ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 12 (1): 36-45, 2013
ISSN 1682-296X / DOI: 10.3923/biotech.2013.36.45

Callus Induction and Somatic Embryogenesis from Leaf and Nodal Explants of *Lycium barbarum* L. (Goji)

[1]Nurul Izzati Osman, [1]Asmah Awal, [2]Norrizah Jaafar Sidik and [1]Shamsiah Abdullah
[1]Faculty of Plantation and Agrotechnology,
[2]Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam,
Selangor, Malaysia

Abstract: *Lycium barbarum* or commonly known as goji or wolfberry is a type of Chinese medicinal herbs which has been consumed since ancient times. In the present study, the best explant and corresponding treatment for callus induction and somatic embryogenesis in this species were determined. Leaves and nodes were used as explants and cultured on different combinations and concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) plant growth regulators. For callus induction, the optimal callus formation effects were found in the treatment of 0.3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} or 0.3 mg L^{-1} BAP in Murashige and Skoog's (MS) media, while 0.1 mg L^{-1} BAP with either 0.3 mg L^{-1} or 0.5 mg L^{-1} 2,4-D in MS media were the optimal treatments in nodal explant. As for somatic embryogenesis, after a series of sequential subculture on MS basal media, the treatment of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media during callus induction phase in leaf explant were identified to be the optimum treatment to produce somatic embryos. Through this study, reliable protocols useful for *L. barbarum* mass propagation through callus culture and somatic embryos formation had been established and verified.

Key words: *Lycium barbarum*, callus induction, somatic embryogenesis, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP)

INTRODUCTION

Since ancient times, plants have been used and associated very well in human life. The incorporation of plants in medicinal approaches has been witnessed to be practically used in various tribes around the world. Chinese tribes are well recognized for their resourcefully use of plant-based medicines as effective treatments for various ailments and supplementation purposes. Traditional Chinese Medicine (TCM) which has evolved for thousands of years has documented a number of plant-based approaches and their corresponding preparations for the maintenance of human health. *Lycium barbarum* L. has been regarded as one of the valuable herbs used in TCM. The species belongs to the family of Solanaceae and commonly known as wolfberry in western countries. It has been acknowledged as an upper class Chinese medicine in Chinese pharmacopoeia (Chang and So, 2008).

Besides gaining popularity in folklore medicines, the uses of *L. barbarum* are undoubtedly proven to be scientifically effective. This is manifested by various studies done in assessing the effectiveness of this species towards certain disorders in various mechanisms of actions. Sorted out from various literatures as well as from traditional beliefs, goji is believed to have prominent antioxidant, antidiabetic (Luo *et al*., 2004; Jing and Yin, 2010; Thomson, 2010) as well as providing excellent effects on cardiovascular system and cholesterol level (Luo *et al*., 2004; Thomson, 2010). Furthermore, the properties of *L. barbarum* as potent antioxidant had been proven in various scientific studies (Wu *et al*., 2004; Li *et al*., 2007; Bucheli *et al*., 2011; Osman *et al*., 2012). In addition, it has been reported by Luo *et al*. (2004) that crude extracts of *L. barbarum* were identified to possess a great capacity of antioxidants by having carotenoids, riboflavin, ascorbic acid, thiamine and nicotinic acid.

Realizing the properties of this medicinal plant species, the initiative of developing protocol to be used for large-scale plant propagation of this species through micropropagation is therefore found to be relevant (Sidhu, 2010). Formation of callus is one of the important aspects in plant biotechnology through tissue culture procedures. Callus is considered to be among the most

Corresponding Author: Nurul Izzati Osman, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, (UiTM), 40450 Shah Alam, Selangor, Malaysia

important subjects in micropropagation technologies. Callus which is formed from the inoculation of explant on callus induction media will further resulted in regeneration of plant through the process of organogenesis or somatic embryogenesis (Yadav and Tyagi, 2006; Sarkar, 2009; Verma *et al*., 2011).

Somatic embryogenesis depicts a sequence of process which is resulting in the formation of somatic embryos. As one of biotechnological approaches in agricultural field, somatic embryogenesis has been acknowledged to possess many advantages for mass propagation. Through somatic embryogenesis, the multiplication rates can be significantly increased and would be able to be potentially scaled-up through liquid suspension culture hence enabling the handling of many embryos at one time (Vannini *et al*., 2012).

Previous findings had documented a number of *L. barbarum* regenerations through callus-mediated pathway. For instance, Tian *et al*. (1993) and Cao *et al*. (1999) were using hypocotyl protoplast and pith tissue, respectively as the explants for callus production in *L. barbarum*. From the research done by Hu *et al*. (2008), callus of *L. barbarum* was successfully produced by using root explants treated in MS media with 0.2 mg dm^{-3} 2,4-D. Through the present study, another reliable protocol for callus induction and somatic embryogenesis in *L. barbarum* is described.

MATERIALS AND METHODS

Sterile *in vitro* seed germination and explant preparation: The study took one year to be accomplished which had started in January 2011. The study was commenced with the germination of goji seeds. Since, goji is not native to Malaysia, therefore the imported dried berries were used as the source of goji seeds. The seeds from dried goji berries were germinated to produce *in vitro* seedlings as the source of explants. The dried berries were sterilized in sodium hypochlorite solution with two drops of Tween 20. This was followed by subsequent three time rinsing procedures with sterile distilled water. The sterilized berries were then excised.

