

Original Research Paper

Enhancement of *in vitro* Rooting through Growth Media, Gelling Agents and Activated Charcoal in *Lycium chinense*

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Abstract: *Lycium chinense*, a traditional Chinese herbal medicine is well known for its medicinal value and composition for centuries in Asia. Plant tissue culture technique has proven as a quick and sustainable ways to regenerate various plants species those are valuable in terms of medicinal as well as ornamental. Here, we investigated the *in vitro* root regeneration and root growth of *L. chinense* in response of different media, gelling agents and activated charcoal. The growth media Schenk and Hildebrandt medium (SH medium) performed the best for being the highest number of roots in each (3.50) and also for the longest root length (33.80 mm) exhibiting 20% and 18% higher roots/explant and root growth, respectively than that of the lowest roots/explant and root growth containing medium of MS. The highest number of roots (3.90) in each explant was observed when phytagar at 6 g L⁻¹ has been used in the culture media producing a 26% higher number of roots in each explant. Phytagar at the lowest concentration (5 g L⁻¹) used in this study produced the longest root length (35.10 mm) exhibiting 20% higher root length than that of the highest concentration (9 g L⁻¹) of phytagar and after that with further increase the concentration of Phytagar the growth of the root length has been decreased. Here concentration of Gelrite at 3 g L⁻¹ responded positively for getting the highest number of roots (4.00) in each explants and the longest root length (35.2 mm). Activated charcoal at 1 g L⁻¹ produced the highest roots (4.10) in each explant and also enhanced to produce the longest (45.40 mm) root length these findings could contribute to enhance rooting ability in any crops especially some medical and ornamental crops and even could provide useful information for future industrial-scale root production of *L. chinense*.

Keywords: Activated Charcoal, Gelling Agents, Growth Media, *In vitro* Rooting Ability, Medicinal Plants

Introduction

Lycium chinense is a shrub belongs to the Solanaceae family. It is a prominent traditional Chinese herbal medicine which has a great variety of benefits especially for the immune modulating activity (Gan *et al.*, 2004), anti-inflammatory effects (Oh *et al.*, 2012), anti diabetic effects (Luo *et al.*, 2004), anticancer properties (Mao *et al.*, 2011) and anti-aging properties (Chang and So, 2008). Several secondary metabolites have been isolated from *L. chinense*, such as alkaloids (Youn *et al.*, 2012), carotenoids

(Bunghetz *et al.*, 2012), lignans (Zhang *et al.*, 2013) and betaine (Shin *et al.*, 1999).

The usual propagating system of any plants either tree, shrub or herb is from the seeds. Besides seeds, plants can also be propagated with other vegetative ways. But regeneration through seeds needed much time and it is also considered as low propagating methods of regeneration. Whereas, tissue culture techniques are just the opposite of traditional regeneration techniques where it is very easily applicable for clonal regeneration, genetically improvement and for the conservation of rare species

which could be done very rapidly with huge propagation rate (Park *et al.*, 2009).

Rooting is the final culture stage prior to acclimatization which is the prime concept of micro propagation system (Ismail *et al.*, 2011; Millán-Orozco *et al.*, 2011). A good rooting system is the prerequisite for survival of *in vitro* grown plantlets in the field depends which might help to absorb water and nutrients from the soil (Benková and Bielach, 2010). Exogenously applied natural or synthetic auxins could help for rooting (Osterc and Štampar, 2011) whereas, other important variables, such as gelling agents, activated charcoal might be important factors for enhancement of rooting in tissue culture systems (Arthur *et al.*, 2006; Thomas, 2008). A gelling agent is often considered to initiate growth support to the culture. It is already reported that increasing the level of agar concentration of the medium resulted in restricted diffusion of macromolecules (Romberger and Tabor, 1971). The aim of this present study was to investigate the response of growth media, gelling agents and activated charcoal on the rooting ability of *L. chinense*.

Materials and Methods

Plant Materials

Young shoots of 1-years-old plants of *L. chinense* were collected and then were grown in the green house of the Chungnam National University, Daejeon, Korea. The leaves of selected young shoots were removed and then cut around 5 cm in length before establishing *in vitro* shoot cultures. Explants were cleaned through tap water for 5-10 min and then were surface sterilized using 70% (v/v) ethanol for 30 sec and then 1% sodium hypochlorite solution was used for 10 min. After that explants were rinsed thoroughly with sterilized distilled water and then incubated on 50 mL of hormone free MS (Murashige and Skoog, 1962) basal medium under the light condition. The basal medium (consisted of mineral salts and vitamins) supplement together with 30 g L⁻¹ of sucrose and 8 g L⁻¹ of Phytagar as a solidifying agent. The pH of the medium was maintained to 5.8 before adding the Phytagar and sterilized by autoclaving at 121°C for 20 min. Four weeks after cultured, the elongated shoots were observed and kept under controlled environmental conditions until further used.

