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Full Length Research Paper

High-frequency shoot regeneration of nodal explants from *Tetrastigma hemsleyanum* Diels et Gilg: A valuable medicinal plant

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This paper describes the shoot regeneration of nodal segments from a medicinal plant, *Tetrastigma hemsleyanum* Diels et Gilg (Vitaceae). The highest number of shoots (7.27 shoots per explant) was observed in MS medium supplemented with 4 mg/l BA after six weeks of inoculation. 2 mg/l BA in combination with 0.1 mg/l NAA not only induced shoot proliferation but also increased shoot length. Well-developed shoots were rooted on half strength MS medium supplemented with 2 mg/l IBA with 100% rooting and 85% of the regenerated plantlets survived before been transferred to field conditions.

Key words: Medicinal plant, nodal explants, shoot regeneration, *Tetrastigma hemsleyanum.*

INTRODUCTION

Tetrastigma hemsleyanum Diels et Gilg (Vitaceae) is a valuable medicinal plant (Editorial Board of Flora of Zhejiang, 1993), native to east, central, south and southwest China. The species is rich in flavanoids (kaempferol, quercetin and kaempferol-3-O-neohespeidoside), sterols (β-sitosterol) and C-glycosylflavones (Liu and Yang, 1999; Liu et al., 2002; Li et al., 2003). As a Chinese folk medicine, the whole plant, including the tubers were reported to possess antipyretic, detoxification, anti-inflammatory, anti-virus and even anti-tumor properties (Liu et al., 2002; Ding et al., 2005; Yang et al., 1989). It is used for the treatment of children fevers, convulsions, pneumonia, asthma, hepatitis, rheumatism, irregular menstruation and sorethroat (State Administration of Traditional Chinese Medicine, 2004).

Raw material of *T. hemsleyanum* is now gotten from the natural populations. Due to over collection and loss of natural habitats in China, wild populations of *T. hemsleyanum* have decreased drastically. Conventional propagation of *T. hemsleyanum* is limited to vegetative means, a difficult and slow process in the production of the commercial quantities required. Hence, large-scale propagation is a prerequisite for meeting future pharmaceutical requirements and to prevent eradication of this highly valuable plant. Except for a preliminary report on plant regeneration via shoot tips and nodal segments (Qian, 2008; Zhong, 2007), no comprehensive *in vitro* studies have been undertaken in *T. hemsleyanum*. Considering the medicinal importance of *T. hemsleyanum*, the present study was undertaken to develop a rapid and efficient *in vitro* multiplication and regeneration system using nodal explants.

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Abbreviations: BA, 6-Benzyladenine; **GA_3,** gibberellic acid; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kn,** kinetin; **NAA,** α-naphthaleneacetic acid; **MS,** Murashige and Skoog medium; **2,4-D,** 2,4-dichlorophenoxyacetic acid.

MATERIALS AND METHODS

Plant material and initiation of *in vitro* shoot cultures

Young *in vivo* shoots with six to eight nodes of *T. hemsleyanum* were collected from wild population in Zhejiang Province, China.

Table 1. Effect of different cytokinins in combination with NAA on shoot regeneration in *T. hemsleyanum*.