Murashige and Skoog's (MS) salt (Duchefa Biochemie, Netherland) was used as basal media and supplemented with 3.0% sucrose (Duchefa Biochemie, Netherland). An amount of 0.8% technical agar (OXOID Ltd., England) was used as the solidifying agent. The pH of the mixed solutions was adjusted to be within 5.6-5.8 and they were sterilized by autoclaving at 121°C (at 1 kg cm^{-2}) for 20 min. The seeds of *L. barbarum* were further cultured on agar-solidified MS media. They were incubated at 25±2°C under continuous daylight fluorescent illumination with 16 h photoperiod. The conditions were maintained for eight weeks and the *in vitro* seedlings obtained from the *in vitro* seed germination steps were used as the source of explants for callus induction.

Media preparation and callus induction: Murashige and Skoog's (MS) salt (Duchefa Biochemie, Netherland) media supplemented with 3.0% sucrose (Duchefa Biochemie, Netherland) and 0.8% technical agar (OXOID Ltd., England) were prepared. Different combinations and concentrations of 2,4-D (0.3, 0.5, 1.0 mg L^{-1}) and BAP (0.1, 0.3, 0.5 mg L^{-1}) were incorporated into the media. A medium devoid of any plant growth regulators was used as control. The explants of *L. barbarum* of about 0.5-1.0 cm were cultured on MS media supplemented with different combinations and concentrations of 2,4-D and BAP. Three explants were cultured per Petri dish and these were replicated by five thus resulted in the total number of 15 inoculated explants. The cultures were then incubated at 25±2°C under continuous daylight fluorescent illumination with a 16 h photoperiod.

Induction of somatic embryogenesis: The calli obtained from callus induction phase were subcultured on MS media (Duchefa Biochemie, Netherland) supplemented with reduced sucrose concentration of 20 g L^{-1} (Duchefa Biochemie, Netherland) for somatic embryogenesis induction. The media devoid of any plant growth regulator were solidified with 8.0 g L^{-1} technical agar (OXOID Ltd., England) and the pH was adjusted to be within 5.6-5.8. They were sterilized by autoclaving at 121°C (at 1 kg cm^{-2}) for 20 min. The calli were regularly subcultured for every three weeks onto fresh MS basal media. The cultures were then incubated at 25±2°C under continuous daylight fluorescent illumination with a 16 h photoperiod.

Data collection and statistical analysis: The assessment on callus induction and formation were done qualitatively by taking into account the degree of callus formation, callus colour, friability and morphology. Quantitative assessment on callus induction was on the other hand done by calculating the percentage of explant successfully initiated callus over the total number of explants cultured for each treatment. The percentage after four weeks of growth was calculated by taking into account the number of explant successfully formed callus per total number of explants inoculated and this was applied for each treatment. On the other hand, for indirect somatic embryogenesis, the intensity of somatic embryos formation were recorded. The percentage of embryogenic

callus in a treatment was also calculated. The SPSS 17.0 program was used for descriptive statistic analysis and the data gathered were analysed according to t-test and analysis of variance, ANOVA methods at 5% level of significance. The value of $p<0.05$ was considered as significant.

RESULTS

Callus induction from leaf and nodal explants of *L. barbarum*: After four weeks on callus induction media, a thorough observation was done. Callus colour and morphology, degree of callus formation and induction percentage were all recorded and calculated accordingly (Table 1). The morphologies and physical characteristics of calli derived from those two explants were found to be different particularly in terms of compactness. From the total of nine treatments used for leaf explant (excluding the control treatment), six out of nine treatments were friable while the rest three were compact. On the other hand, the findings in nodal explants were completely vice-versa in which only three treatments produced friable calli while the rest six were compact.

In the present study, there was a marked difference in the duration for the callus induction to be first initiated in those two different explants. With regard to explant responsiveness, nodal explant was seen to be more responsive in terms of time taken to induce callogenesis in which the calli were started to be induced after 6-7 days of first culturing procedure. On the other hand, the calli were only seen after 10-14 days in leaf explant.

The MS media added with 0.3 mg L^{-1} 2,4-D was found to produce the best callus induction in leaf explant when combined with either 0.1 mg L^{-1} BAP or 0.3 mg L^{-1} BAP. After four weeks in culture, both treatments gave 100% callus induction with profuse callus formation. The calli produced from those treatments were greenish and friable likewise shown in Fig. 1a. It was clearly seen in the study that the incorporation of 0.3 mg L^{-1} 2,4-D in the MS media regardless of BAP concentration produced greenish calli while the rest of the treatments yielded yellowish calli. It was also noted that the incorporation of 0.5 mg L^{-1} BAP in the MS media initiated the formation of compact calli regardless of 2,4-D concentration.

The callus induction potency in leaf explant was ranging from 83.3-100.0%. Only two treatments out of nine were successfully generated 100.0% callus formation. Nevertheless, only two treatments gave profuse callus formation while the rest produced moderate calli and only one treatment generated minor calli.

