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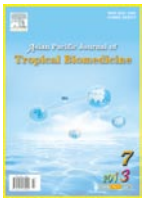

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Effect of cytokinins on *in vitro* multiplication of *Sophora tonkinensis*

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PEER REVIEW

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Comments

This is an excellent study on an important medicinal plant in which the authors have studied and determined the most effective cytokinin and its concentration for the tissue culture and *in vitro* propagation of *S. tonkinensis* in terms of shoot growth and multiplication. The results obtained are interesting and have shown that 2-isopentyladenine (2iP) is the optimal plant growth regulator for *Sophora* multiplication.
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ABSTRACT

Objective: To determine the effects of different cytokinins at various concentrations on *in vitro* shoot multiplication of an important medicinal plant. **Methods:** Nodal explants (1.5-2.0 cm) of *Sophora tonkinensis* were used. Multiple shoots were induced from nodal explants cultured on the Murashige and Skoog (MS) medium supplemented with 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 μmol 2-isopentyladenine (2iP), N6 benzyladenine, kinetin or thiadiazuron. **Results:** Among the four investigated cytokinins, 2iP showed the best response for shoot multiplication. Maximum shoot induction (75%) was achieved on the MS medium supplemented with 2.0 μmol 2iP, with a mean number of 5.0 shoots per explant. In comparison to other cytokinins tried, 2iP showed the highest shoot elongation with a mean shoot length of 4.8 cm. Root initiation was observed within 15 d within the transfer of shoots onto the MS basal medium, and the rooting percentage was 100% with a mean number of 5.4 roots per shoot and root length of 6.2 cm over a period of 4 weeks. The healthy plants, hardened and transferred to a greenhouse for proper acclimatization, exhibited 100% survival. **Conclusions:** It can be summarized that 2iP is the optimal plant growth regulator for *Sophora* multiplication.

KEYWORDS
Herbaceous legumes, Nodal explants, Shoot regeneration, 2-isopentyladenine, Kinetin

1. Introduction

The genus *Sophora* (Fabaceae) is composed of about 70 species. They are widespread in warm and dry habitats, including Asia, North and South Americas, and New Zealand[1]. *Sophora* roots have been known to be effective for the relief of abdominal pains and the treatment of tumors and to act as antidotes, *etc*[2].

Sophora tonkinensis (*S. tonkinensis*), commonly known as Shan-Dou-Gan, is herbaceous legume, widely used in traditional Chinese medicine. The dried roots and rhizomes of this species are used to treat fever, acute pharyngolaryngeal infections and sore throats. Phytochemical investigations have revealed that the plant

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accumulates isoprenyl-substituted flavonoids and lupin alkaloids as its main constituents[3]. As the compounds isolated from *S. tonkinensis* have been proven to demonstrate various medicinal properties and uses, it acts as the driving force behind current study to micropropagate the plant using tissue culture techniques.

In vitro propagation method is widely used for mass multiplication and ex-situ conservation of medicinal plants[4]. Direct regeneration in plant tissue culture is a requirement for any successful *in vitro* multiplication and transformation programs, as the regeneration through callus is known to induce somaclonal variations[5]. Axillary bud multiplication is an effective alternative for clonal propagation.

Cytokinin is one of the plant hormones crucial for plant growth and development and is known to promote cell division and differentiation[6]. Among cytokinins, zeatin and 2-isopentyladenine (2iP) are naturally occurring cytokinins, whereas, N^6 benzyladenine (BA), 6-furfuryl-aminopurine (kinetin, Kin), and [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)] urea (thidiazuron, TDZ) are synthetic cytokinins. Various types of cytokinins can also stimulate lateral bud growth and thus can cause multiple shoot formation by breaking shoot apical dominance[7].

Hence, taking all these into account, the objective of this study was to select the most effective cytokinin and its concentration for *in vitro* propagation of *S. tonkinensis* in terms of shoot growth and multiplication.

