In vitro bulblet induction from nodules of *Charybdis numidica*

Anupan Kongbangkerd*[1], Christoph Wawrosch[2] and Brigitte Kopp[2]

[1]Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science Naresuan University, Muang, Phitsanulok, 65000 Thailand
[2]Institute of Pharmacognosy, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria
*Corresponding author. E-mail: anupank@nu.ac.th

ABSTRACT

The *in vitro* bulblet induction from meristematic nodules of *Charybdis numidica* on semisolid media was studied. Nodules induced from leaf segments in liquid MS Murashige and Skoog, (1962) medium with 20 µM 6-Benzyladenine (BAP) under dark conditions. Nodules were cultured on semisolid medium with different concentrations of α-naphthaleneacetic acid (NAA, 0-5 µM) under 16 hour photoperiod. Bulblet induction was observed in all media after 10 weeks of culture. The highest total number of bulblets was induced on the medium without NAA and significantly decreased with an increasing concentration of NAA. The number of bulblets formed in different size classes in all of the media was no significantly different whereas the number of rooted bulblets was significantly increased when cultured on the medium with 0.5 µM NAA. Rooted bulblets could be directly transplanted to soil and unrooted bulblets could either produce vigorous roots on basal solid MS medium without growth regulators or directly produced roots in the soil. All rooted bulblets were successfully acclimatized in the greenhouse with high survival rate.

Keywords: Squill, bulblet, micropropagation, meristematic nodules

INTRODUCTION

Charybdis, a genus of Hyacinthaceae, is a perennial, bulbous plant contains large quantities of the cardiac glycosides scilliroside and scillaren A which have been used since ancient times as a rodenticide (Verbiscar *et al*, 1986). The recent results also revealed that the glycoside proscillaridin A showed strong immunoregulatory activities (Terness *et al.*, 2001). Since natural vegetative propagation does not make up a sufficient number of bulbs collected every season, the conventional cultivation of *Charybdis* using bulb sections was performed. However, the vegetative propagation by bulb offsets is very slow (Verbiscar *et al*, 1986). Nowaday, *in vitro* propagation seems to be the alternative way to support the production of crude drug. Micropropagation of *Charybdis* by mean of tissue culture has been improved for more rapid shoot multiplication (Stojakowska, 1993) and bulblet induction (El Grari and Backhaus, 1987). Large numbers of adventitious shoot formation through nodule culture has also been reported (Wawrosch *et al*., 2001). However, the final product to be transplanted to the field should be the bulbs since they are easier to handle and the survival rate after transplantation is much better when transferred to soil than rooted shoots. In other bulbous species, bulblet induction *in vitro* was influenced by many factors, including light, temperature, culture medium and plant growth regulators. Several papers have been reported about the effect of NAA on bulblet induction. The

objective of our work was to determine the effect of NAA on bulblet induction from nodules of *Charybdis numidica* of selected clone UN26.

MATERIALS & METHODS

Meristematic nodules of the selected clone UN26 (*Charybdis numidica*) induced from leaf cutting of stock plants *in vitro* were used for the experiment (Fig. 1A). Nodules were multiplied in liquid MS basal medium (Murashige and Skoog, 1962) with 3% sucrose and 20 µM BAP in the dark. For the experiment, nodules (with an average 0.4 g of initial fresh weight per nodule) were cultured on semisolid MS medium supplemented with 0.8% agar, 3% sucrose and various concentrations of NAA (0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 µM). Forty milliliters of medium were filled in baby food jars and closed with Magenta B caps. The pH of the media was adjusted to 5.7 prior to autoclaving at 121^{o}C for 20 min. The cultures were incubated at 25±1^{o}C under 16 hour photoperiod with a total irradiance of 60 µmoles.m^{-2}s^{-1} (OSRAM BIOLUX tubes) and 60% relative humidity. Experiments were conducted using 10 replicates per treatments and each replicate consisted of 4 nodules. The experiment was repeated. Number of bulblets per nodule, mean fresh weight of bulblet, mean number of bulblets in different size classes (diameter) and external morphology of bulblet derived from nodules were observed and recorded after 10 weeks of culture.