Meanwhile, in the nodal explant, five treatments were successfully generated 100.0% callus formation. The range of percentage for callus formation was ranging from 60.0-100.0%. A distinctive trend of callus morphology was seen in nodal explant whereby all treatments consisted of 0.3 mg L^{-1} 2,4-D in MS media yielded yellowish green and compact calli with considerable degree of callus formation from moderate to profuse clumps likewise in Fig. 1b. It was also noted that all the treatments consisted of 0.1 mg L^{-1} BAP in MS media were found to produce profuse callus formation in nodal explant. Nonetheless, combination of 0.1 mg L^{-1} BAP with either 0.3 mg L^{-1} 2,4-D or 0.5 mg L^{-1}

Table 1: Callus induction from leaf and nodal explants of *L. barbarum* after 4 weeks of culture in MS medium supplemented with different combinations of BAP and 2,4-D treatments

	Combination of 2,4-D and BAP (mg L^{-1})				
Explant	2,4-D	BAP	Percentage of callus formation (%)	Colour and morphology of callus	Degree of callus formation
Leaf	0.0	0.0	0.0	No callus formed	-
	0.3	0.1	100.0	Greenish, friable	++++
	0.3	0.3	100.0	Greenish, friable	++++
	0.3	0.5	83.3	Greenish, compact	+++
	0.5	0.1	86.7	Yellowish green, friable	+++
	0.5	0.3	80.9	Yellowish, friable	+++
	0.5	0.5	94.4	Yellowish, compact	+++
	1.0	0.1	85.7	Yellowish, friable and watery	+++
	1.0	0.3	94.4	Yellowish green, friable	+++
	1.0	0.5	100.0	Yellowish, compact	++
Node	0.0	0.0	0.0	No callus formed	-
	0.3	0.1	100.0	Yellowish green, compact	++++
	0.3	0.3	100.0	Yellowish green, compact	+++
	0.3	0.5	100.0	Yellowish green, compact	+++
	0.5	0.1	100.0	Yellowish, greenish, friable and watery	++++
	0.5	0.3	60.0	Yellowish, friable	+++
	0.5	0.5	100.0	Greenish, compact	+++
	1.0	0.1	83.3	Yellowish, friable	++++
	1.0	0.3	83.3	Yellowish brown, compact	+++
	1.0	0.5	90.5	Brownish, compact	+

-: No calli, +: Very few calli, ++: Minor calli, +++: Moderate calli, ++++: Profuse calli

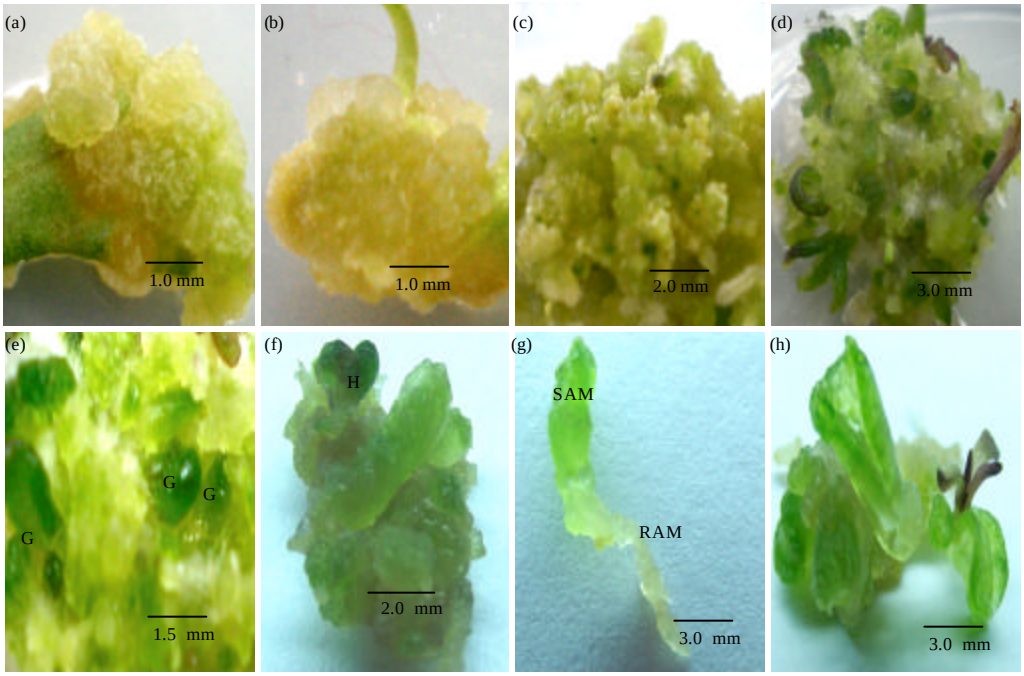

Fig. 1(a-h): (a) Profuse, greenish and friable callus formed from leaf explant, (b) Profuse, yellowish green and compact calli formed from nodal explant, (c) The presence of green and white spots on the surface of callus and some of the white spots had turned into roots at the base of the callus clump, (d) A type of embryogenic callus after 15 weeks in culture, The callus was green and nodular with the presence of abundant somatic embryos, Adventitious shoots and roots were also noted, (e) Globular-stage embryo (G) derived from the initial treatment of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media, (f) Heart-shaped embryo (H) isolated from a callus clump derived from the initial treatment of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media, (g) Torpedo-stage embryo with Shoot Apical Meristems (SAM) and Root Apical Meristems (RAM) clearly visible from the initial treatment of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media and (h) A developed progress of somatic embryogenesis isolated from a clump of callus after the torpedo-stage embryo during the cotyledonary phase

2,4-D in MS media were evaluated to be the optimal combinations for callus induction in nodal explant as they were profusely produced calli together with 100.0% callus induction percentage.