2. Materials and methods

2.1. Chemicals

The hormones were purchased from Sigma-Aldrich Co., St. Lo. Mo. and chemicals from Duchefa Biochemie B.V., Haarlem, the Netherlands.

2.2. Explant source

Actively growing shoots were collected from mature greenhouse-grown plants to be used as explants source and were washed thoroughly under running tap water for 15 min. They were treated with few drops of Tween 20 for 5 min followed by washing under tap water, then rinsed 3 times with sterile distilled water. Thereafter, in aseptic conditions, the explants were disinfected in 70% (v/v) ethanol for 2 min and 1.5% (v/v) sodium hypochlorite for 10 min followed by 3 rinses with sterile distilled water. Nodal segments (1.5-2.0 cm) were inoculated on the Murashige and Skoog (MS) medium with or without cytokinins[8].

2.3. Medium and culture conditions

The culture medium consisted of the MS basal salts and vitamins supplemented with 3% (w/v) sucrose. The medium was gelled with 0.8% (w/v) agar. The pH of all media was adjusted to 5.8 using 0.1 mol/L NaOH or 0.1 mol/L HCl before autoclaving at 121 °C for 15 min. Thidiazuron was filter sterilized and added to the autoclaved medium. Other cytokinins were added to the basal medium prior to pH adjustment and sterilization. All cultures were maintained at (25±2) °C under a 16 h photoperiod with 45 μmol/(m^2·s) irradiance provided by cool white fluorescent light (40 W tubes, Philips).

2.3.1. Effects of cytokinin on axillary shoot multiplication

For shoot multiplication, nodal segments were inoculated on the MS medium supplemented with 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 μmol 2iP, BA, Kin or TDZ. The number of explants initiating shoots and the average number of shoots per explant were recorded after 4 weeks.

2.3.2. In vitro rooting and acclimatization

The elongated shoots (4-5 cm) were excised individually and transferred to the MS medium without any supplemented plant growth regulators for rooting. Data on percentage of rooted shoots, number of roots per shoot, and root length were recorded after 4 weeks of transfer. Plantlets with well developed shoots and roots were removed from the culture medium, washed gently under running tap water, and were planted in 72-cell plug trays containing a greenhouse medium (Tosilee medium, Shinan Precision Co., Jinju, Korea). Then they were placed in a mist chamber (10 second every 10 min during the day time) in a greenhouse and irrigated at every alternative day with quarter-strength MS salts solution for 2 weeks. After 2 weeks, plants were transferred to the greenhouse bench and watered daily for 3 weeks. The survival rate was recorded at 5 weeks after transplanting. The greenhouse temperature and relative humidity set points were (20±5) °C and 66% respectively. The greenhouse was cooled by ventilation of an air flow of 1.3 m/s at the same temperature. Mean, maximum, and minimum daily air temperatures were recorded during the experimental period by digital thermometers (Thermo recorder TR-71S, T&D Corp., Matsumoto, Japan) were 20.2 °C, 25.0 °C, and 13.0 °C respectively.

2.4. Statistical analysis

In all experiments, each treatment consisted of 15 replicates, and each experiment was repeated 3 times. Data were statistically analyzed by ANOVA. Significant differences

between means were assessed using Duncan's multiple range test (DMRT) at 5% probability level. Data analysis was performed using SAS computer package (Release 9.1, SAS Institute Inc., Cary, USA).

3. Results

3.1. Shoot proliferation

The described disinfection method yielded 95% aseptic explants. Nodal explants developed a single shoot on the plant growth regulator-free medium. Table 1 shows the formation of axillary shoots from nodal explants grown on the MS medium supplemented with different types and concentrations of cytokinin. Regenerated shoots from the nodal explants became evident after about 7 d. Nodal explants cultured on the MS medium supplemented with 2iP induced more axillary shoot proliferation when compared with the control. An increase in 2iP concentration from 0.5 to 2.0 μmol resulted in an increase in the induction of axillary shoots. Maximal shoot regeneration (75%) was achieved on the MS medium containing 2.0 μmol 2iP with an average of 5.0 shoots per explants (Figure 1A).