RESULTS & DISCUSSION

A method of direct *in vitro* bulblet induction from *Charybdis* nodules has been studied. Nodules when transferred to culture on semisolid MS medium either with or without NAA produced various sizes of both rooted and unrooted bulblets (Figure 1(A-B)). The highest total numbers of bulblets per nodule (14.3) was obtained when cultured on semisolid MS medium without growth regulators (Table 1). The total number of bulblets per nodule significantly reduced when NAA was added. The total number of bulblets slightly decreased with an increasing NAA concentration. Rooted bulblets could be produced in all media. Highest number of rooted bulblets per nodule was obtained when 0.25 µM NAA was added to the medium. Most bulblets initiated in all media were small (<2 mm) size in diameter. The highest number of bulblets formed in small (10.6) and medium size (3.1) was found on medium without NAA while the highest number of large size (>5 mm) of bulblets (0.9) was found on medium with 0.5 µM NAA (Table 1). However, the number of large bulblets showed no significantly different in all media. The mean number of bulblets of small size (<2 mm) formed in all media was higher than the medium (2-5 mm) and large size (>5 mm).

The growth of bulblets as measured by the fresh weight was also effected by NAA. The mean fresh weight of both medium (2-5 mm) and large (>5 mm) size of unrooted bulblets showed no significantly different in all media. In contrast, mean fresh weight of both medium (2-5 mm) and large (>5 mm) size of rooted bulblets slightly increased when NAA was added into the medium (Table 2). Rooted bulblets seemed to promote higher fresh weight than unrooted bulblets in all media. The highest mean fresh weight of unrooted bulblets of medium size (2-5 mm in diameter) was found on media with 5.0 µM NAA (42.6 mg) whereas the highest mean fresh

weight of rooted bulblets was observed on media with 2.5 µM NAA (65.7 mg). The highest mean fresh weight of both rooted and unrooted bulblets of large size (>5 mm in diameter) was obtained on media with 1.0 µM NAA (173.1 and 81.5 mg respectively). Root initiation was observed in bulblets induced in all media after 4 weeks of culture. These induced roots were creamy, thick, smooth and vigorous. The mean root length measuring after 10 weeks was up to 5 cm with 2-3 numbers of roots per bulblet. Rooted bulblets which are small (<2 mm), medium (2-5 mm) and large (>5 mm) size in diameter could be directly transplanted into soil with high survival rate (Figure 2(A-B)).

Table 1 Regeneration of bulblets from nodules of *Charybdis numidica* cultured on solid MS media with various concentrations of NAA after 10 weeks.

NAA (µM)	Total no. bulblets per nodule[a]	No. rooted bulblets per nodule	Mean no. bulblets formed in different size[a] (diameter, mm)		
			<2 mm	2-5 mm	>5 mm
0[b]	14.3±0.8d[c]	2.91±0.29a[c]	10.6±0.8d[c]	3.1±0.3d[c]	0.6±0.1ab[c]
0.05	10.9±0.6c	2.39±0.22a	8.0±0.5c	2.4±0.2abc	0.5±0.1ab
0.1	10.1±0.7bc	2.69±0.28a	7.3±0.6bc	2.3±0.2abc	0.5±0.1ab
0.25	11.4±0.7c	4.58±0.39b	8.2±0.6c	2.9±0.2cd	0.4±0.1a
0.5	9.4±0.4abc	2.96±0.25a	5.9±0.4ab	2.6±0.2abcd	0.9±0.1b
1.0	9.6±0.9abc	3.33±0.39a	6.0±0.7ab	2.9±0.3bcd	0.7±0.1b
2.5	7.6±0.5a	2.83±0.33a	4.8±0.4a	2.2±0.2ab	0.6±0.1ab
5.0	8.8±0.6ab	2.95±0.36a	6.0±0.5ab	2.1±0.2a	0.8±0.1b

[a]Values are mean±standard error of two independent experiments
[b]Control = MS medium without any hormone
[c]Means in each column followed by different letters are significantly different at 5% level (Duncan's multiple range test)

Table 2 Mean fresh weight (mg) of bulblets regenerated from nodules of *Charybdis numidica* on solid MS media with various concentrations of NAA.