Somatic embryogenesis from leaf and nodal explants of *L. barbarum*: After identification of optimum treatment in callus induction potency in both explants had been performed, the subculturing procedures were then carried out after four weeks. Before being subcultured, the weights of the calli formed were weighed accordingly by considering the fresh weight of the calli per explant in each treatment (Table 2). The treatment of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media were found to produce the greatest amount of mean callus weights in both of the explants with 1.123±0.158 and 0.760±0.032 g in leaf and nodal explants, respectively. The isolated calli gathered from each treatment were then subcultured onto fresh MS basal media. The frequency of subculturing procedures was performed in a predetermined regularity of three weeks interval. During the first subculturing cycle, the calli were inoculated on the fresh MS basal media and the diameter measurement of callus clump was recorded for each treatment. The measurements of clump's diameters were made in order to note and compare any progress in callus development (Table 2).

After a total of 12 weeks in culture, the growth progress of the calli were further observed and recorded. Upon observation, most of the calli were friable and the formation of green and white spots were abundant and common (Table 3). The formations of white spots which were noted to be commonly found on the surface of calli were observed to be subsequently developed into roots Fig. 1c. On the other hand, it has been observed that the

Table 2: Callus weight and size of *L. barbarum* after 5 weeks in culture at 25±2°C under 16 h photoperiod

Leaf explant				Nodal explant			
Treatment of 2,4-D+BAP (mg L^{-1})	Callus weight per explant (g)	Callus diameter during first subculture (cm)	Callus diameter after three weeks of subculture (cm)	Treatment of 2,4-D+BAP (mg L^{-1})	Callus weight per explant (g)	Callus diameter during first subculture (cm)	Callus diameter after three weeks of subculture (cm)
0.0+0.0	N.A	N.A	N.A	0.0+0.0	N.A	N.A	N.A
0.3+0.1	0.673±0.669	2.00	2.50	0.3+0.1	0.557±0.333	2.00	2.80
0.3+0.3	0.763±0.134	2.00	3.00	0.3+0.3	0.303±0.038	1.50	1.50
0.3+0.5	0.477±0.062	1.00	1.00	0.3+0.5	0.363±0.064	2.00	2.30
0.5+0.1	0.870±0.149	2.00	2.50	0.5+0.1	0.387±0.028	2.00	2.50
0.5+0.3	0.683±0.023	2.00	2.50	0.5+0.3	0.467±0.186	1.50	2.00
0.5+0.5	0.347±0.096	2.00	2.70	0.5+0.5	0.377±0.019	2.00	2.50
1.0+0.1	1.123±0.158	1.00	1.20	1.0+0.1	0.760±0.032	2.00	2.50
1.0+0.3	0.603±0.087	2.00	2.40	1.0+0.3	0.100±0.023	1.50	1.80
1.0+0.5	0.593±0.059	2.00	2.30	1.0+0.5	0.057±0.007	N.A	N.A

Table 3: Callus induction progress of *L. barbarum* after 12 weeks in culture at 25±2°C under 16 h photoperiod, subcultured on MS basal media

Leaf explant		Nodal explant	
Treatment of 2,4-D+BAP (mg L^{-1})	Colour and morphology of callus	Treatment of 2,4-D+BAP (mg L^{-1})	Colour and morphology of callus
0.0+0.0	Nil	0.0+0.0	Nil
0.3+0.1	Green callus with white and green spots and hairy root structures	0.3+0.1	Green callus with white and green spots
0.3+0.3	Green callus with white and green spots	0.3+0.3	Yellow callus with white spots.
0.3+0.5	Nil	0.3+0.5	Green and brown, friable and watery callus
0.5+0.1	Green and nodular callus with very few green spots	0.5+0.1	Dark brown and compact callus. No progress
0.5+0.3	Brown callus with very few green spots	0.5+0.3	Yellowish green callus with white spots
0.5+0.5	Brown and nodular callus	0.5+0.5	Nil
1.0+0.1	Green callus with white and green spots and hairy root structures.	1.0+0.1	Green callus with white and green spots and hairy root structures
1.0+0.3	Brown and compact callus. No progress	1.0+0.3	Nil
1.0+0.5	Yellowish green callus with white spots	1.0+0.5	Nil

Table 4: Callus induction and somatic embryogenesis in *L. barbarum* from embryogenic calli after 16 weeks in culture at 25±2°C under 16 h photoperiod

Explant	Treatment of 2,4-D+BAP (mg L^{-1})	Percentage of callus induction (%)	Total No. of adventitious shoot per treatment	Percentage of embryogenesis (%)[a]	Degree of embryos formation per treatment
Leaf	0.3+0.1	100.0	10	70	+++
	0.3+0.3	100.0	44	40	+++
	1.0+0.1	85.7	140	90	++++

+++: Abundant, approximately 50-100, ++++: Profusely abundant, approximately 100-200, [a]:Number of callus clumps with somatic embryos over the total number of callus clumps cultured

presence of tiny projection of shoots was preceded by the formation of green spots on the calli clumps (Fig. 1c).

