EFFECTSOF NITROGEN SOURCEAND WAVELENGTH OF LED-LIGHT ON MICROPROPAGATION OF *LYSIONOTUS PAUCIFLORUS* MAXIM.

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Abstract

Effect of nitrogen sources on *in vitro* culture of *Lysionotuspauciflorus*Maxim. was investigated using 4 kinds of media each with varied nitrogen composition and concentration based on Murashige and Skoog (MS) medium; MS (1650 mg l^{-1} NH_4NO_3 + 1900 mg l^{-1} KNO_3), MS3B (825 mg l^{-1} NH_4NO_3 + 995 mg l^{-1} KNO_3), MS4 (1900 mg l^{-1} KNO_3), and MS5 (1900 mg l^{-1} KNO_3 + 1751 mg l^{-1} $NaNO_3$). When leaves were cultured on these media each containing 1 mg l^{-1} 6-benzylaminopurine (BAP) and 0.5 mg l^{-1} α-naphthaleneacetic acid (NAA), shoot regeneration frequencies of MS, MS3B, MS4 and MS5 were 66.7, 53.3, 33.3 and 0%, respectively, and average numbers of shoots per explant were 8.9, 5.0, 0.9 and 0, respectively. On the other hand, when shoot apices were cultured on MS, MS3B, and MS4 without supplementation of any plant growth regulator,they elongated to give node numbers of 4.4 – 4.6, whereas MS5 gave less growth with node number of 2.4. Consequently, averages of shoot length were 23.6, 28.7, 27.8, and 4.7 mm in MS, MS3B, MS4, and MS5, respectively. Effect of light quality on *in vitro* culture of leaf explants was also examined on MS

containing 0.5 mg l^{-1} NAA in combination with or without 1 mg l^{-1} BAP by incubating at 25±2°C under continuous light from light emitting diodes (LEDs) with peak wavelengths of 470 nm (blue), 590 nm (orange), 625 nm (red), and from white LEDs at 40 µmol m^{-2} s^{-1} or darkness.Shoots were regenerated from all leaf explants cultured under LED-light, whereas shoot regeneration was completely inhibited under the dark condition. The highest number of shoots regenerated was 30.4 when cultured on medium supplemented with 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA under red LED-light. The highest frequency of long shoot (> 5 mm) formation was also obtained under red LED-light. Under orange LED-light, shoot differentiation was suppressed as compared with that under the other peak-wavelength LED-light.

Keywords: Gesneriaceae, lighting, root regeneration, shoot regeneration.

Running title: Effect of nitrogen and lighting on propagation of *Lysionotus*.

INTRODUCTION

Lysionotus pauciflorus has a high ornamental value such as a beautiful attractive flower.Moreover, it hasattracted high attention as amedicinal plant, which has been usedfor lymph node tuberculosis, cough with tachypnoea and rheumatic pains (Liu et al., 1996; 1998) by Yi minority ofYunnanProvince in Chinarather thanas an ornamental plant. Recently over collection from the natural habitat for using as a medicine these is becoming a problem. Therefore, it is necessary to produce large amount of nursery plants through *in vitro* culture to ~~fulfil~~fulfill the demand in China. However, micropropagation with tissue culture technique has only been reported on *Lysionotuspauciflorus*Maxim. native to Japan (Haruki and Inamura, 2003) and Yunnan Province, China (Lu et al., 2006; Godo et al., 2010).

For improving productivity of nursery plant by tissue culture technique, many researchers have studied on culture conditions such as kind of basal medium, combination and concentration of plant growth regulators, kind and concentration of sugars, culture temperature and illumination. Nitrogen is one of the main elements contributing to the growth of plant. The form of nitrogen, as NH_4^+ or NO_3^-, has a dramatic

influence on the morphogenic response of plant tissue culture (Bhojwani and Razdan, 1996). Halperin and Wetherell (1965) reported that wild carrot calli induced on a medium with KNO_3 as the sole source of nitrogen faild to form somatic embryo. Hassan et al. (1991) described that little or no shoots regenerated from leaf explants of pear on media lacking NH_4^+. In contrast, Ivanova and Van Staden (2009) described negative effects of NH_4^+on regeneration and growth of new shoots of *Aloe polyphylla*.