Table 1

Effect of different types and concentrations of cytokinin on shoot induction from nodal explant of *S. tonkinensis* recorded after 30 d.

PGR's (μmol)				Shoot induction (%)	Mean no. of shoots/explant	Mean shoot length (cm)
BA	Kin	2iP	TDZ	–		
0.5	–	–	–	24.8±0.6^{e}	1.00±0.01^{d}	2.6±0.7^{b}
1.0	–	–	–	37.5±0.8^{c}	1.00±0.01^{d}	2.4±0.9^{b}
2.0	–	–	–	50.80±0.73^{b}	2.50±0.08^{a}	2.5±1.2^{b}
4.0	–	–	–	–	–	–
8.0	–	–	–	–	–	–
16.0	–	–	–	–	–	–
–	0.5	–	–	24.2±0.5^{e}	1.00±0.01^{d}	1.8±1.1^{d}
–	1.0	–	–	25.2±0.7^{d}	1.00±0.01^{d}	2.1±1.0^{c}
–	2.0	–	–	25.6±0.9^{d}	1.50±0.09^{c}	2.3±0.9^{b}
–	4.0	–	–	–	–	–
–	8.0	–	–	–	–	–
–	16.0	–	–	–	–	–
–	–	0.5	–	37.5±0.1^{c}	1.50±0.05^{c}	4.0±0.5^{a}
–	–	1.0	–	51.5±0.7^{b}	2.50±0.07^{a}	4.4±0.7^{a}
–	–	2.0	–	75.2±0.6^{a}	5.00±0.01^{a}	4.8±0.7^{a}
–	–	4.0	–	25.9±0.3^{d}	1.00±0.01^{d}	4.5±0.9^{a}
–	–	8.0	–	–	–	–
–	–	16.0	–	–	–	–
–	–	–	0.5	24.2±0.7^{e}	1.00±0.01^{d}	1.6±0.8^{c}
–	–	–	1.0	24.9±0.5^{e}	1.50±0.06^{c}	1.4±1.1^{e}
–	–	–	2.0	27.5±0.3^{d}	1.00±0.01^{d}	1.2±1.2^{e}
–	–	–	4.0	–	–	–
–	–	–	8.0	–	–	–
–	–	–	16.0	–	–	–

Means followed by the same letters within a column are not significantly different ($P \leqslant 0.05$).