Plant growth regulator	Number of shoots per explant			Shoot length (cm)		
	0 mg/l NAA	0.1 mg/l NAA	0.5 mg/l NAA	0 mg/l NAA	0.1 mg/l NAA	0.5 mg/l NAA
0 mg/l BA	1.05±0.05^{e}			1.70±0.13^{ab}		
0.5 mg/l BA	2.26±0.14^{d}	2.25±0.25^{b}	1.40±0.24^{abc}	1.95±0.05^{a}	2.69±0.19^{a}	1.12±0.12^{b}
1 mg/l BA	2.93±0.22^{c}	2.50±0.19^{b}	1.43±0.20^{ab}	1.79±0.09^{a}	1.31±0.09^{cd}	1.17±0.09^{b}
2 mg/l BA	4.10±0.38^{b}	4.00±0.52^{a}	1.67±0.17^{a}	0.95±0.14^{c}	1.25±0.16^{cd}	0.92±0.10^{b}
4 mg/l BA	7.27±0.66^{a}	4.13±0.48^{a}	1.33±0.21^{abc}	0.48±0.09^{d}	1.06±0.15^{d}	0.58±0.05^{c}
0.5 mg/l Kn	1.06±0.06^{e}	1.30±0.15^{c}	1.00±0.0^{c}	1.69±0.17^{ab}	1.88±0.24^{b}	0.97±0.04^{b}
1 mg/l Kn	1.10±0.07^{e}	1.20±0.20^{c}	1.18±0.12^{bc}	1.30±0.14^{bc}	1.62±0.21^{bc}	1.73±0.15^{a}
2 mg/l Kn	1.13±0.13^{e}	1.14±0.14^{c}	1.00±0.0^{c}	1.08±0.12^{c}	1.11±0.06^{cd}	0.62±0.05^{c}
4 mg/l Kn	1.21±0.11^{e}	1.13±0.13^{c}	1.00±0.0^{c}	1.35±0.11^{bc}	1.89±0.18^{b}	0.50±0.04^{c}

Values are given as the mean ± standard error of three experiments; each experiment consisted of 15 to 20 explants. Values within column followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple range test. The data were recorded after six weeks of the culture

Nodal segments (1 to 1.5 cm long) with a single node (without leaf) were excised and washed thoroughly in running tap water for 30 min. The explants were rinsed in 70% ethanol for 1 min, followed by a 15 min surface-sterilization with 0.1% (w/v) mercuric chloride solution containing two to three drops of Tween-20, and subsequently rinsed five times with sterile water and inoculated in glass tubes (3 × 10 cm) containing approximately 15 ml MS (Murashige and Skoog, 1962) basic medium supplemented with 0.5 mg/l BA to promote sprouting of axillary buds.

Adventitious shoot regeneration from axillary bud explants

When axillary shoots reached 2 to 5 cm long from the aseptic nodal segments after culture for six weeks, they were cut into segments about 0.5 cm long and the leaves were removed. The explants were inoculated in a vertical upright position in 235 ml glass jars (7 × 11 cm) with four explants per jar, each containing 50 ml of MS basal medium with BA and Kn at concentrations of 0.5, 1, 2 or 4 mg/l alone or in combination with NAA at concentrations of 0, 0.1 or 0.5 mg/l. After six weeks of culture, the number of shoots per explant and the length of the shoots were recorded to evaluate the cytokinin efficacy on shoot proliferation and growth. A medium without plant growth regulators was used as a control. All culture media were supplemented with 3% (w/v) sucrose and 0.62% (w/v) agar. The pH was adjusted to 5.8 before autoclaving at 121°C (104 kPa) for 20 min. Cultures were incubated under a 16/8 h light/dark photoperiod provided by cool white fluorescent lamps at a light intensity of 36 µmol m^{-2} s^{-1} and the culture room temperature was 25 ± 2°C .

Rooting and acclimatization

The shoots (2 to 3 cm long) were transferred to rooting medium consisting of half-strength MS medium supplemented with 2% sucrose and 0.7% agar. 2,4-D at different concentrations (0.1, 0.5 and 1 mg/l), IAA, NAA and IBA at different concentrations (0.5, 1 and 2 mg/l) were compared for rooting. Data on the percentage of rooting and the mean number of roots were recorded after 4 weeks. The well rooted shoots were washed thoroughly under running tap water and transplanted to pots containing peat and perlite (2:1), hardened in an acclimatization chamber for 2 months, then transferred to field conditions.

Statistical analysis

Each experiment was repeated three times and consisted of 15 to 20 explants each. Data were analyzed by one way analysis of variance (ANOVA). Differences between means were scored using Duncan's multiple range test at P≤0.05 (Duncan, 1955).