Light isalso an important environmental factor affecting plant growth, thus the influence of light on*in vitro*plant growth has been investigated intensively. Since the light emitting diode (LED) has recently showed drastic development, it has become possible to evaluate the influence of light quality on plant growth by using several types of LEDs each with a different peak wavelength. However, much of the research onthe wavelengthof lighthas beencarried outso far by using onlyred andblue LEDs (Poudel et al. 2008, Baque et al. 2010; 2011, Lian et al. 2002, Moreira da Silva et al. 1997, Shin et al. 2008).

This report describes the effects of nitrogen source and wavelength of LED-light on tissue cultures for micropropagation of *L. pauciflorus* native to Yunnan Province, China.Establishment of the micropropagation method of high productivity is not only useful for production of a medicinal plant but useful for conservation of the species by reducing the collection from wild. Furthermore, if light environment effective in mass propagation can be found out, it will also become possible to produce a medicinal plant in a plant factory.

MATERIALS AND METHODS

Plant materials

In vitro plants of*Lysionotuspauciflorus* (Gesneriaceae), native to Yunnan Province, China, were used in this study.They were maintained in glass tubes with 2g/l gellan gum (Phytagel; Sigma Chemical Co., St. Louis, USA)-solidified 1/2 MS (Murashige and Skoog, 1962) medium (half strength MS micro- and macroelements, full strength MS organic constituents, 20 g/l sucrose) without supplementation of any plant growth regulators (PGRs). These cultures were incubated at 25±2ºC under 16 h fluorescent lighting at 40 µmol m^{-2} s^{-1}.

Kommentar [I1]: I would be very careful with this statement, because in vitro conditions with heterotrophic growth are hardly to compare with photoautotrophic conditions.

Kommentar [P2]: If this is standard in your media it is sufficient to notice it ones here and not later on.

Culture medium for nitrogen source experiment

Four kinds of media, MS, MS3B, MS4 and MS5, all of which were purchased fromDuchefaBiochemie B.V., Netherlands, were used as basal media. MS was normal composition of Murashige and Skoog (1962) medium. MS3B, MS4, and MS5 were modified MS mediawith different combination and concentration of three nitrogen sources, NH_4NO_3, KNO_3, and $NaNO_3$ (Table 1). The pH of all media used in this study was adjusted to 5.8 before autoclaving at 121ºC for 15 min.

Leaf explant culture for nitrogen source experiment

Leaves excised from *in vitro* plants were individually placed onto 10 ml of 2 g/l gellan gum-solidified MS, MS3B, MS4, and MS5 mediaeach supplemented withor without 1mg l^{-1} 6-benzylaminopurine (BAP) and 0.5 mg l^{-1}α-naphthaleneacetic acid (NAA) in each glass tube (20 × 140 mm) covered with polypropylene cap. Totally 15 leaf explants were prepared for each medium, and each explant was inoculated into each glass tube. The cultures were incubated at 25±2ºC under 16 hphotoperiodwithwhite fluorescent lamps at 40 µmol m^{-2} s^{-1}. Frequency of shoot regeneration, number of regenerated shoots, and frequency of root regeneration from leaf explants were counted after one month of culture.

Shoot tip culture for nitrogen source experiment

Shoot tipswith two nodes were excised from *in vitro* plants,and six or seven shoot tips were placed on 100 ml of 2 g/l gellan gum-solidifiedand PGR-free MS, MS3B, MS4, and MS5 media without any PGRs in a 450 ml glass bottle. Totally more than 40 shoot tips were inoculated in each medium. These glass bottles were covered with a methylpentene cap and cultured at 25±2ºC under 16 h photoperiod withfluorescent lamps at 40 µmol m^{-2} s^{-1}.Node number and shoot length were measured after 10 weeks of culture.

Leaf explant culture for LED lighting experiment

Two kinds of MS medium supplemented with (1) 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, and (2) 1 mg l^{-1} BAP alone, were used in the LED lighting experiment. The pH of all media was adjusted to 5.8, and added with2g/l

Kommentar [I3]: The molar concentration of NO3 and NH4 should be calculated maybe the affects the discussion. And also different K concentrations are to consider. ... [1]

Kommentar [I4]: Solidified with?

Kommentar [I5]: Leaf or leaf explants? Which size? Describe the explants. ... [2]

Kommentar [I6]: Bring this part to the paragraph above ... [3]

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Kommentar [I7]: One?

