Journal of Phytology 2024, 16: 8-12
doi: 10.25081/jp.2024.v16.8793
https://updatepublishing.com/journal/index.php/jp

Research Article

ISSN: 2075-6240

Influence of medium and gelling agents concentration on *in vitro* rooting of *Polygonum tinctorium*

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ABSTRACT

Polygonum tinctorium has long been employed in the medical and dye industries in many nations, particularly in Korea and Japan, for traditional fabric staining as a source of blue color. The plant tissue culture method has proven to be a rapid and sustainable approach for the regeneration of various plant species, particularly those with medicinal or ornamental value. In this study, we explored the in vitro root regeneration and growth of *P. tinctorium* in response to different growth media and gelling agents. Among the tested media, Schenk and Hildebrandt medium (SH medium) demonstrated superior performance, resulting in the highest number and length of roots per explant. SH media produced about 53 and 18% higher root per explant and a 40% and 14% increase in root length compared to B5 and MS media, respectively. Half strength of SH medium proved to be the optimal condition for both root number (7.46 roots per explant) and root length (3.81 mm). Among the gelling agents, 2 g/L of Gelrite medium was most effective in promoting the highest and tallest roots. These findings have the potential to enhance rooting abilities in various crops, particularly in the case of medicinal and ornamental plants, and may offer valuable insights for future industrial-scale root production of *P. tinctorium*.

KEYWORDS: Gelling agents, Root regeneration, Growth media, *In vitro* rooting ability

Received: July 15, 2023
Revised: January 24, 2024
Accepted: January 26, 2024
Published: February 07, 2024

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INTRODUCTION

The annual plant *P. tinctorium* has long been employed in the medical and dye industries (Cooksey, 2007; Hirota *et al.*, 2014). It is also often used in many nations, particularly Korea and Japan, for traditional fabric staining as a source of blue color (Chung *et al.*, 2005; Hirota *et al.*, 2016). A substance called indigo, which was discovered from *P. tinctorium*, is widely used in traditional Chinese medicine and as a natural color in denim manufacturing (Kukuła-Koch *et al.*, 2013). The greatest quantities of indigo for therapeutic purposes have been extracted from naturally existing plant material using water extraction and alkaline precipitation (Campeol *et al.*, 2006).

Plant tissue culture methods are now widely employed as a valuable tool for fundamental and applied research as well as for the large-scale production of commercially significant crops (Shasmita *et al.*, 2017; Ozdemir & Budak, 2018). All plants, whether they may be trees, shrubs, or herbs, are often propagated from their seeds. Plants can be multiplied by different vegetative methods in addition to seeds. However, seed regeneration requires a long period and is regarded as a low-propagation mode of regeneration. On the other hand, tissue culture methods are the exact reverse of conventional regeneration methods in that they may be quickly and massively multiplied for clonal regeneration, genetic enhancement, and the conservation of endangered species (Park *et al.*, 2009).

The fundamental concept in a micropropagation system revolves around acclimation, with the final stage of the culture process referred to as rooting (Ismail *et al.*, 2011; Millán-Orozco *et al.*, 2011). In order to thrive in natural settings, in vitro-generated plantlets need a robust root system that contributes to the soil's absorption of water and nutrients (Benková & Bielach, 2010). Application of exogenous natural or synthetic auxins may facilitate the rooting process (Osterc & Štampar, 2011),

while additional influential factors, such as the composition of the medium and the choice of gelling agents, could play crucial roles in enhancing rooting within tissue culture systems (Arthur *et al.*, 2006; Thomas, 2008).

Gelling agents are frequently thought of as starting points for the culture's development support. Increases in the medium's agar content have previously been shown to limit the diffusion of macromolecules (Romberger & Tabor, 1971). The current study sets out to find out how growth media and gelling agents affected *P. tinctorium*'s capacity to root. In this work, we developed a method for *P. tinctorium* plant regeneration employing internode explants and varying gelling agents and medium concentrations.