Upon the observation after 16 weeks in culture, leaf explants which were treated with 0.3 mg L^{-1} of each 2,4-D and BAP in MS media as well as those treated with 0.3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media during callus induction phase were producing a great amount of somatic embryos. However, the most optimum treatment identified to produce a significantly huge amount of somatic embryos were found from leaf explant treated with 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media (Table 4). This treatment also found to be the treatment which produced the greatest weight of callus (Table 2) before induction of somatic embryos formation. The percentage of embryogenesis in a treatment was obtained by considering the number of callus clumps with somatic embryos over the total number of callus clumps cultured. After 16 weeks of observation period, it had been clearly demonstrated that the leaf explants treated with 0.3 mg L^{-1} of each 2,4-D and BAP in MS media as well as the treatment with 0.3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media were producing a significantly profuse amount of calli and similarly, they produced a great amount of somatic embryos as well. The rest of the treatments which were found to be optimal during callus induction phase were seen to be only productively inducing non-embryogenic calli. In conclusion, they were seen to be ineffective in producing somatic embryos under the present research conditions.

Upon Scanning Electron Microscopy (SEM) view of the embryogenic callus, apparent heart-shaped (Fig. 2a) and torpedo-stage embryos (Fig. 2b) could be observed. The fact was clearly observed and agreed in the present research in which at certain particular time, there were

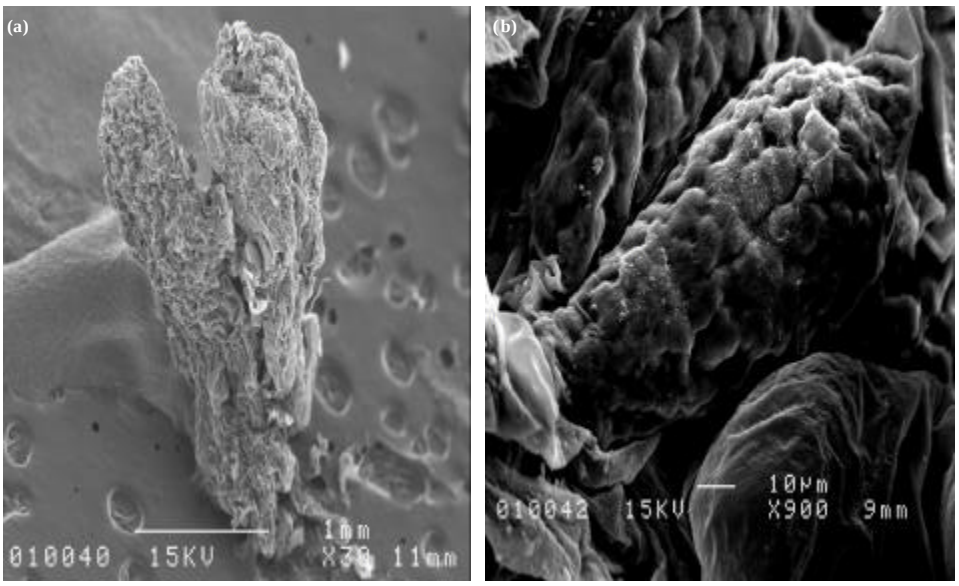

Fig. 2(a-b): (a) Image of heart-shaped embryo captured by Scanning Electron Microscope under 30× magnifications, (b) Image of torpedo-stage somatic embryos captured by Scanning Electron Microscope under 900× magnifications

various types of developmental stages in somatic embryos found in a callus clump (Fig. 1d). All stages and forms of somatic embryogenesis which are globular (Fig. 1e), heart-shaped (Fig. 1f), torpedo (Fig. 1g) as well as cotyledonary (Fig. 1h) could be observed in the present study.

DISCUSSION

Callus induction from leaf and nodal explants of *L. barbarum*: In the present study, there was a marked difference in the duration for the callus induction to be first initiated in those two different explants. Nodal explant was seen to be more responsive in terms of time taken to induce callogenesis whereby it took only 6-7 days whereas, leaf explants required 10-14 days to show initial response. Such variations in the responsiveness may be attributed to the properties of the corresponding explants. Sridhar and Naidu (2011) reported that the formation of callus was influenced by the factors of wound reactions or exogenous plant growth regulators. However, the present study found that the callus formation was influenced by both factors synergistically. This is proven by the findings that the callus formation was completely absent in the hormone-free media both in the leaf and nodal explants whereas the explant wounding was there.

In terms of different explants responsiveness, some factors which are possibly related to the callus induction properties in different explants include the content of endogenous phytohormones in the explants (Gupta *et al*., 2010; Tang *et al*., 2010), their hormonal uptakes as well as types of auxin and cytokinin hormones used and their different mode of actions (Gupta *et al*., 2010). The importance of explant source factor in callus induction was also agreed by Nikam and Shitole (1998) as well as by Pal *et al*. (2007). A finding by Osman *et al*. (2010) on another species from Solanaceae family which is *Lycopersicon esculentum* Mill. or tomato, also revealed that both factors which are type of explants and plant growth regulators impart essential effects on callus induction potency. Therefore, it was apparently verified that those factors exert synergistic effects on callus formation.