Kommentar [I8]: Type, brand?

Kommentar [I9]: Why after one month and not after two months as in LED experiment? ... [4]

Kommentar [I10]: Length?

Kommentar [I11]: From your experiment or from the stock plants? ... [5]

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gellan gum for solidification. After autoclaving at 121ºC for 15 min., fifty ml of the media was placed in each Petri dish (9 cm diameter). Leaves excised from *in vitro* plants were cut perpendicularly into two segments at the midrib, and ten for lighting condition and twenty for dark condition segments were cultured under light and dark conditions, respectively for each medium.

After inoculating the explants as described above,Petri dishes were sealed with Parafilm™ (American Can Company, Chicago, IL, USA) and incubated at 25±2°C under continuous light of 40 µmol m^{-2} s^{-1} from LEDs with peak wavelengths of 470 nm (blue), 590 nm (orange), 625 nm (red), and from white (sharp peak at 460 nm with a broad peak at 560 nm) LEDs or darkness (Fig. 1). Frequency of explants with shoot regeneration, number of regenerated shoots, and frequency of explants with root regeneration werecountedafter 2 months of culture.

Statistical analysis

Data were analyzed using ANOVA and correlation analysis was performed using software (Statview; Abacus Concepts, Inc., Berkeley, CA, USA). Fisher's PLSD was used to compare means.

RESULTS AND DISCUSSION

Effect of nitrogen source on leaf culture

Shoot regeneration from leaf explants was observed within 2 weeks after initiation of culture.The frequencies of explants with shoot regeneration on PGR-free media of MS, MS3B MS4, and MS5, were 13.3, 6.7, 0, and 0%, respectively,and the averages of shoot numberper explant were1, 0.9, 0 and 0, respectively (Table 1). In contrast, on mediacontaining 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, the frequencies of shoot regeneration of MS, MS3B, MS4, and MS5 were 66.7, 53.3, 33.0 and 0%, respectively, and the averages of shoot numberper explant were8.9, 5.0, 0.9 and 0, respectively (Table 1). These results suggest that the mostsuitablebasal medium forshoot regeneration from leaf explants of *L. pauciflorus*was MS with standard combination and concentration of nitrogen source (1650 mg l^{-1} NH_4NO_3 + 1900 mg l^{-1} KNO_3),regardless of existence ofPGRs(Table 1). Tsai and Saunders (1999) also described that the mixed nitrogen source of MS

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Kommentar [I12]: Why ten and twenty?

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was superior or equal to any single nitrogen source for shoot and leaf disk cultures of sugar beet.

The number and length of shoots regenerated from cotyledonary explants of mungbean were reduced by high concentration ofNH_4^+ (Gulati and Jaiwal 1994). Ivanova and Van Staden (2009) also described that NH_4^+used as a sole source of nitrogen appeared to have a negative effect on regeneration and growth of shoots of *Aloe polyphylla*.In the present study, however, both the regeneration frequency and the number of shootsproduced on NH_4^+-lacking media (MS4 and MS5) were low compare to those on NH_4^+-containing media (MS and MS3B) (Table 1). Thus, NH_4^+ might bean important element for shoot regeneration from leaf explants of *L. pauciflorus*.Essential role ofNH_4^+on shoot regenerationhas also been reported inapple (Fasolo et al. 1989; Predieri et al. 1989), garlic (Nagakubo et al. 1993), and pear (Hassan et al. 1991) although the responses of tissue culture to NH_4^+ varied from species to species.

Root formation was observed on all media without applying any PGRs, but was inhibited on mediumcontaining 1 mg l^{-1} BA and 0.5 mg l^{-1} NAA (Table 1). MS4, which included only KNO_3 as a nitrogen source,showed a high root formation frequency of 53.3%, and the frequencies of the other media, containingNH_4NO_3 or $NaNO_3$, were low. These results suggestthe negative effect of NH_4^+ and Na^+ on root formation of *L. pauciflorus*.

