BIOLOGIA PLANTARUM 52 (2): 329-333, 2008

BRIEF COMMUNICATION

Cryopreservation of cell suspension cultures of *Taxus* × *media* and *Taxus floridana*

K. ŠKRLEP[1], M. BERGANT[1,2,5], G.M. DE WINTER[2], B. BOHANEC[3], J. ŽEL[1], R. VERPOORTE[4], F. VAN IREN[2] and M. CAMLOH[1]*

National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia[1]
Institute of Biology, Leiden University, Wassenaarseweg 64, NL-2333 AL Leiden, The Netherlands[2]
Biotechnical faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia[3]
Institute of Biology, Leiden University, Einsteinweg 55, NL-2333 CC Leiden, The Netherlands[4]
University of Nova Gorica, Vipavska 13, P.O. Box 301, SI-5001 Nova Gorica, Slovenia[5]

Abstract

Different lines of cell suspension cultures of *Taxus* × *media* Rehd. and *Taxus floridana* Nutt. were cryopreserved with a two-step freezing method using a simple and inexpensive freezing container instead of a programmable freezer. Four to seven days old suspension cell cultures were precultured in growth medium supplemented with 0.5 M mannitol for 2 d. The medium was then replaced with cryoprotectant solution (1 M sucrose, 0.5 M glycerol and 0.5 M dimethylsulfoxide) and the cells incubated on ice for 1 h. Before being plunged into liquid nitrogen, cells were frozen with a cooling rate of approximately -1 °C per min to -80 °C. The highest post-thaw cell viability was 90 %. The recovery was line dependent. The cryopreservation procedure did not alter the nuclear DNA content of the cell lines. The results indicate that cryopreservation of *Taxus* cell suspension cultures using inexpensive freezing container is possible.

Additional key words: cryo-storage, flow cytometry, genetic stability, yew.

Taxus species are the main source of taxane diterpenes – taxanes among which paclitaxel (*Taxol*®) with its antitumor activity is the most important. Because of its carcinostatic properties it has been used since 1992 for treating various types of cancer. For the current requirements of taxol the initial acquisition from the bark of *Taxus brevifolia* has been replaced with a semi-synthesis from a 10-deacetyl baccatin III (Zhong 2002). Because of its unique mechanism of action the demand for taxol is increasing and so therefore is the need for alternative sources (Kadkade 2003). One of the approaches available to provide large amounts of taxanes is yew cell suspension culture (Jaziri *et al.* 1996, Ketchum *et al.* 1998, Tabata 2004). During the past decade great advances in taxol production in cell cultures have been achieved, including the use of inducing factors such as jasmonic acid (Baebler *et al.* 2002) or fungal elicitors (Yu *et al.* 2005). Examples of industrial use have also been published (Zhong 2002, Tabata 2004). Despite these achievements prolonged maintenance of high producing *Taxus* cells in suspension culture leads to accumulation of spontaneous mutations which can result also in reduction or loss of biosynthetic capacity for product formation (Kadkade 2003). Furthermore, flow cytometric analysis of nuclear DNA content revealed genomic instability of *Taxus* × *media* (Baebler *et al.* 2005). Cryo-preservation is one of the most convenient methods to avoid such problems. Although cryopreservation conserves genetic and phenotypic properties of the cells and allows long-term storage of plant material (Reed 2002, Menges and Murray 2004), it is desirable to assess the genetic integrity of plant material after cryopreservation (Harding 2004).

Received 25 November 2005, *accepted* 20 January 2006.
Abbreviations: DAPI - 4,6-diamidino-2,5-phenylindole; 2,4-D - 2,4-dichlorophenoxyacetic acid; DMSO - dimethyl sulfoxide; FDA - fluorescein diacetate; NAA - α-naphthaleneacetic acid.
Acknowledgements: We would like to thank Špela Baebler and Matjaž Hren for their help and discussion on the maintenance of *T.* × *media* cell suspension cultures. This work was supported by Ministry of Higher Education, Science and Technology of the Republic of Slovenia.
* Corresponding author; fax: (386) 1 2573 847, e-mail: marjana.camloh@nib.si