The portion of incorporation of PGR is a great deal which determines the texture, colour and morphology of callus induced in each treatment. PGR and their corresponding concentrations impart a great influence on the pattern of growth and physiological well-being of plant cultures particularly grown *in vitro*. According to Juan *et al*. (2010), callus growth *in vitro* is physiologically affected by plant hormones and they are being among the most important factors. In addition, Skoog and Miller (1957) stated that the major differences in the

response of different plants and different explants to tissue culture conditions depend on the ratio of auxins to cytokinins. Upon statistical analysis, there was a significant difference in the treatments of various media ($p<0.05$). However, different explants were seen to exert less significant effect on the percentage of callus induction ($p>0.05$).

Somatic embryogenesis from leaf and nodal explants of *L. barbarum*: According to Yadav and Tyagi (2006), somatic embryogenesis is one of the methods used in plant transformation studies. Somatic embryogenesis involves the usage of somatic cells to produce the embryo-like structure and depicts a sequence of process which is resulting in the formation of somatic embryos.

In the present study, after 3 weeks of first subculture, the calli were further subcultured on MS basal media without PGR with reduced sucrose concentration for the subsequent cycle. Those which were already turned blackish brown and showed no further progress were discarded. According to Kaparakis and Alderson (2008), the presence of auxin in the media for subsequent cycle in somatic embryogenesis process will cause the inhibition of somatic embryos formation. In addition, in their review as well, they revealed that the presence of BAP will reduce the embryogenic potential for somatic embryogenesis to take place.

The presence of white and green spots on the calli surface after a total of 12 weeks of culture had been previously observed and described to be found in the callus cultures of *Vigna unguiculata* L. by Soh *et al.* (1998). In addition, similarly they found that those spots were gradually transformed to white nodular structures which subsequently formed adventitious roots. Another finding by Thadavong *et al.* (2002) had also suggested the similar developing progress of green spots which were eventually developed into shoots and plantlets. The occurrence of such phenomena signified that the process of indirect organogenesis has taken place in the cultures. In order to regenerate a new whole plant through somatic embryogenesis, several stages are involved. After the formation of embryogenic callus, further development is the formation of globular somatic embryo stage, followed by heart then torpedo stage and eventually the cotyledonary stage before regenerating a new plantlet. Similarly in the present study, it had been observed that the somatic embryos formation which is derived from embryogenic callus were firstly formed globular embryos (Fig. 1e), then followed by heart-shaped embryos (Fig. 1f) and further transformed into torpedo-stage embryos with the presence of Root Apical Meristem (RAM) and Shoot Apical Meristem (SAM) (Fig. 1g) (Sarkar, 2009). The RAM and SAM were further developed into cotyledonary phase (Fig. 1h). A small population of cells from which the root system originated is the one which is termed as RAM. The RAM developed at the basal end of the plant embryo (Twyman, 2003). On the other hand, the SAM which is developed during somatic embryogenesis would further give rise to the above-ground portion of the plant from which the lateral and basal organs will be produced such as leaves and stems, respectively (Bowman and Eshed, 2000).

Another finding in the present research had discovered that the treatment in leaf explant which composed of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media gave less optimum callus induction effects with moderate formation of calli. Nevertheless, during somatic embryogenesis induction phase, the combination of such explant with corresponding treatment was found to give the most optimum result in producing somatic embryos in this species which was justified to provide the greatest percentage of somatic embryogenesis (Table 4). The effects of different explants were found to be significant in the induction of somatic embryogenesis process ($p<0.05$). Similarly, different treatments provided a significant difference ($p<0.05$) in the potential of somatic embryogenesis induction.

The removal of auxin and cytokinin for subsequent induction of somatic embryos were considered in the present research. A review by Zimmerman (1993) had highlighted a concern regarding the importance of auxin pulse for only a short while in embryogenesis. For instance, in alfalfa species, a very brief period for about a few minutes to a few hours of auxin supply is required before initiation of embryogenesis in hormone-free media. In addition, it has been verified that the embryogenesis program will proceed following auxin removal due to inactivation of a number of gene which favours embryogenesis program to develop. Halperin and Wetherell (1964) as well as Borkird *et al.* (1986) agreed that the transition phase to the heart stage embryo will only possible upon the presence of new gene products. The new gene products however could only be synthesized when exogenous auxin is removed. A loss of embryogenic capability had been reviewed by Sarkar (2009) upon maintenance of cultures on high auxin media which is also being the cause of embryo development to be arrested.

Apart from that, the levels of cytokinin in the media might also influence the possibility and responsiveness of somatic embryogenesis to occur. A study done by Wenck *et al.* (1998) on orchard grass had observed that a considerably higher levels of cytokinins was conversely related to the tendency of somatic embryogenesis to occur since it had been found that a greater level of

cytokinin was identified in those which were difficult to induce. Furthermore, a study carried out on *Capsicum annum* L. by Kaparakis and Alderson (2008) had revealed that the presence of cytokinins such as kinetin, BAP and zeatin had given either no significant effects on somatic embryogenesis or even reduced the embryogenic potential.

In the present research, the concentration of sucrose in the MS media was adjusted to be 2.0 or 20.0 g L^{-1} (w/v) for the induction of somatic embryogenesis procedure. The MS basal media supplemented with 2.0% sucrose were used to be the media onto which a series of subculturing procedures to be performed. The use of such media had successfully proven to be effective in inducing somatic embryogenesis. This is in accordance to Cunha and Fernandes-Ferreira (1999) in which they had revealed that lower concentration of disaccharides (1.0 and 2.0%) would be more effective in inducing somatic embryogenesis than monosaccharide.