MATERIALS AND METHODS

Plant Materials

The seeds of *Polygonum tinctorium* were procured from the experimental farm at Chungnam National University in Daejeon, Korea. Seed disinfection involved a 30 second exposure to a 70% (v/v) ethanol solution, followed by a 15 minute immersion in a 4.5% (v/v) aqueous NaOCl solution containing Tween 20. Following this, the treated seeds underwent aseptic rinsing with sterilized distilled water and were placed on a solid basal medium for germination. The basal medium employed was Murashige and Skoog (MS), supplemented with salts and vitamins, along with the addition of 3% sucrose, pH adjustment to 5.7-5.8, and incorporation of 0.7% (w/v) plant agar. The entire medium was sterilized through autoclaving at 121 °C for 20 minutes. Each Petri dish received approximately 10 sterilized seeds and was then incubated in a growth chamber at 25±1 °C, with illumination at 35 µmol s-1 m-2, maintaining a 16 hour photoperiod. After one week, the seedlings were transferred to Magenta boxes (Magenta LLC, Chicago, IL, USA) with the same MS solid medium, and plant growth was sustained for an additional 4 weeks (Figure 1). Following this 4-week cultivation period, elongated shoots were observed, and these plants were kept under controlled environmental conditions for subsequent utilization.

In vitro Rooting using Different Media

In each Magenta box, six shoot explants, approximately 2.0 cm long, were placed. The Magenta boxes were filled with 50 mL of hormone-free full-strength basal media, specifically Murashige and Skoog (MS) (Murashige & Skoog, 1962), SH (Schenk & Hildebrandt, 1976), and B5 (Gamborg *et al.*, 1976). These basal media were solidified using 0.8% Phytagar and supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 before the addition of Phytagar, and then the entire medium was autoclaved at 121 °C with a pressure of 1.1 kg cm^{-2} for 20 minutes. Cultures were maintained at a temperature of 25±1 °C, with a 16 hour photoperiod provided by standard cool and white fluorescent tubes. Each experiment was conducted in five replicates. After a 4-week incubation period, rooting efficiency, the number of roots per explant, and the length of the roots were quantified.

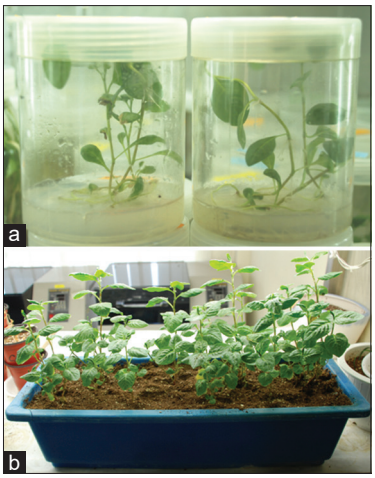

Figure 1: (a) *In vitro* germinated plantlet of *P. tinctorium* in Magenta box with MS solid medium and (b) The rooted plants of *P. tinctorium* in the pots containing autoclaved vermiculite soil

Determination of SH Medium Concentration

The most effective medium identified in the preliminary experiment was chosen for further investigation. Following this selection, root regeneration was evaluated using different concentrations (1/4 SH, 1/2 SH, SH, and 2 SH) of the designated medium. Cultivation involved placing approximately 2 cm segments from six shoots on the respective media. The sterilization process and cultural conditions, as outlined in the initial experiment, were consistently applied in this subsequent investigation.

Promoting Root Regeneration with Gelling Agent

A range of gelling agents, activated charcoal, and diverse concentrations, spanning from 5 to 9 g/L for Phytagar and 1 to 5 g/L for Gelrite, were utilized in combination with 1/2 SH medium to improve the effectiveness of root regeneration in *P. tinctorium*. Six segments of shoots, each 2 cm in length, were placed in Magenta boxes, with each box containing 50 mL of the respective medium. The media were sterilized by autoclaving, following the same procedure outlined earlier for plant materials. Each treatment was replicated three times, and data were gathered four weeks after the culture initiation.

Transferring Plantlets to Green House Conditions

The regenerated plantlets were consistently kept in controlled in vitro conditions until their roots were well-established. After a period of 6 weeks, the plantlets with well-established roots were transplanted into pots filled with autoclaved vermiculite soil (Figure 1). Following transplantation, both the plants and pots were covered with a moistened polythene bag to prevent desiccation. To minimize abrupt shock, the pots were situated in a growth-controlled room for 7-15 days. The polythene bags were gradually punctured at 2-3 day intervals, allowing the plants to acclimate gradually to the natural environment. Complete removal of the bags took place after 10-15 days, coinciding with the point when the plantlets exhibited self-supportiveness. At

this stage, the plantlets were exposed to the natural environment for 3-10 hours daily. Finally, after 15-20 days, they were transferred to greenhouse conditions.