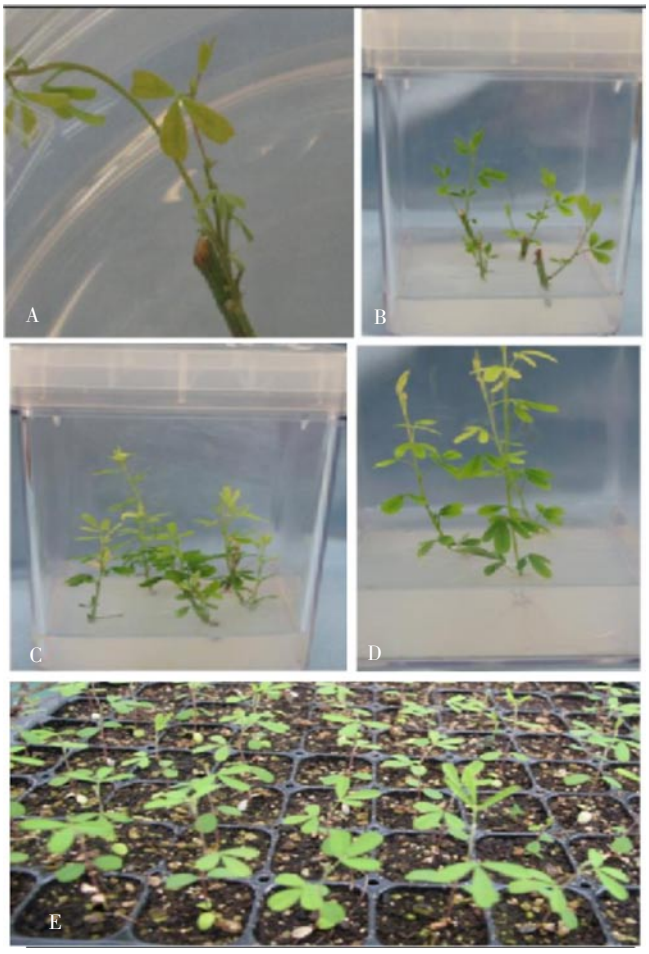

Figure 1. Axillary shoot multiplication from the nodal explant of *S. tonkinensis*.

A: multiple shoots induction on MS medium with 2.0 μmol 2iP after 2 weeks; B: formation of shoots on the MS medium with 2.0 μmol BA after 2 weeks; C: shoot elongation on MS medium with 2.0 μmol 2iP after 30 d; D: rooting of *in vitro* raised shoots on the full strength MS medium after 4 weeks; E: hardened plants in plug trays.

In the present study, increase in concentration of 2iP beyond the optimal level (2.0 μmol) resulted in low shoot multiplication frequency at 4.0 μmol, and on increasing concentration to 8.0 or 16.0 μmol, shoot induction was inhibited (Table 1). On the MS medium with BA, the best shoot induction (50.8 %) achieved was at 2.0 μmol with mean 2.5 shoots per explants (Figure 1B). However, with Kin in the medium, the frequency of shoot induction and mean number of shoots per explant at 2.0 μmol was 25.6 and 1.5, respectively (Table 1). On the MS medium with 2.0 μmol TDZ, the shoot induction percentage and mean number of shoot was 27.5 and 1.0, respectively (Table 1). In the present study, when the different concentrations of TDZ were incorporated to the medium, callus formation has been observed at the base of nodal explants even at low concentrations.

On the MS media with BA (0.5-2.0 μmol), there was not much difference in the mean shoot length and it varied between 2.4-2.6 cm. In the case of kinetin, the shoot length increased on increasing concentration 0.5-2.0 μmol Kin (Table 1). With 2iP, the shoot length increased with increasing concentration till 2.0 μmol (Figure 1C) and then it decreased. With TDZ, the

mean shoot length decreased on increasing concentration (0.5-2.0 μmol). However, the shoot length was smaller when compared to others. Among shoot elongation, 2iP showed the best mean length of 4.8 cm at 2.0 μmol 2iP, and it was followed by BA (2.5 cm), Kin (2.3 cm), and TDZ (1.2 cm) each at 2.0 μmol concentration (Table 1). Cytokinin alone did not induce multiple shoot formation remarkably in *S. tonkinensis*. Induction of shoots was not stimulated by increasing the concentration (8-16 μmol) of all the cytokinins tried.

3.2. Root induction

Rooting was achieved with the full strength MS medium without any plant growth regulator within 15 d. The highest rooting percentage was 100% with a mean number of 5.4 roots per shoot and 6.2 cm root length over a period of 4 weeks (Figure 1D).

3.3. Hardening

Healthy plantlets with well developed roots were planted in 72-cell plug trays containing a greenhouse medium. They were irrigated with quarter-strength MS salts (Tosilee medium) and acclimatized in a mist chamber in the greenhouse for 2 weeks, and the survival rate was 100% (Figure 1E). The plantlets thus developed from nodal explants were uniform and identical to donor plants with respect to morphological and growth characteristics.

4. Discussion

Development of single shoot on nodal explants on plant growth regulator-free medium was also reported in *Sterculia urens*[9]. The stimulatory effect of 2iP on multiple shoot formation has been reported earlier in *Caralluma adscendens* var. attenuate[10], *Scrophularia takesimensis* Nakai[4], *Sterculia urens* Roxb[9], and *Gossypium hirsutum*[5]. Contrary to the present findings, a high percentage of shoot induction (60%) has been reported at 24.6 μmol 2iP from nodal explants of *Ceropegia elegans*[11]. The lesser number of shoots induced by Kin in the present study also coincides with the reports in *Caralluma adscendens*[10]. The mean number of shoots induced per explant was 0.76, 1.33, and 1.83 at 1.0, 2.0, and 3.0 mg/L Kin, respectively.