NAA (µM)	Mean fresh weight of bulblets formed in different size[a] (mg)			
	2-5 mm		>5 mm	
	Unrooted	Rooted	Unrooted	Rooted
0[b]	35.9±3.4ab[c]	44.7±4.1a[c]	53.3±10.3a[c]	80.4±12.6a[c]
0.05	39.1±2.0ab	50.8±3.6ab	49.8±7.9a	92.4±14.9ab
0.1	31.0±3.0a	57.4±4.1abc	52.5±7.5a	116.1±21.1ab
0.25	38.7±1.7ab	64.7±3.5c	51.0±7.7a	97.3±17.0ab
0.5	41.4±3.2b	65.4±2.9c	79.0±12.1a	133.4±14.9abc
1.0	41.0±3.4b	62.5±4.9bc	81.5±11.5a	173.1±22.2c
2.5	37.6±3.4ab	65.7±5.4c	81.1±10.5a	118.0±18.9ab
5.0	42.6±2.3b	58.0±5.7bc	78.0±10a	141.8±19.2bc

[a]Values are mean±standard error of two independent experiments
[b]Control = MS medium without any hormone
[c]Means in each column followed by different letters are significantly different at 5% level (Duncan's multiple range test)

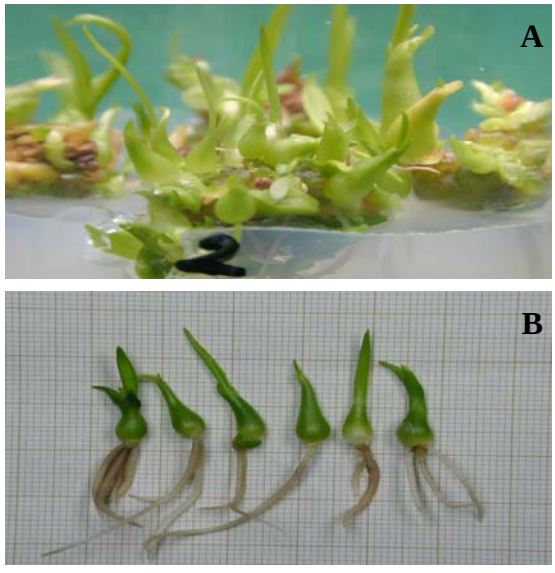

Figure 1(A-B) *In vitro* direct bulblet induction from nodules of *Charybdis numidica* of clone UN26 on solid MS medium. Nodules used as starting explants (A). Bulblets induced on MS medium supplemented with 0.5 μM NAA (B).

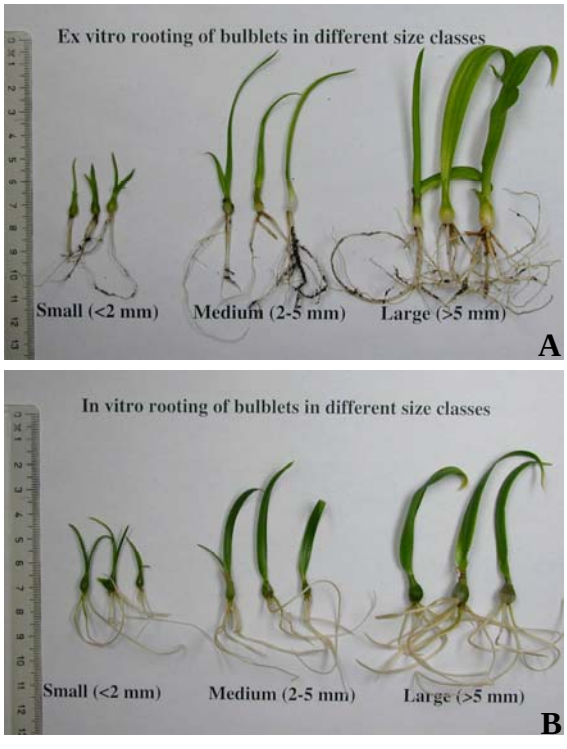

Figure 2(A-B) *In vitro* direct bulblet induction from nodules of *Charybdis numidica* of clone UN26 on solid MS medium. Rooted bulblets derived from nodule and unrooted bulblets could produce roots under *ex vitro* (A) and *in vitro* conditions (B).