In vitro Rooting using Different Media

Seven shoot explants were put in each magenta box which contains 50 mL of hormone free full strength basal MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972) and B5 (Gamborg *et al.*, 1972) media, respectively. These basal media were solidified with 0.3% Gelrite and supplemented with 3% (w/v) sucrose. The medium pH was settled down to 5.8 prior to the addition of Gelrite and autoclaved at 121°C with the

pressure of 1.1 kg cm⁻² for 20 min. Cultures were then incubated maintaining a temperature of 25±1°C along with a 16 h photoperiod per day under the standard cool and white florescent tubes. All experiments were replicated thrice. Efficiency of rooting, number of roots in each explant and root length were measured 4 weeks after incubation.

Determination of SH Medium Concentration

The most suitable medium was chosen from the preliminary experiment. Then, root regeneration was determined by using different concentration (1/4SH, 1/2 SH, SH and 2SH) of the medium. About 1~2 cm of seven shoot segments were cultured on the respective medium. The same procedure for sterilizing the medium and culture conditions, mentioned in the first experiment, was applied.

Promoting Root Regeneration with Gelling Agent and Activated Charcoal

Different gelling agents and activated charcoal and their different concentrations of 5, 6, 7, 8, 9 g L⁻¹ Phytagar, 1, 2, 3, 4, 5 g L⁻¹ Gelrite and 0.0, 0.1, 0.5, 1, 3 g L⁻¹ activated charcoal were used in combination with SH medium for efficient root regeneration in *L. chinense*. Five segments of 1~2 cm long shoots were put on Magenta box containing 50 mL each medium. Media were sterilized by autoclaving as the same procedure applied above for plant materials. Each treatment was replicated three times and data were collected after two weeks of culture.

Statistical Analysis

The data obtained was analyzed as mean ± standard deviation from 50 shoot explants tested.

Results

Effect of Different Media on Root Generation and their Growth

Three different media i.e., SH, B5 and MS were used to investigate the variation of root regeneration as well their growth of *Lycium chinense*. Root regeneration and growth of root length significantly differed due to different growth media used in this study (Table 1). From the results of this study, it was found that SH medium performed the best for producing the highest root number in each explant and also for the highest root length, followed by B5 and MS basal medium. The SH medium produced 20 and 13% higher root per explant than that of MS and BS medium, respectively. Similarly, the SH medium exhibited 18 and 14 % higher root length compared to MS and B5 medium, respectively. For the best performance of both number of roots and root length

SH medium was selected as a suitable basal medium for the root development and growth of *L chinense*.

Effect of Concentrations of SH Medium on Root Generation and Growth

To determine the effect of different strength of SH medium, explants were cultured for four weeks on basal media of $\frac{1}{4}$ SH, $\frac{1}{2}$ SH, SH and 2SH. The results showed that a significant variation was observed both for producing roots and their root length due to the influence of different strength of SH media (Table 2). Both number of root and root length full strength of SH medium responded as the best condition for establishing the maximum number of root per explant (3.5) and also for the longest root length (33.80 mm) followed by $\frac{1}{2}$ SH, 2SH and $\frac{1}{4}$ SH media. The SH medium gave 35, 13, 3% higher root per explant than that of $\frac{1}{4}$ SH, 2SH and $\frac{1}{2}$ SH media, respectively. On the same way, the SH medium produced exhibited 22, 18 and 9% higher root length than that of $\frac{1}{4}$ SH, 2SH and $\frac{1}{2}$ SH media, respectively.

Gelling Agents on the Regeneration and growth of Roots

To enhance the regeneration and growth of roots gelling agents (Phytagar and Gelrite) were used to the excised stem of *Lycium chinense* and were harvested after four weeks of *in vitro* culture. Both Phytagar and Gelrite showed significant variation for the production of roots per explant and their root growth (Table 3). The range of number of roots/explant among the concentration of phytagar was 3.10 to 3.90. Through the application of phytagar at 6 g L^{-1} the maximum number of roots/explant were observed and thereafter with increase the concentration of phytagar, number of roots/explant have been decreased. At this concentration (6 g L^{-1}) Phytagar showed 26% higher number of roots/explant from the lowest roots produced concentration of Phytagar (9 g L^{-1}). The range of root length among the concentration of phytagar was 29.20 to 35.10 mm. Phytagar at the lowest concentration (5 g L^{-1}) used in this study produced the longest root length (35.1 mm) exhibiting 20% higher root length than that of the highest concentration (9 g L^{-1}) of phytagar and after that with further increase the concentration of Phytagar the growth of the root length has been decreased.