Effect of nitrogen source on shoot apex culture

Shoots cultured on MS, MS3B, and MS4 elongated to give node numbers of 4.4–4.6, whereas MS5 gave less growth with node number of 2.4 after one month of culture (Table 2, Fig. 2). The averages of shoot length were 23.6, 28.7, 27.8 and 4.7 mm in MS, MS3B, MS4, and MS5, respectively (Table 2, Fig. 2). These results indicate that $NH_4^+$is not indispensable forshoot growthof *L. pauciflorus* (Table 2) although it isan importantelement for shoot regeneration inleaf explants culture in this study (Table 1).The growth of shoots on MS5 was strongly inhibitedlike as the result of leaf explants culture. Since $NaNO_3$was only used in MS5, it is possible that Na^+strongly inhibited shoot growth of *L. pauciflorus*. Although the difference inadventitious root formation from leaf explants was observed amongthe culture media(Table 1), shootsexcised from leaf explants rooted easily on all mediaand developedenough root system, irrespective of

Kommentar [I15]: Here you did not measure shoot growth

Kommentar [I16]: Describe the size and quality of these adventitious shoot. Why you did not use the shoot from apical shoot tips for rooting?

157 the kind of nitrogen source (Fig. 2). In *Aloe polyphylla*, shoots of good quality, high multiplication rate, and
158 low hyperhydricity were produced on medium including NO_3^- as the sole nitrogen source (Ivanova and Van
159 Staden, 2009). In the present study, however,MS and MS3B media showed good performance for shoot
160 growth, regardless of the involvement ofNH_4^+asa nitrogen source (Table 2).
161
162 ***Effect of wavelength of LED-light on leaf culture***
163 Shoots were regenerated from all leaf explants cultured under lighting from all types of LED, but shoot
164 regeneration wascompletely inhibited under the dark condition (Table 3, Fig. 3). The negative effect of
165 darkness on shoot regeneration was also reported in lettuce (Hunter and Burritt 2004) and
166 *Cattleya*(Cybularz-Urban et al. 2007). More than 20 shoots were regenerated per leaf explant under white
167 (460, 560 nm), blue (470 nm), and red (625 nm) LED-light, whereasshootregenerationwas suppressed under
168 orange (590nm)-LED light (Table 3).The highest number of regenerated shoots was obtained, when leaf
169 explants were cultured on medium supplemented with 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA under red LED-light.
170 Similarly, the highest number of shoot per explant was also obtained under red light in two cultivars of lettuce
171 (Hunter and Burritt 2004) and*Cattleya*hybrid(Cybularz-Urban et al. 2007),while significant difference was
172 not recognized between red and white LED-light in the other two cultivars of lettuce (Hunter and Burritt
173 2004). The number of long shoot (> 5 mm) was also highunder red LED-light, although these shoots showed
174 abnormal morphology with spindly growth (Table 3, Fig. 3). ~~Superiority~~Superiority of red LED-light
175 overwhite or blue LED-light for shootlength was also reported in*Cattleya* hybrid (Cybularz-Urban et al.
176 2007), *Calanthe* hybrids (Baque et al. 2011), chrysanthemum (Kim et al. 2004), and grape (Poudel et al.
177 2008). Elongated shoots were easily rooted *in vitro* and convenient for micropropagation, andabnormal
178 morphologywith shoot elongation turns tonormalby movingtounder fluorescent (data not shown).
179 Consequently, red LED is a useful lighting source for micropropagation of *L.pauciflorus*.
180 Root regeneration was not observed in all the explants cultured on medium supplemented with BAP and
181 NAA, but occurred in all explants cultured on medium supplemented with only NAAunder light condition
182 irrespective of wavelength of LED-light (Table 3, Fig. 3). In the culture under darkness, roots were

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regenerated from all explantson both the media (Table 3), butthose regenerated on medium supplemented with BAP and NAAwere short (Table 3).

The results of the present suggestthat NH_4^+has a stimulative effect on shoot regeneration inleaf explant culture, but thatNH_4^+ and Na^+act inhibitorily for root formation of *L. pauciflorus*.Red LED is a useful light source for micropropagation of *L.pauciflorus*, whereas shoots regeneration wascompletely inhibited under the dark condition. In conclusion, it is important to choose the optimal nitrogen source and light condition in addition to the concentration of PGRs,andculture temperature for the micropropagation of *L. pauciflorus*.

Kommentar [I18]: Conclusions?