It has been acknowledged that the process of somatic embryogenesis is closely related and very much attributed to a series of mechanisms which occurs as a result of stress-induced responses (Ikeda-Iwai *et al*., 2003; Zavattieri *et al*., 2010). However, apart from that, the effects of carbohydrate as a source of carbon for the media are also essential in somatic embryogenesis. Sugars especially glucose and sucrose are always supplied in cultural media to assist and encourage the growth of cells (Nambiar *et al*., 2012). Nonetheless, Salaj *et al*. (2004) stated that higher concentration of carbohydrates may impart negative effects on somatic embryos growth. This is due to degeneration and subsequent necrosis of pre-cotyledonary somatic embryos which may be resulted in following higher concentrations of carbohydrate sources. The incorporation of carbohydrates into the media may impair the maturation of somatic embryos hence will result in the degeneration of early stages of development (Salaj *et al*., 2004).

CONCLUSION

An efficient callus induction protocol for *L. barbarum* has been developed from these findings. The treatments of 0.3 mg L^{-1} 2,4-D and 0.1 mg L^{-1} or 0.3 mg L^{-1} BAP in MS media were found to be the optimal hormonal combinations for callus induction in leaf explant while 0.1 mg L^{-1} BAP with either 0.3 mg L^{-1} or 0.5 mg L^{-1} 2,4-D in MS media were found to be the optimal treatments in nodal explants. This protocol is essential to assist in identifying the best treatment for proliferation and the establishment of mass propagation of *L. barbarum* through callus culture. As for the induction of somatic embryogenesis, the leaf explants treated with 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP in MS media gave the most optimum somatic embryos formation. Indeed, the efficacy of MS basal media with reduced sucrose concentration (2.0%) to be solely used in the production of somatic embryos during the somatic embryogenesis induction procedures was verified.

ACKNOWLEDGMENT

The heartily thanks are expressed to the Research Management Institute (RMI), Universiti Teknologi MARA (UiTM) for the partial funding of this research through Excellence Fund allocation with grant number 600-RMI/ST/DANA/5/3/Dst (70/2011).

REFERENCES

Borkird, C., J. Choi and H. Sung, 1986. Effect of 2,4-dichlorophenoxyacetic acid on the expression of embryogenic programme in carrot. Plant Physiol., 81: 1143-1146.

Bowman, J.L. and Y. Eshed, 2000. Formation and maintenance of the shoot apical meristem. Trends Plant Sci., 5: 110-115.

Bucheli, P., K. Vidal, L. Shen, Z. Gu, C. Zhang, L.E. Miller and J. Wang, 2011. Goji berry effects on macular characteristics and plasma antioxidant levels. Optom. Vis. Sci., 88: 257-262.

Cao, Y.L., F. Chen, Q. Luo and L. Qu, 1999. *In vitro* culture of pith tissue and high-frequency plant regeneration of *Lycium barbarum*. Guangxi Zhiwu, 19: 239-242.

Chang, R.C.C. and K.F. So, 2008. Use of anti-aging herbal medicine, *Lycium barbarum*, against aging-associated diseases. What do we know so far? Cell Mol. Neurobiol., 28: 643-652.

Cunha, A. and M. Fernandes-Ferreira, 1999. Influence of medium parameters on somatic embryogenesis from hypocotyl explants of flax (*Linum usitatissimum* L.): Effect of carbon source, total inorganic nitrogen and balance between ionic forms and interaction between calcium and zeatin. J. Plant Physiol., 155: 591-597.

Gupta, P., S. Sharma and S. Saxena, 2010. Callusing in *Stevia rebaudiana* (Natural Sweetener) for steviol glycoside production. Int. J. Agric. Biol. Sci., 1: 30-34.

Halperin, W. and D.F. Wetherell, 1964. Adventive embryony in tissue cultures of the wild carrot, Daucus carota. Am. J. Bot., 51: 274-283.

Hu, Z., Y. Hu, H.H. Gao, X.Q. Guan and D.H. Zhuang, 2008. Callus production, somatic embryogenesis and plant regeneration of *Lycium barbarum* root explants. Biol. Plantarum, 52: 93-96.

Ikeda-Iwai, M., M. Umehara, S. Satoh and H. Kamada, 2003. Stress-induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. Plant J., 34: 107-114.

Jing, L. and L. Yin, 2010. Antihyperglycemic activity of polysaccharide from *Lycium barbarum*. J. Med. Plants Res., 4: 23-26.

Juan, L., W. Lihua, L. jing and W. Junhui, 2010. Effect of different plant growth regulators on callus induction in *Catalpa bungei*. Afr. J. Agric. Res., 5: 2699-2704.

Kaparakis, G. and P.G. Alderson, 2008. Role for cytokinins in somatic embryogenesis of Pepper (*Capsicum annuum* L.). J. Plant Growth Regul., 27: 110-114.

Li, X.M., Y.L. Ma and X.J. Liu, 2007. Effect of the Lycium barbarum polysaccharides on age-related oxidative stress in aged mice. J. Ethnopharmacol., 111: 504-511.

Luo, Q., Y.C.J. Yan, M. Sun and H. Corke, 2004. Hypoglycemic and hypolipidemic effects and antioxidant activity of fruit extracts from *Lycium barbarum*. Life Sci., 76: 137-149.