Callus induction at the base of nodal explants has also been reported in *Scrophularia takesimensis* Nakai[4]. The inhibitory effect of TDZ has also been reported in *Cotoneaster wilsonii*[12], *Psoralea corylifolia*[13], and *Eclipta alba*[14]. The present results are in accordance with reports on *Sterculia urens* Roxb[9] and *Hymenocallis littoralis*[7] which also support that 2iP is the most effective one for shoot elongation.

The 2iP supplemented cultures showed elongated shoot length in comparison to other cytokinins tried. In the present study, among the four investigated cytokinins, 2iP was more effective for shoot multiplication. Suitable cytokinin type and concentrations stimulate shoot induction and elongation which varies depending upon plant's species; this might be also the case in *Sophora*.

In conclusion, analysis of the effect of all four cytokinins on shoot induction of *S. tonkinensis* infers that 2iP gives the best results. Our further investigation will be based on the study of the effect of combination of two cytokinins as well as auxin and cytokinin combinations in order to enhance shoot multiplication of this important medicinal plant. Hence, this paper supports the rapid multiplication of this medicinally important plant by an *in vitro* culture technique which can be further used in transformation studies.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Direct regeneration in plant tissue culture provides a great opportunity for the mass multiplication, basis for the establishment of a good transformation protocol, and ex-situ conservation of medicinal plants. Moreover, it is really vital to reduce somoclonal variation in these *in vitro* raised plantlets by the method of nodal explants for axillary bud multiplication with the help of different cytokinins. Different cytokinins have been used for different medicinal plants. The most suitable cytokinin and the most efficient concentration for the micropropagation of specific medicinal plants have to be optimized and will provide a platform to understand the tissue culture of important medicinal plants in terms of shoot growth and multiplication.

Research frontiers

Studies are being done to establish standardized protocols for the *in vitro* propagation of rare and important medicinal plants. The effect of different plant growth regulators especially cytokinins have been done for the tissue culture of *S. tonkinensis*. Multiple shoot generation has been studied in nodal explants by using MS medium supplemented with different concentrations of 2-isopentyladenine (2iP), N^6 benzyladenine (BA), kinetin (Kin) or thiadiazuron (TDZ).

Related reports

The stimulatory effect of 2iP on multiple shoot formation and the lesser effect of kinetin reported in this study are in accordance with other related studies. The results also showed that 2iP is the most effective one for shoot elongation of *Sophora* in comparison to other cytokinins tried which is in accordance with such data available for some other medicinal plants. So studies on specific cytokinins which are known to stimulate shoot induction and elongation provide results which vary depending upon plant's species and this might be also the case in *Sophora*.

Innovations and breakthroughs

According to data regarding the micropropagation of *S. tonkinensis*, it is very less to use tissue culture techniques. This study is very novel and is the first one to study the most effective cytokinin and its concentration for the *in vitro* propagation of *S. tonkinensis* in terms of shoot growth and multiplication. The results obtained have clearly shown that 2iP is the optimal plant growth regulator for *Sophora* multiplication.

Applications

Such investigations are of great importance for practice, because cultured cells, tissues and organs of *Sophora* may be used for production of very important medicinal compounds. These standardized protocols in terms of effective cytokinin selection may be useful for further genetic transformation studies in this plant and enable us to enhance the *in vitro* culture, shoot generation of these cells and increased productivity of this plant and the medicinal compounds present in it.

Peer review

This is an excellent study on an important medicinal plant in which the authors have studied and determined the most effective cytokinin and its concentration for the tissue culture and *in vitro* propagation of *S. tonkinensis* in terms of shoot growth and multiplication. The results obtained are interesting and have shown that 2-isopentyladenine (2iP) is the optimal plant growth regulator for *Sophora* multiplication.

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