RESULTS AND DISCUSSION

Establishment of aseptic plantlets from nodal explants

78% of aseptic nodal explants was obtained within 10 days inoculation on MS basal medium with 0.5 mg/l BA. Axillary buds sprouted from the aseptic nodal explants with 95% and developed into plantlets of 2 to 5 cm in length within six weeks. Nodal explants from those plantlets were subsequently used for the following experiments.

Shoots growth and multiplication

The effects of BA, Kn alone and in combination with NAA on shoot multiplication is presented in Table 1. Nodal explants cultured on plant growth regulator-free MS medium showed reduced shoot formation. Without the addition of NAA, the optimum number of regenerated shoots (7.27) was observed when 4 mg/l BA was added to the medium. There were fewer shoots (1.06 to 1.21) per explant when the medium was supplemented with all concentrations of Kn. The results showed that BA was more effective than Kn in inducing shoot development and multiple shoot induction. With the addition of NAA, BA at all concentrations together with 0.1 mg/l NAA did not show a marked effect on shoot number, but increased shoot length. Both shoot number and shoot length were inhibited when 0.5 mg/l NAA was added in the medium

Table 2. Effect of various concentrations of 2,4-D, IAA, NAA and IBA on root formation from regenerated shoots on half-strength MS medium of *T. hemsleyanum*.

Auxin	Percentage of rooting (%)	Number of roots per shoot
(Control)	57^{d}	1.85±0.20^{g}
0.1 mg/l 2,4-D	93^{ab}	3.88±0.18^{ef}
0.5 mg/l 2,4-D	81^{c}	3.10±0.25^{fg}
1 mg/l 2,4-D	77^{c}	3.00±0.42^{fg}
0.5 mg/l IAA	77^{c}	4.10±0.54^{def}
1 mg/l IAA	97^{ab}	6.32±0.43^{bc}
2 mg/l IAA	100^{a}	6.38±0.49^{bc}
0.5 mg/l NAA	83^{c}	5.52±0.37^{cd}
1 mg/l NAA	90^{b}	4.96±0.40^{cde}
2 mg/l NAA	80^{c}	4.88±0.53^{de}
0.5 mg/l IBA	100^{a}	7.31±0.38^{b}
1 mg/l IBA	100^{a}	8.75±0.31^{a}
2 mg/l IBA	100^{a}	8.91±0.72^{a}

Values are given as the mean ± standard error of three experiments, each experiment consisted of 15 to 20 explants. Values within column followed by the same letter are not significantly different at the 0.05 level by Duncan's multiple range test. The data were recorded after four weeks of culture.

combined with BA. The positive effects of BA on bud proliferation and multiple shoot formation were recently reported for the propagation of other plants, including *Helicteres isora* L. (Shriram et al., 2008), *Trichodesma indicum* (Linn) R.Br. (Verma et al., 2008), and *Spilanthes acmella* Murr. (Singh et al*.,* 2009). Our results also showed that 2 mg/l BA in combination with 0.1 mg/l NAA can not only induce shoot proliferation but also improve shoot length. The results are in agreement with previous observations on *T. hemsleyanum* (Qian, 2008), while Zhong (2007) revealed that 0.2 mg/l NAA was favorable for shoot multiplication in *T. hemsleyanum*. Kn alone or in combination with NAA was less effective in shoot propagation in *T. hemsleyanum*. Similar results were also published for *Justicia gendarussa* (Thomas and Yoichiro, 2006) and *Embelia ribes* (Dhavala and Rathore, 2010). In this study, BA alone induced multiple shoot bud proliferation, but the multiple shoots obtained at the concentration of 4 mg/l of BA failed to elongate on the same medium. High concentrations of BA have often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Brassard et al., 1996; Figueiredo et al., 2001; Purkayastha et al., 2008).

