1 *In Vitro* Regeneration of *Lycium Barbarum* (Goji)

Indirect organogenesis pathway had been observed in the study whereby the *in vitro* regeneration process was preceded by the formation of callus in all MS treatments. Nodal explants were identified to be more responsive than the leaf explants since the latter took two weeks to produce callus while the former took only a week for callus formation to be initiated. It had been observed that during initial phase of up to 6 weeks in culture, the leaf explants regenerated roots while the nodal explants regenerated shoots and leaves. After 14 weeks in culture, a thorough observation on the *in vitro* regeneration was performed and recorded (Table 1). Even though the treatment with 0.1 mg/L NAA and 1.0 mg/L BAP in nodal explant was seen as the best treatment with respect to the shooting response and formation of leaves, but until week 14, the plantlets formed were still regenerated with very few numbers of roots and some were almost absent. By referring to the final observation, it is conclusive that the treatment with 0.5 mg/L NAA and 0.5 mg/L BAP in leaf explant is the best treatment for *in vitro* regeneration of *L. barbarum.* It was verified as such by having 7.60±2.80 as the average number of leaves per plantlet and at the same time they were rooted very well (Figure 1a). It had been noted that the treatment of 0.5 mg/L NAA and BAP each in leaf explants produced a balance of both *in vitro* roots and leaves regeneration.

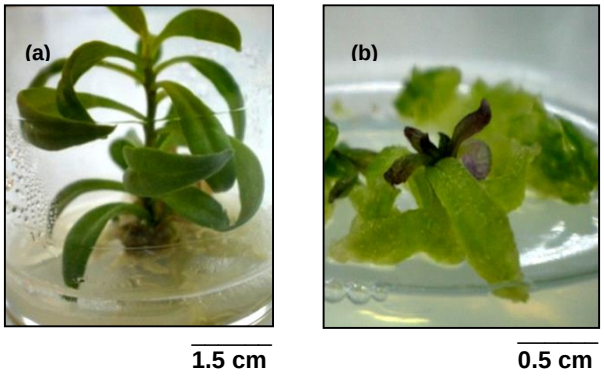

Figure 1 Plantlets regenerated *in vitro* from (a) the most optimum treatment consisted of 0.5 mg/L NAA and BAP each in leaf explants and (b) the 0.1 mg/L NAA and 1.0 mg/L BAP in nodal explants (less efficient in regenerating roots)

3.2 Antioxidant Properties of *Lycium Barbarum* (Goji)

Generally, all samples were seen to exert a considerable antioxidant activity. A graph was constructed for each developmental age consisted of all organs involved in the study to figure out the EC_{50} values from graphical interpolation. It was found that the free radical scavenging activities of all samples were directly related to the concentration of extracts. From the analysis done, the EC_{50} value of the positive standard (ascorbic acid) was found to be 0.08 mg/ml. Among all the samples assessed, the most optimum sample with optimum antioxidant activity was seen to be in the methanolic extract of the two-month old leaves and stems of *L. barbarum* manifested by the lowest EC_{50} values comparable to that of ascorbic acid (0.08 mg/ml).

Table 2 The EC_{50} values (mg/ml) of each plant organ in each developmental age

Developmental ages (months old)	EC_{50} values (mg/ml) according to plant organs		
	Leaf	Stem	Root
2	0.08	0.08	0.22
3	0.10	0.12	0.10
4	0.11	0.11	0.10
5	0.42	0.12	0.11

From the results obtained in leaf extracts, the highest EC_{50} value was found in the five month old leaf extract (0.42 mg/ml). Therefore, this suggested that the lowest ability of free radical scavenging activity was found in leaves at that particular age as compared to its younger counterparts. In contrast to the leaf and stem extracts, a different finding was found in which the two month old root extract exhibited the lowest activity as free radical scavenger with the EC_{50} value of 0.22 mg/ml as compared to its older counterparts. Thus, the findings verified that the age from which the extracts obtained is significant in determining the capacity of antioxidant activity.

4.0 CONCLUSION

The results from present research revealed that *L. barbarum* could potentially be micropropagated with the use of appropriate explant and its corresponding treatment. In addition, *L. barbarum* also has a considerable antioxidant activities with the leaf and stem extracts of two month old seedlings that exerted the greatest radical scavenging activity comparable to that of ascorbic acid as positive standard.

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