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Table 1 Effects of type and concentration of nitrogen sources and plant growth regulators on regeneration of shoot and root from leaf explants of *Lysionotuspauciflorus*.

medium	NH_4NO_3 (mg l^{-1})	KNO_3 (mg l^{-1})	$NaNO_3$ (mg l^{-1})	BAP (mg l^{-1})	NAA (mg l^{-1})	% of explants with shoots	No. of shoots per explant	% of explants with roots
MS	1650	1900	-	-	-	13.3	1.0±1.6c	6.7
				1	0.5	66.7	8.9±9.9a	0
MS3B	825	950	-	-	-	6.7	0.9±1.2c	13.3
				1	0.5	53.3	5.0±4.3b	0
MS4	-	1900	-	-	-	0	0c	53.3
				1	0.5	33.3	0.9±1.8c	0
MS5	-	1900	1751	-	-	0	0c	13.3
				1	0.5	0	0c	0

Means in the column followed by the same letter are not significantly different at $p < 0.05$.

Kommentar [I20]: In 16h light and after one month

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Kommentar [I21]: I wonder that this is different from 8.9 because of the large standard deviation.

Table 2 Effects of type and concentration of nitrogen sources on shoot growth of *Lysionotuspauciflorus*

medium*	NH_4NO_3 (mg l^{-1})	KNO_3 (mg l^{-1})	$NaNO_3$ (mg l^{-1})	No. of node	Length of shoot (mm)
MS	1650	1900	0	4.6±0.7a	23.6±7.5b
MS3B	825	950	0	4.6±1.0a	28.7±8.7a
MS4	0	1900	0	4.4±0.8a	27.8±4.4a
MS5	0	1900	1751	2.4±0.6b	4.7±1.0c

Means in the column followed by the same letter are not significantly different at $p < 0.05$.

Kommentar [I22]: Of shoot tips?

After .. month? I light?

Table 3 Effect of wavelength of LED-light on shoot and root regeneration from leaf explants of *Lysionotuspauciflorus*.

LED	Peak wavelength (nm)	1 mg l^{-1} BAP+0.5 mg l^{-1} NAA				0.5 mg l^{-1} NAA			
		% of explants with shoot regeneration	No. of shoots per explant	No. of long (>5 mm) shoots	% of explants with root regeneration	% of explants with shoot regeneration	No. of shoots per explant	No. of long (>5 mm) shoots	% of explants with root regeneration
White	(460, 560)	100	20.4±7.2b	3.6±1.4b	0	100	23.1±6.0a	4.6±2.1b	100
Blue	470	100	23.6±8.7ab	3.4±2.8b	0	100	25.6±8.8a	0.8±1.1c	100
Orange	590	100	17.2±8.6b	1.7±1.2bc	0	100	11.6±3.4b	3.3±1.9b	100
Red	625	100	30.4±7.5a	8.1±3.0a	0	100	23.9±6.4a	7.2±3.9a	100
Dark		40	5.4±3.8c	0.7±c	100	5	0.1±0.2c	0±c	100

Different letters among the ~~mearns~~means in the same column indicate significant differences at the 0.05 level.

Kommentar [I23]: After 2 months

Legend to figures

Fig. 1 Spectral profiles of white, blue, orange and red light emitting diodes at 40 μmol m^{-2} s^{-1}.

Fig. 2 Plantlets of *Lysionotuspauciflorus* on four kinds of media containing different kind and concentration of nitrogen source without plant growth regulatorsten weeks after culture of shoot tipswith two nodes were excised from *in vitro* plants cultured on 1/2 MS medium without supplementation of any plant growth regulators. A: MS medium, B: MS3B medium, C: MS4 medium, D: MS5 medium. The bar indicates 2 cm.

Fig. 3 Effect of wavelength of LED-light on shoot and root regeneration from leaf explants of *Lysionotuspauciflorus* after 2 months of culture. The bar indicates 1 cm.

Seite 4: [1] Kommentar [I3]	Ina	03.11.2013 13:44:00

The molar concentration of NO3 and NH4 should be calculated maybe the affects the discussion. And also different K concentrations are to consider.

Seite 4: [2] Kommentar [I5]	Ina	03.11.2013 13:17:00

Leaf or leaf explants? Which size? Describe the explants.

Seite 4: [3] Kommentar [I6]	Ina	03.11.2013 13:11:00

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Seite 4: [4] Kommentar [I9]	Ina	03.11.2013 13:18:00

Why after one month and not after two months as in LED experiment?

Seite 4: [5] Kommentar [I11]	Ina	03.11.2013 13:14:00

From your experiment or from the stock plants?