The most frequently used method for cryopreservation of plant cell cultures is a two-step procedure consisting of pre-culture with sugars and polyalcohols and treatment with chemical cryoprotectants, combined with slow cooling of cells to subzero temperatures before storage in liquid nitrogen. There are many factors that can influence post-thaw recovery. The main ones are cell size, the stage in the growth cycle, the combination and concentration of sugars and polyalcohols in the precultivation medium, the composition of cryoprotectants, and the conditions of freezing and thawing (Kim *et al.* 2001, Kadkade 2003). There are only a few reports of successful cryopre-servation by two-step freezing of plant cell suspensions using a simple and cheap freezing container as opposed to a programmable freezer (Schrijnemakers and Van Iren 1995, Menges and Murray 2004). Methods used for plant cells are limited and often characterised by low viability, long lag periods before recovery of rapidly growing cell suspension and the requirement for specialised equipment to provide a controlled, slow cooling rate (Menges and Murray 2004). For *Taxus* species there are only two publications on cryopreservation of *T. chinensis*, one on two-step freezing with the use of programmable freezer (Kim *et al.* 2001) and the other using a vitrification method (Kadkade 2003). Our aim was to develop a simple protocol for efficient cryopreservation of various lines of *Taxus* × *media* and *Taxus floridana* cell suspension cultures with a two-step freezing method using a freezing container instead of a programmable freezer. The genetic stability of the plant material before and after freezing was determined.

Taxus × *media* Rehd. suspension cell cultures from the collection of the National Institute of Biology of Slovenia were used. They were initiated in 1998 from different parts of seedlings. Cell cultures have been maintained in modified B5 medium (Gamborg *et al.* 1968) supplemented with 20 g dm^{-3} sucrose, 0.1 g dm^{-3} ascorbic acid, 2 mM glutamine, 10 μM kinetin and 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Cell cultures were grown in growth chambers at 23 ± 1 °C in the dark on a gyratory shaker at 100 rpm and sub-cultured every two weeks (Baebler *et al.* 2002, 2005).

Taxus floridana Nutt. cell suspensions were established from seedlings in the Department of Plant Metabolomics and Pharmacognosy, IBL, and were maintained in modified B5 medium (Gamborg *et al.* 1968) supplemented with 20 g dm^{-3} sucrose and 10 μM α-naphthaleneacetic acid (NAA). Cell cultures were grown at 25 ± 1 °C in 16-h photoperiod with irradiance of 20 ± 4 μmol m^{-2} s^{-1} on a gyratory shaker at 100 rpm and were sub-cultured every three weeks by dilution 1/1 with fresh medium.

Two-step freezing was based on a modified Schrijnemakers and Van Iren (1995) procedure for the cryopreservation of cell suspensions. 4 to 7-d-old cell suspension cultures were left to settle down and, after the medium was removed, cells of *Taxus* × *media* were precultured for 2 d in precultivation medium, medium containing 0.5 M mannitol (growth medium with 1 M mannitol instead of sucrose was mixed 1/1 with cell suspension). For the *T. floridana* cells, 0.33 and 0.5 M mannitol were applied and the preculture time was 4 or 7 d.

After preculture under the standard regime, cells were allowed to settle down for 20 min on ice. The medium was removed, and the same volume of cryoprotectant solution containing 2 M sucrose, 1 M glycerol and 1 M dimethylsulfoxide (DMSO) was added. Cell suspensions were then incubated on ice on a gyratory shaker at 100 rpm for 1 h. Cryoprotected cell suspensions were transferred into 2 cm^3 cryovials and placed in a freezing container (*Nalgene® Mr. Frosty*, *Nalge Nunc International Corporation*, Rochester, USA) filled with isopropanol at -80 °C for 120 - 150 min. Under these conditions the initial cooling rate (down to about -40 °C) was close to 1 °C per min and the terminal temperature close to -80 °C. After slow cooling, cryovials were rapidly plunged into liquid nitrogen and kept there for at least 1 h.

After storage in liquid nitrogen the cryovials were quickly transferred into a 35 °C water bath and gently turned over until their contents completely melt. Each cryovial was then immediately placed on ice. To remove the cryoprotectant solution, cryovials were centrifuged at 100 *g* 2 min at 0 °C. For *T.* × *media* cells, the medium was removed, cells were resuspended in fresh precultivation medium and centrifuged again. The medium was removed and the cells transferred in clusters (5 to 10 per experiment) to solid medium with two filter-paper discs. For *T. floridana*, the cells were allowed to settle down for *ca*. 1 min, most of the supernatant medium was removed and the cell mass was transferred to solid medium with two filter paper discs. After 3 or 4 d the upper filter paper disc with cells was transferred to fresh medium. *T.* × *media* cell cultures were cultivated 1 week in the dark and, subsequently, under standard conditions. *T. floridana* cells received standard conditions, but 1 layer of filter-paper was put over the dishes. After 3 weeks the cell masses were transferred to liquid medium. Post-thaw cell viability was estimated after thawing by FDA staining assay (Wildholm 1972).