The multiple shoots obtained with higher concentrations of BA elongated when they were transferred to hormone-free medium, lower concentrations of BA or after GA_3 was added (Purkayastha et al., 2008). In this study, all shoots of 0.4 to 0.8 cm in length grew to 2 to 4 cm long within three weeks when transferred to MS medium with 0.5 mg/l of GA_3 or 0.5 mg/l of BA in combination with 0.1 mg/l NAA. The single node excised from the regenerated shoots continued to produce shoots during successive subculture on MS medium supplemented with 2 mg/l BA in combination with 0.1 mg/l NAA. The mother explants could be subcultured four times without affecting their shoot forming potential (data not shown). It is a desirable regeneration system that can be continuously multiplied to produce a regular supply of shoots for field transfer.

Rooting and acclimatization

Regenerated shoots (2 to 3 cm long) were excised and cultured on half-strength MS medium containing different concentrations of 2.4-D, IAA, NAA or IBA. The rooting response to different auxins is shown in Table 2. Root induction occurred in 57% of the cuttings in the control treatment to which no auxin was added to the medium, but the roots were longer, thicker and more robust when IBA or IAA was present. From the four auxins tested, IBA was most effective and the percentage of rooting formation was observed to be 100% at all concentrations. 2 mg/l IBA produced a maximum of 8.91 ± 0.72 roots per shoot within 4 weeks. The roots were induced directly from the shoot base without an intervening callus phase on the medium supplemented with IBA. More shoots produced roots when the IAA concentration was increased, such that 77% of the explants formed roots at 0.5 mg/l as compared to 100% at 2 mg/l IAA. Lower concentration of 2,4-D (0.1 mg/l) produced 93% rooting frequency with a maximum of 3.88 ± 0.18 roots per shoot. The percentage of rooting formation decreased with an