Gelrite influenced positively to increase number of roots and their growth. With increase the concentration of Gelrite, both number of roots and root length increased up to 3 g L^{-1} and then it started to decrease with further increase the concentration of Gelrite (Table 3). Among the concentration of gelrite the range of number of roots/explant was 3.30 to 4.00. Here concentration of Gelrite at 3 g L^{-1} responded well and helped to give the maximum roots number/explant (4.00) showing 21% higher number of roots/explant than that of the lowest number of roots/explant at the highest Gelrite concentration treatment (5 g L^{-1}). The range of root length among the concentration of Gelrite was 30.20 to 35.20 mm. At the same concentration (3 g L^{-1}), the longest root length (35.2 mm) was observed showing 17% higher root length from the lowest root length containing Gelrite treatment (5 g L^{-1}).

Activated Charcoal on the Regeneration and Growth of Roots

Finally our efforts go to improve the rooting ability and root growth by using activated Charcoal to the excised stem of *Lycium chinense* and were harvested after four weeks of *in vitro* culture. Activated Charcoal highly influenced for the the production of roots per explant and their root growth (Table 4). The variation of root number and their root growth was much higher among the treatments used in this study. The range of number of roots/explant among the concentration of activated Charcoal was 3.30 to 4.10. Here also with increase the concentration of activated Charcoal, increased root number and their root length up to 1 g L^{-1} and then it decreased very rapidly with increase the concentration of Charcoal (Table 3). At the concentration of 1 g L^{-1} of activated Charcoal, the maximum roots/explant (4.10) and the highest root length (45.40 mm) were observed. At this concentration (1 g L^{-1}) activated Charcoal produced 24% higher number of roots/explant from the lowest roots produced concentration of activated Charcoal (3 g L^{-1}). The range of root length among the concentration of activated Charcoal was 33.80 to 45.40 mm. Activated Charcoal at the concentration (1 g L^{-1}) used in this study produced the longest root length (45.40 mm) exhibiting 34% higher root length compared to control treatment.

Table 1. Response of different growth media on regeneration of roots and their growth from excised stem of *Lycium chinense* after four weeks of *in vitro* culture

Medium	No. of root/explant	Root length (mm)
SH	3.50±0.13	33.80±3.26
B5	3.10±0.11	29.60±3.20
MS	2.90±0.10	28.70±3.59

Table 2. Response of concentrations of SH media on regeneration of root and their growth from excised stem of *Lycium chinense* after four weeks of *in vitro* culture

SH Medium	No. of root/explant	Root length (mm)
1/4 SH	2.60±0.12	27.80±2.25
1/2 SH	3.40±0.16	31.10±2.77
SH	3.50±0.13	33.80±3.26
2SH	3.10±0.11	28.70±2.95

Table 3. Response of gelling agents on root regeneration and their growth from the excised stem of *Lycium chinense* after four weeks of *in vitro* culture

	Gelling agent (g/L)	No. of root/explant	Root length (mm)
Phytagar	5.0	3.80±0.13	35.1±2.38
	6.0	3.90±0.11	34.6±2.46
	7.0	3.70±0.12	34.2±2.15
	8.0	3.50±0.13	33.8±3.26
	9.0	3.10±0.11	29.2±2.90
Gelrite	1.0	3.60±0.17	33.8±3.58
	2.0	3.80±0.15	34.8±2.66
	3.0	4.00±0.12	35.2±2.66
	4.0	3.70±0.13	33.5±3.27
	5.0	3.30±0.14	30.2±3.58

Table 4. Response of activated charcoal on root regeneration and their root growth from the excised stem of *Lycium chinense* after four weeks of *in vitro* culture

Activated charcoal (g/L)	No. of root/explant	Root length (mm)
0.0	3.50±0.13	33.80±3.26
0.1	3.80±0.12	34.50±2.27
0.5	4.00±0.11	38.40±3.53
1.0	4.10±0.11	45.40±3.95
3.0	3.30±0.14	36.40±3.24