In initial experiments, growth was observed only visually. In later experiments the fresh mass of callus was determined. The fresh mass of control and cryopreserved cells was measured before the calli were transferred to solid medium and after 3, 6 and 9 weeks of cultivation on solid medium. Cells which were not cryopreserved but were otherwise treated as cryopreserved cells were used as the controls in all experiments.Two-tailed Student's *t*-test was used for the statistical analysis of regrowth of cryopreserved and control samples. The alpha value vas set at the 0.05.

Relative nuclear DNA content was measured with a *PAS III* flow cytometer (*Partec*, Munster, Germany). Calli regenerated after cryopreservation and non-cryopreserved calli of the same lines were protoplasted with water solution of 0.03 % macerozym, 2 % rhozyme,

2 % cellulase R-10, 5 mM $CaCl_2$ and 0.5 M mannitol at pH 5.8. Approximately 0.5 g of callus tissue was incubated in the enzyme mixture for 4 h at 28 °C on a rotary shaker at 35 rpm. The mixture was filtered through a 100 μm stainless steel mesh sieve. The filtrate was centrifuged at 1 000 *g* for 5 min. The pellet was washed twice in nuclei-releasing solution consisting of 21 g dm^{-3} citric acid and 5 g dm^{-3} *Tween 20*. For flow cytometric measurement nuclei were filtered through 30 μm nylon mesh sieve and a 4-fold staining solution (modified from *Partec* protocol 1988) containing 4 mg dm^{-3} 4,6-diamidino-2,5-phenylindole (DAPI) and 71 g dm^{-3} $Na_2HPO_4 \cdot 2\ H_2O$ was added to the nuclear suspension. The relative nuclear DNA content was calculated using pea (*Pisum sativum*) cv. Ctirad as internal standard. All experiments were repeated at least twice with reproducible results.

Table 1. Viability and regrowth of different lines of cryopreserved *Taxus* × *media* cells. Viability was estimated after thawing by FDA staining. Regrowth was determined 9 weeks after cryopreservation [– - no, + - poor (achieved at least in one experiment), ++ - fair, +++ - good]. Regrowth of all control cells was good.

Line	Age [d]	Viability [%]	Regrowth
3	7	80 - 90	+++
3	15	40 - 60	+++
14	7	50 - 90	+
15	7	50 - 80	++
27	7	30 - 45	–

In cryopreservation experiments, the cell lines 3, 14, 15 and 27 of *T.* × *media* Rehd. cell suspension cultures which differed in morphology, growth rate and yield of taxanes were used (Hren *et al*. 2006). For the first three lines, post-thaw cell viability was at least 50 % and, in some experiments, 80 or 90 %, which is higher than those observed by other authors (Table 1). The average viability after cryopreservation of *T. chinensis* was only 30 - 40 % although a controlled freezer was used (Kim *et al.* 2001). The higher viability in our experiments could be due to the different conditions of freezing and thawing used, including the terminal temperature which was lower than that recommended and obtained with the programmable freezer (Schrijnemakers and Van Iren 1995, Kim *et al*. 2001, Kadkade 2003).

Although lines 3, 14 and 15 had comparable viability they differed in regrowth (Table 1). The best regrowth was observed with cell line 3 where the growth of cryopreserved cells was comparable with control ($P > 0.05$). It is interesting, that for line 3 cryopreservation was successful, and regrowth was obtained even if the cell suspension culture was 15-d-old. However, its growth was slower than that of the control (Fig. 1*A*, $n = 6$, $P < 0.01$ for samples 6 weeks, and $P < 0.05$ for samples 9 weeks after cryopreservation). Similar results were reported also for an embryogenic cell suspension of grapevine, where increase of the fresh mass of cryopreserved cells was only half of that obtained with control cells (Wang *et al.* 2002). For *T. chinensis* a long lag phase in the early culture period was shown. Furthermore, the increase in dry mass in the cryopreserved culture after 35 d of culture was approximately 30 % lower than that of the control (Kim *et al.* 2001).