Statistical Analysis

The data collected was subjected to analysis, and the results are presented as the mean value along with the corresponding standard deviation. The analysis was based on observations from 50 shoot/explants that were tested.

RESULTS

Effect of Different Media on Root Generation and their Growth

Three distinct media, namely SH, B5, and MS, were utilized to examine the variations in root regeneration and growth of *P. tinctorium*. The results indicated significant differences in both root regeneration and root length growth among the different growth media employed in this study (Table 1). The findings revealed that the SH medium outperformed, producing the highest root number per explant and the greatest root length, followed by MS and B5 basal media. Specifically, the SH medium resulted in 53% and 18% more roots per explant compared to the B5 and MS media, respectively. Similarly, the SH medium demonstrated 40% and 14% greater root lengths compared to the B5 and MS media, respectively. Based on the optimal performance in both root number and root length, the SH medium was identified as a suitable basal medium for the root development and growth of *P. tinctorium*.

Effect of Concentrations of SH Medium on Root Generation and Growth

To assess the impact of different concentrations of SH medium, explants were cultivated for a duration of four weeks on basal media of 1/4 SH, 1/2 SH, SH, and 2 SH. The results indicated significant variations in both root production and root length influenced by the different strengths of SH media (Table 2). Regarding both the number of roots and root length, half-strength SH medium responded as the optimal condition, establishing the maximum number of roots per explant (7.46) and the longest root length (3.81 mm), followed by SH, 1/4 SH, and 2 SH media. The 1/2 SH medium exhibited 31%, 17%, and 32% higher root production per explant compared to 1/4 SH, SH, and 2 SH media, respectively. Similarly, the 1/2 SH medium demonstrated 19%, 8%, and 15% higher root length compared to 1/4 SH, SH, and 2 SH media, respectively.

Gelling Agents on the Regeneration and Growth of Roots

Both Phytagar and Gelrite showed significant variation in the production of roots per explant and their root growth (Table 3). The range of the number of roots/explants among the concentration of phytagar was 4.45 to 7.63. Through the application of phytagar at 6 g/L, the maximum number of roots/explants was observed and thereafter with an increase in the concentration of phytagar, the number of roots/explants was decreased. At this concentration (6 g/L) Phytagar showed a 51% higher number of roots/explants from the lowest roots produced concentration of Phytagar (9 g/L). The range of root length among the concentrations of phytagar was 2.29 to 4.46 mm. Phytagar at the lowest concentration (6 g/L) used in this study produced the longest root length (4.46 mm) exhibiting 53% higher root length than that of the highest concentration (9 g/L) of phytagar and after that, with further increase in the concentration of phytagar, the growth of the root length has been decreased.

Gelrite influenced positively to increase the number of roots and their growth. With an increase in the concentration of Gelrite, both the number of roots and root length started to decrease (Table 3). Among the concentration of gelrite the range of the number of roots/explants was 4.21 to 8.28. Here the concentration of Gelrite at 2 g/L responded well and helped to give the maximum roots number/explant (4.00) showing a 21% higher number of roots/explant than that of the lowest number of roots/explants at the highest Gelrite concentration treatment (5 g/L). The range of root length among the concentration of Gelrite was 2.61 to 5.28 mm. At the same concentration (2 g/L), the longest root length (5.28 mm) was observed but the lowest root length was found in Gelrite treatment (5 g/L) (Table 4).

Table 1: Response of different growth media on regeneration of roots and their growth from the excised stem of *P. tinctorium* after four weeks of *in vitro* culture

Medium	No. of root/explant	Root length (cm)
B5	4.31 ± 0.33^{c}	2.72 ± 0.17^{b}
MS	5.62 ± 0.46^{b}	3.34 ± 0.21^{a}
SH	6.62 ± 0.39^{a}	3.82 ± 0.32^{a}

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

SH Medium	No. of root/explant	Root length (mm)
1/4 SH	5.69 ± 0.52^{b}	3.19 ± 0.36^{b}
1/2 SH	7.46 ± 0.46^{a}	3.81 ± 0.19^{a}
SH	6.38 ± 0.42^{b}	3.54 ± 0.24^{ab}
2SH	5.65 ± 0.35^{b}	3.05 ± 0.18^{b}

Table 3: Response of phytagar on root regeneration and their growth from the excised stem of *P. tinctorium* after four weeks of *in vitro* culture on 1/2 SH medium