Discussion

In this study, media, gelling agents and activated Charcoal had significant influence for the production of roots and their growth. It is mentionable that this is the first study for *in vitro* rooting enhancement in *Lycium chinense* through media, gelling agents and activated charcoal. It was shown that full strength SH media responded the best for giving the maximum roots and their growth in *Lycium chinense* among the growth media used in this study. There were some studies those were carried out before for different medicinal plants where it was reported that SH medium performed the best, for producing the maximum number of roots and root length, followed by B5 and MS basal medium in *Rehmannia glutinosa* (Thwe *et al.*, 2013). The root length in *Lycium chinense* which was found from this study was higher than that of root length of *Rehmannia glutinosa* by the influenced of SH media (Thwe *et al.*, 2013). It was also proclaimed that with increase the strength of SH medium decreases the regenerated root number as well their root growth (Thwe *et al.*, 2013). Quarter strength MS medium along with 10 µM IBA was effective for rooting of the shoots (Shinde *et al.*, 2016) of *Artemisia nilagirica*. MS medium promotes the highest growth of *Cladanthus mixtus* with an average of 2.75±0.12 cm shoot length and 2.60±0.29 shoots per

explants and the mean number of roots achieved 3.33±0.17 root per explants with a length of 2.42±0.16 cm (Harras and Lamarti, 2014). The best rooting rate was achieved in *Astragalus membranaceus* by B5 medium among three different medium (B5, MS and WPM) and 0.1 mg L⁻¹ IBA treatment induced the highest (80%) rooting ratio (Han *et al.*, 2014).

Among the gelling agent, 3 g L⁻¹ of Gelrite medium responded the best for producing the highest roots (4.0 per explant) and the longest root length (35.20 mm). Similar trend was observed by some other researchers (Park *et al.*, 2009; Thwe *et al.*, 2015) in *R. glutinosa* where it was reported that 3 g L⁻¹ of Gelrite performed the best for shoot organogenesis. It is mentionable that the effect of gelling agent was higher both for root regeneration and root growth in *Lycium chinense* compared to other plant species i.e., *R. glutinosa*. Gelling agent also significantly enhanced for *invitro* seed germination, shoot differentiation and rooting of *Albizialebeck* (Raina and Babbar, 2011). It was reported that Gelrite has better performance than phytagar for the regeneration of shoot in apple reported by Saito and Suzuki (1999; Shrivastava and Rajani, 1999). Increasing concentration of Phytagar from 5 to 7 g L⁻¹ enhanced the rooting percentage, root number and root length (Thwe *et al.*, 2015) and after that a further increase in concentration of agar (i.e., to 8 and 9 g L⁻¹)

did not improve the rooting response. In another study it was also observed that *in vitro* rooting has been increased in *Syzygium alternifolium* when the concentration of agar ranged from 0 to 0.8% and after further increasing the agar level from 1.0 to 1.2% no response was observed from (Sha Valli Khan *et al.*, 1999). From the gelling agents related studies it can be suggested that increased level of agar showed negative role in rooting and their root growth. Activated Charcoal (AC) has growth regulatory effect on *in vitro* rooting of many plant species which was reported by several studies. In this study the activated charcoal has more positive effect on both for root generation and root growth compared to any other plant species studied before. With addition of AC in the rooting medium had a positive effect on number of roots per rooted shoot in Carrizo Citrange (Montoliu *et al.*, 2010). From a very recent study positive effects on rooting and the development of active carbon was observed in *Myrtus communis* (Kaçar *et al.*, 2017). From another study it was reported that addition of AC encouraged the secondary and tertiary roots formation in date palm (Abul-Soad and Jatoi, 2014). Growth of roots in *Rehmannia glutinosa* markedly increased through addition of activated charcoal at concentrations of 0.1-0.3% (Paek *et al.*, 1995). The multiplication ratio, elongation and average number of roots increased by application of 0.5 g L⁻¹ activated charcoal in date palm root culture (Wahed, 2013). Addition of AC promotes roots induction and formation from *in vitro* shoots in Brahmi (Priya Dharishini *et al.*, 2015).

Conclusion

Plant tissue culture in combination with genetic engineering is very useful in gene transfers which might acted for genetic transformation leading to plant improvement. Therefore, efficient protocols are crucial for saving time and cost associated with molecular works. Establishment of reliable protocols are the prerequisite for root regeneration. In this study, we worked out to find out the most efficient medium and concentration, best gelling agent and influence of activated charcoal in *L. chinense* for the first time. This information provides a useful indication for further study in promoting commercial root production via gene transformation in the future. Further study is needed on root regeneration of *L. chinense* by using other different concentration and combination of plant hormones for advanced root regeneration.

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Author's Contributions

Jae Kwang Kim: Performed the experiments and analyzed the data.

Sung Un Park: Designed the experiments and wrote the manuscript

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