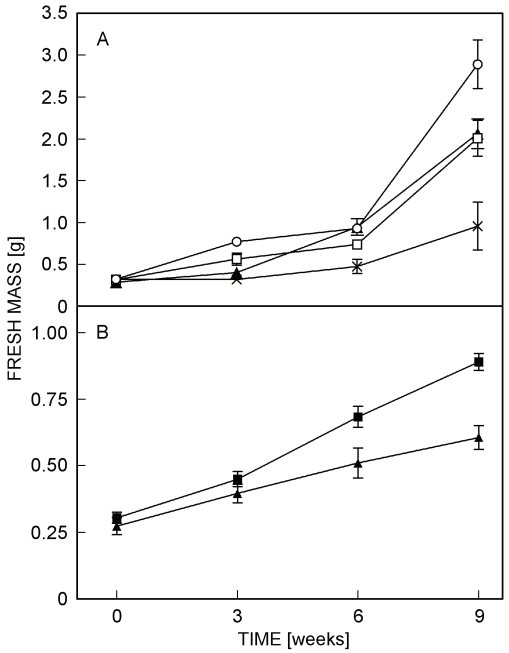

Fig. 1. Regrowth of two lines of *T.* × *media* Rehd.: *A* - line 3 control, non-cryopreserved cells, 7-d-old at the beginning of the experiment (*empty circles*); cryopreserved cells 7-d-old at the beginning of the experiment (*empty squares*); control, non-cryopreserved cells, 15-d-old at the beginning of the experiment (*filled triangles*); cryopreserved cells, 15-d-old at the beginning of the experiment (*crosses*). *B* - line 15, control, non-cryopreserved cells (*filled squares*); line 15, cryopreserved cells (*filled triagles*). Means of 5 - 10 replicates ± SE.

Regrowth of line 15 was successful after cryopreservation, however the growth of control and cryopreserved cells of this line was significantly slower than that of line 3 (Fig. 1*B*, $n = 6$, $P < 0.001$ for the cryopreserved samples 6 weeks, and $P < 0.001$ for the cryopreserved and control samples 9 weeks after cryopreservation). This could be due to differences in genotype and physiological status of the lines.

For line 14 regrowth of cells was obtained in only one out of 4 experiments, and was relatively poor, despite high post-thaw cell viability (Table 1). From these results it is evident that the cryopreservation procedure itself was successful for this line, however, the recovery conditions differed from those of line 3. Since individual cell lines of *Taxus* cultures vary in their preference for growth medium constituents (Kadkade 2003) it can be assumed that the cryopreserved cells of line 14 require some modification of the regrowth medium. Recently it was reported that preparative procedure and culture medium

affect the cryostorage of shoot tips of *Holostemma annulare* (Decruse *et al.* 2004). Furthermore, it has been shown for different species that ammonium nitrate in the culture medium reduces post-freeze recovery (Ryynanen and Häggman 2001).

In line 27 the post-thaw viability was lower than for other lines and regrowth of cryopreserved cells was not observed. It is known that the conditions for cryopreservation differ from species to species (Menges and Murray 2004), and that the ability of some cultures to tolerate cryopreservation can be genotype dependent (Häggman *et al*. 1998). Our previous results also showed line specific requirements for cryopreservation (Van Iren *et al.* 1996). These findings could explain the low post-thaw cell viability and no regrowth in line 27 obtained in our experiments.

Cells of *T. floridana* precultured for 4 d in medium supplemented with 0.33 or 0.5 M mannitol recovered rapidly from cryopreservation. Growth could be observed visually after 2 weeks, and the cells could be brought back into suspension after only 3 weeks. Subculture frequency was gradually increased, and after 12 weeks routine subculture could be resumed. The same held for cells pretreated for 7 d on 0.33 M mannitol, but for the same period on 0.5 M mannitol recovery was much slower.

Relative nuclear DNA content of *T.* × *media* Rehd. was monitored by flow cytometry. The protocol developed for these studies was found to be very efficient since *a*) protoplasts were released from cell lines in large amounts and *b*) isolation of nuclei and staining with DAPI produced histograms of low coefficient of variation. Results showed that the relative nuclear DNA content of cryopreserved plant cells of line 3 was not altered by the cryopreservation procedure (Fig. 2). Similar results were obtained also for line 15 (data not shown). This finding was in contrast to the high variability of relative nuclear DNA content found among different lines of *Taxus* in a previous study (Baebler *et al.* 2005) and in other species (Van Iren *et al.* 1996). The maintenance of genetic stability after cryopreservation was also shown with RAPD analysis for some other coniferous species (Häggman *et al*. 1998).