Nambiar, N., C.S. Tee and M. Maziah, 2012. Effects of organic additives and different carbohydrate sources on proliferation of protocormlike bodies in *Dendrobium* Alya Pink. Plant Omics J., 5: 10-18.

Nikam, T.D and M.G. Shitole, 1998. *In vitro* culture of *Safflower* L. cv. Bhima. Initiation, growth optimization and organogenesis. Plant Cell Tissue Organ. Cult., 55: 15-22.

Osman, M.G., E.A. Elhadi and M.M. Khalafalla, 2010. Callus formation and organogenesis of tomato (*Lycopersicon esculentum* Mill, C.V. Omdurman) induced by thidiazuron. Afr. J. Biotechnol., 9: 4407-4413.

Osman, N.I., A. Awal, N.J. Sidik and S. Abdullah, 2012. Antioxidant activities of *in vitro* seedlings of *Lycium barbarum* (Goji) by diphenyl picrylhydrazyl (DPPH) assay. Int. J. Pharm. Pharm. Sci., 4: 137-141.

Pal, S.P., I. Alam, M. Anisuzzaman, K.K. Sarker, S.A. Sharmin and M.F. Alam, 2007. Indirect organogenesis in summer squash (*Cucurbita pepo* L.). Turk. J. Agric. For., 31: 63-70.

Salaj, T., R. Matusova and J. Salaj, 2004. The effect of carbohydrates and polyethylene glycol on somatic embryo maturation in hybrid fir *Abies alba?abies numidica*. Acta. Biol. Cracov. Bot., 46: 159-167.

Sarkar, A., 2009. Plant Stem Cells. 1st Edn., Discovery Publishing House, New Delhi, ISBN-13: 9788183564076, Pages: 324.

Sidhu, Y., 2010. *In vitro* micropropagation of medicinal plants by tissue culture. Plymouth Student Scientist, 4: 432-449.

Skoog, F. and C.O. Miller, 1957. Chemical regulations of growth and organ formation in plant tissue cultured *in vitro*. Symp. Soc. Exp. Biol., 11: 118-140.

Soh, W.Y., P.S. Choi and D.Y. Cho, 1998. Effects of cytokinin on adventitious root formation in callus cultures of *Vigna unguiculata* (L.) walp. *In vitro* Cell. Dev. Biol. Plant, 34: 189-195.

Sridhar, T.M. and C.V. Naidu, 2011. An efficient callus induction and plant regeneration of *Solanum nigrum* (L.)-an important antiulcer medicinal plant. J. Phytol., 3: 78-82.

Tang, Y., J. Liu, B. Liu, X.M. Li, J. Li and H.X. Li, 2010. Endogenous hormone concentrations in explants and calluses of bitter melon (*Momordica charantia* L.). Interciencia, 35: 680-683.

Thadavong, S., P. Sripichitt, W. Wongyai and P. Jompuk, 2002. Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. Kasetsart J. (Nat. Sci.), 36: 334-344.

Thomson, G.E., 2010. Further consideration of Asian medicinal plants in treating common chronic diseases in the West. J. Med. Plants Res., 4: 125-130.

Tian, H.Q., Y.H. Xiao and W.F. Liu, 1993. Plant regeneration from hypocotyl protoplasts of *Lycium barbarum* L. Shi Yan Sheng Wu Xue Bao, 26: 89-93.

Twyman, R.M., 2003. Genetics of primary root development. pp: 1096-1101, http://www.writescience.com/RMT%20PDFs/Elsevier/eaps%20rootdev.pdf

Vannini, C., M. Campa, F. Sassi and M. Bracale, 2012. Medicinal Plants: Molecular Biology/Biotechnology Approach. In: Herbal Medicines: Development and Validation of Plant-Derived Medicines for Human Health, Bagetta, G., M. Cosentino, M.T. Corasaniti and S. Sakurada (Eds.). CRC Press/Taylor and Francis Group, Boca Raton, FL., USA., pp: 51-77.

Verma, S.K., B.B. Yucesan, S. Gurel and E. Gurel, 2011. Indirect somatic embryogenesis and shoot organogenesis from cotyledonary leaf segments of *Digitalis lamarckii* Ivan., an endemic medicinal species. Turk. J. Biol., 35: 743-750.

Wenck, A.R., B.V. Conger, R.N. Trigiano and C.E. Sama, 1998. Inhibition of somatic embryogenesis in orchard grass by endogenous cytokinins. Plant Physiol., 88: 990-992.

Wu, S.J., L.T. Ng and C.C. Lin, 2004. Antioxidant activities of some common ingredients of traditional chinese medicine, *Angelica sinensis, Lycium barbarum* and *Poria cocos*. Phytother. Res., 18: 1008-1012.

Yadav, P.R. and R. Tyagi, 2006. Biotechnology of plant tissue. Discovery Publishing House, New Delhi, India, pp: 1-2.

Zavattieri, M.A., A.M. Frederico, M. Lima, R. Sabino and B. Arnholdt-Schmitt, 2010. Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron. J. Biotechnol., Vol. 13, No. 1. 10.2225/vol13-issue1-fulltext-4

Zimmerman, J.L., 1993. Somatic embryogenesis: A model for early development in higher plants. Plant Cell, 5: 1411-1423.