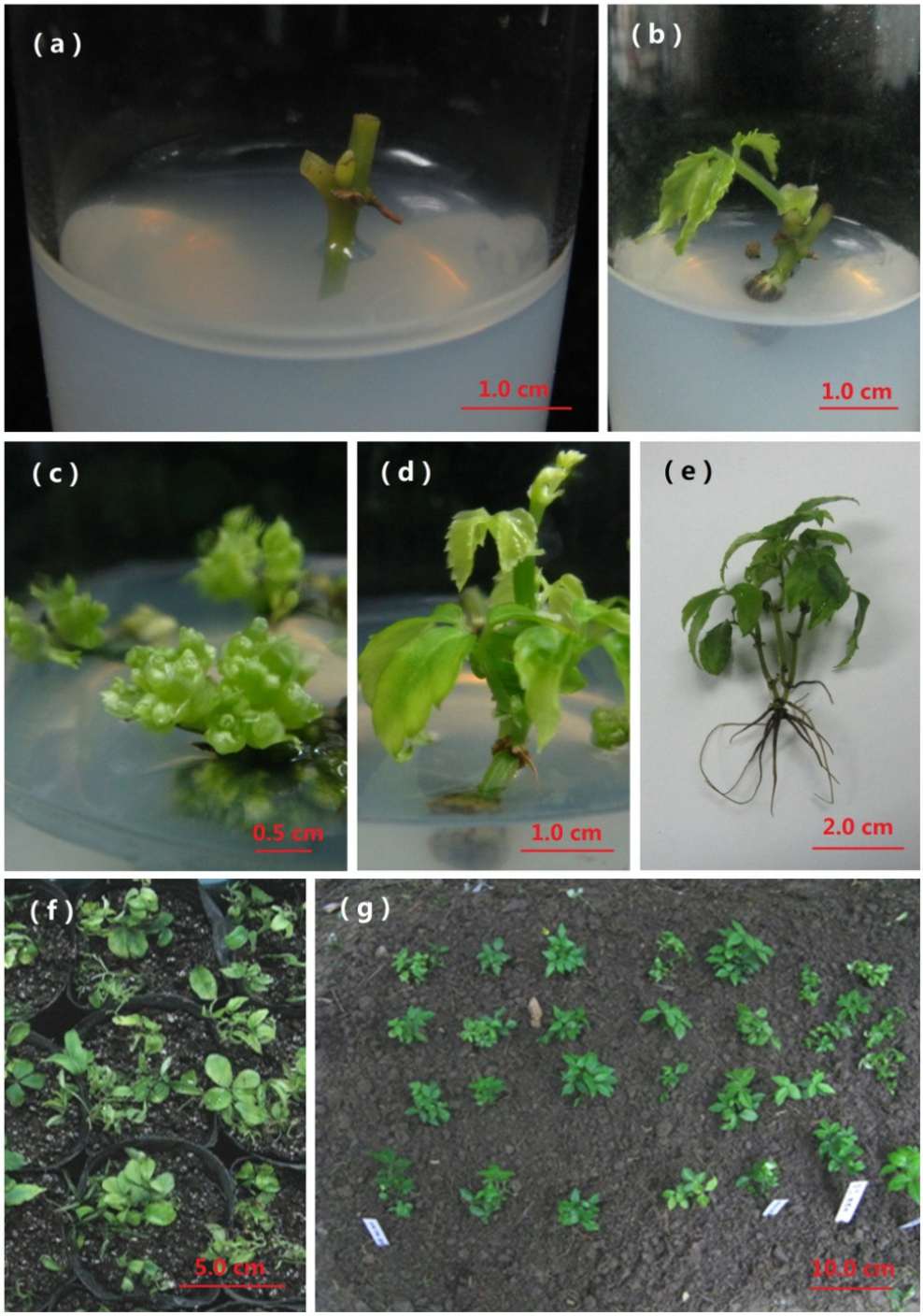

Figure 1. Regeneration of shoots from nodal segment of *T. hemsleyanum*. (a) nodal segment used as explants; (b) axillary buds sprouted and elongated from nodal segment on the induction medium containing 0.5 mg/l BA; (c) multiple shoot induction and proliferation from nodal explants within six weeks of culture on MS supplemented with 4 mg/l BA; (d) elongated shoot developed on MS medium containing 0.5 mg/l GA_3; (e) plantlet with well–developed roots; (f) acclimatized plant growing in pot for one month; (g) plants growing in the field, at the Botanical Garden of Zhejiang University.

increase in 2,4-D concentration. 0.5 to 2 mg/l NAA resulted in 80 to 90% root formation with an average of 4.88 to 5.52 roots per shoot.

The roots were shorter and swollen as compared to those on media containing IBA at the same concentration. IBA was reported to be the most effective auxin for

rooting with some medicinal plants in other studies, such as in *Isodon wightii* (Thirugnanasampandan et al., 2010), *Andrographis paniculata* (Purkayastha et al., 2008), *Phellodendron amurense* Rupr. (Azad et al., 2005), *Mucuna pruriens (L.) D.C.* (Faisal et al., 2006), *Balanites aegyptiaca* L. (Siddique and Anis, 2009) *and Centella asiatica* L. (Mohapatra et al., 2008). Zhong (2007) showed that 0.5 mg/l IBA in combination with 1 mg/l NAA was good for inducing root formation in *T. hemsleyanum.* Qiang (2008) suggested that half-strength MS medium supplemented with 0.2 mg/l NAA is helpful for rooting in *T. hemsleyanum.* In our study, NAA was not so good for rooting, because callus formation was stimulated on the base of the seedlings, which is in agreement with the results in *Vitis vinifera* cv. *Pinot noir* (Heloir et al., 1997) and *Andrographis paniculata* (Purkayastha et al., 2008).

The rooted plantlets (root length at least 2 cm) were transferred to pots with a 2:1 mixture of peat and perlite and hardened in an acclimatization chamber (Figure 1f). Plantlets were watered 3 or 4 times every day. After 2 months, approximately 85% of the regenerated plants survived. The surviving plants were then transferred to field conditions (Figure 1g).

Conclusion

This study established a protocol for direct organogenesis in *T. hemsleyanum* through nodal segments of field-grown plants. The use of shoot nodal explants ensures clonal multiplication of selected species and helps to maintain good traits, which is important for the quality of medicines. Using this regeneration method, we can quickly obtain a large number of plantlets for medicinal use. This method is beneficial for the protection of wild resources.

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