Gelling agent (g/L)	No. of root/explant	Root length (mm)
Phytagar		
6.0	7.63 ± 0.48^{a}	4.46 ± 0.38^{a}
7.0	6.79 ± 0.63^{ab}	3.82 ± 0.33^{b}
8.0	6.47 ± 0.52^{b}	3.63 ± 0.26^{b}
9.0	4.45 ± 0.38^{c}	2.92 ± 0.23^{c}

DISCUSSION

The formation of roots and their development were significantly influenced in this study by the medium and gelling agents. It

Table 4: Response of gelrite on root regeneration and their growth from the excised stem of *P. tinctorium* after four weeks of *in vitro* culture on 1/2 SH medium

Gelling agent (g/L)	No. of root/explant	Root length (mm)
Gelrite		
2.0	8.28 ± 0.74[a]	5.28 ± 0.42[a]
3.0	7.36 ± 0.59[a]	4.52 ± 0.36[b]
4.0	6.23 ± 0.48[b]	3.35 ± 0.27[c]
5.0	4.21 ± 0.27[c]	2.61 ± 0.15[d]

was demonstrated that, of the growth media utilized in this study, half-strength SH medium reacted the best in terms of providing the greatest number of roots and their development. Prior research on other medicinal plants revealed that, when it came to *Rehmannia glutinosa*, SH medium outperformed B5 and MS basal media in terms of growing the greatest number of roots and root length (Thwe *et al.*, 2013). Because of the impact of SH media, the study's findings about the root length of *Lycium chinense* revealed that it was longer than the root length of *R. glutinosa* (Thwe *et al.*, 2013). Additionally, it was declared that when SH medium strength increases, so does the quantity of regenerated roots and their development (Thwe *et al.*, 2013). Shinde *et al.* (2016) found that quarter-strength MS medium combined with 10 µM IBA was successful in rooting *Artemisia nilagirica* shoots. The most growth is accomplished by *Cladanthus mixtus* in MS media, with an average shoot length of 2.75±0.12 cm and 2.60±0.29 shoots per explant. The mean number of roots per explant is 3.33±0.17, with a length of 2.42±0.16 cm (Harras & Lamarti, 2014). According to Han *et al.* (2014), *Astragalus membranaceus* exhibited the highest rooting ratio (80%) when treated with 0.1 mg/L IBA, out of three distinct medium types (B5, MS, and WPM). The B5 medium yielded the best rooting rate.

To promote the growth of the tallest and most extensive roots among the various gelling agents, the use of 2 g/L of Gelrite medium proved to be the most effective. Similar observations have been made by other researchers working with *R. glutinosa*, where 3 g/L of Gelrite was identified as the most optimal for shoot organogenesis (Park *et al.*, 2009). Notably, in comparison to other plant species like *R. glutinosa*, the impact of the gelling agent was more pronounced for both root development and regeneration in *Lycium chinense*. Furthermore, Gelrite significantly enhanced *Albizzia lebbeck* seed germination, shoot differentiation, and in vitro rooting (Raina & Babbar, 2011). In the case of apple shoot regeneration, Gelrite was found to function better than Phytagar (Saito & Suzuki, 1999; Shrivastava & Rajani, 1999). Enhancements in rooting percentage, number, and length of plants were observed by elevating the Phytagar concentration from 6 to 9 g/L (Thwe *et al.*, 2015). However, increasing the agar concentration did not improve rooting response. Another study noted that in vitro rooting in *Syzygium alternifolium* increased when agar concentration ranged from 0% to 0.8%, with no further improvement observed after increasing agar levels from 1.0 to 1.2% (Khan *et al.*, 1999). These gelling agent-related studies suggest that higher levels of agar play a negative role in rooting and root growth.

CONCLUSION

In this research, we aimed to identify the most effective medium and concentration, optimal gelling agent, and the impact of activated charcoal on *P. tinctorium*, marking the first investigation of its kind. The results demonstrated that half-strength SH medium exhibited the highest efficiency in generating the maximum number of roots and promoting their growth compared to the other growth media examined in this study. Additionally, among the various gelling agents, 2 g/L of Gelrite medium proved to be the most effective for producing the greatest number of roots and achieving the longest root length. This data offers a helpful guide for future research aimed at advancing the application of gene modification to increase commercial root output. More research is required on *P. tinctorium* by root regeneration utilizing various plant hormone concentrations and combinations for enhanced root regeneration.

ACKNOWLEDGMENT

This work was supported by BK21 FOUR Program by Chungnam National University Research Grant, 2022.

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