Unrooted bulblets were either subcultured onto semisolid MS medium without hormone for *in vitro* rooting or directly transplanted into soil for *ex vitro* rooting and rooted bulblets were directly transplanted into soil. Unrooted bulblets cultured on semisolid MS medium without growth regulators could produce vigorous roots which could be transferred to grow in greenhouse without after 8 weeks of culture.

In vitro bulblet formation has been observed in growth regulator-free media or when transferring to media containing lower levels of NAA in a number of bulb crops (Sutter, 1986, Chang *et al*., 2000 and Peak and Murthy, 2002). Lower number of well shaped bulblets of *Charybdis maritima* induced from bulb scales on medium supplemented with NAA alone has also been presented (El Grari and Backhaus, 1987). However, bulblet formation from bulb-scale segments required two steps for bulblet induction and root formation, respectively. Our present study showed that the one step of bulblet induction from nodules obtained a higher number than that from bulb scales as previously reported by El Grari and Backhaus (1987). Due to of its high regeneration potential, nodule culture has now been proved to be efficient for mass propagation in several plants. Allacher (1999) found that the outer surface of *Charybdis* nodules contained a large number of meristematic cells resulted in a high shoot multiplication rate. Thus, it might be of interest to produce a large number of bulblets from nodules. Bulblets induced from nodules could produce roots and directly transplanted into soil. Rooted bulblets could grow well and unrooted bulblets could also produce adventitious roots both *in vitro* and *ex vitro* rooting with a satisfied survival rate when transplanting to soil directly. To our knowledge, this is the first report of successful bulblet induction from nodules of *Charybdis numidica*.

ACKNOWLEDGEMENTS

The authors are thankful to the Austrian Academic Exchange Service for the financial support provides through a scholarship for Anupan Kongbangkerd.

REFERENCES

Allacher, P. (1999).Einsatz der Biotechnologie zur Massenvermehrung von *Urginea maritima*agg. Sowie Überprüfungen der Bufadienolidsynthese und des Chromosomenstatus der Kulturen. Thesis Univ. Wien.

Chang, C., Chen, C.T., Tsai, Y.C. and Chang, W.C. (2000). A tissue culture protocol for propagation of a rare plant, *Lilium speciosum* Thunb. var. *gloriosoid es* Baker. *Botanical Bulletin of Academic Sinica*, *41*, 139-142.

El Grari, R. and Backhaus, R.A. (1987). In vitro propagation of red squill, *Urginea maritima* Baker. *Plant Cell Tissue and Organ Culture*, *10*, 65-71.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, *15*, 473-497.

Paek, K.Y. and Murthy, H.N. (2002). High frequency of bulblet regeneration from bulb scale sections of *Fritillaria thunbergii*. *Plant Cell Tissue and Organ Culture*, *68*, 247-252.

Stojakowska, A. (1993). Micropropagation of *Urginea maritima* (L.) Baker s. str. *Acta Societika and Botanica Polonica*, *62*, 11-15.

Sutter, E.G. (1986). Micropropagation of *Ixia viridifolia* and a *Gladiolus x Homoglossum* hybrid. *Scientia Horticulturae*, *29*, 181-189.

Terness, P., Navolan, D., Dufter, Ch., Kopp, B. and Opelz, G. (2001). The T-cell suppressive effect of bufadienolides: structural requirements for their immunoregulatory activity. *International Immunopharmacology*, *1*, 119-134.

Verbiscar, A.J., Atel, J., Banigen, T.F. and Opelz, G. (1986). Scilliroside and other Scilla compounds in red squill. *Journal of Agricultural and Food Chemistry*, *34*, 973-979.

Wawrosch, Ch., Kongbangkerd, A. and Kopp, B. (2001). Micropropagation of *Charybdis* sp. (Hyacinthaceae) through nodule culture. COST843 Working Group2 2nd Meeting, Thessaloniki, Greece, Abstract Book (pp. 41-42).