In conclusion, a two-step freezing method with a simple and inexpensive freezing container has been shown to provide high post-thaw viability of *T.* × *media* and *T. floridana* cell suspension cultures. With this protocol regrowth was successful for 3 out of the 4 *T.* × *media* lines tested and for the *T. floridana* line tested. This is remarkable, because all lines are slowly growing, and, as a rule of thumb, such lines are not expected to be cryopreserved relatively easily (Van Iren *et al*. 1996). We believe that the stability of nuclear DNA content found in cell lines that passed cryopreservation is also significant, particularly in view of the previous finding that, in general, relative nuclear DNA content of *Taxus* cell lines was highly variable. As this freezing method was successful for 4 suspension cell lines belonging to 2 *Taxus* species with quite different characteristics, application to other *Taxus* species appears promising.

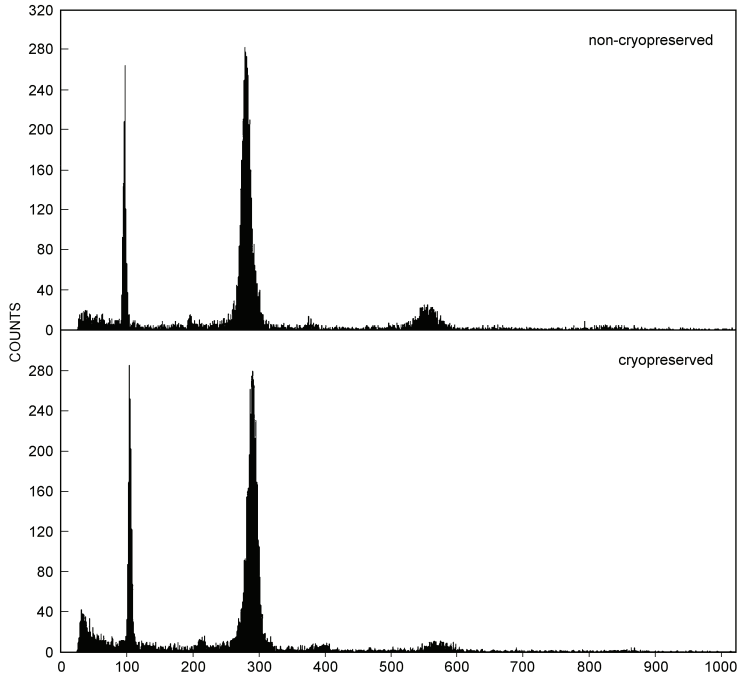

Fig. 2. Histograms showing almost identical positions of relative DNA amount of nuclei released from non-cryopreserved and cryopreserved cells of line 3 (*left peak*- internal standard *Pisum sativum*, peaks at channel 280 and 560 - *T.* × *media* G1 and G2, respectively).

References

Baebler, Š., Camloh, M., Kovač, M., Ravnikar, M., Žel, J.: Jasmonic acid stimulates taxane production in cell suspension culture of yew *(Taxus × media)*. - Planta med. **68**: 475-476, 2002.

Baebler, Š., Hren, M., Camloh, M., Ravnikar, M., Bohanec, B., Plaper, I., Ucman, R., Žel, J.: Establishment of cell suspension cultures of yew *(Taxus × media* REHD*)* and assessment of their genomic stability. - *In vitro* cell. dev. Biol. Plant **41**:338-343, 2005.

Decruse, S.W., Seeni, S., Nair, G.M.: Preparative procedures and culture medium affects the success of cryostorage of *Holostemma annulare* shoot tips. - Plant Cell Tissue Organ Cult. **76**:179-182, 2004.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 150-158, 1968.

Häggman, H.M., Ryynänen, L.A., Aronen, T.S., Krajnakova, J.: Cryopreservation of embryogenic cultures of Scots pine. - Plant Cell Tissue Organ Cult. **54**: 45-53, 1998.

Harding, K.: Genetic integrity of cryopreserved plant cells: a review. - Cryo-Letters **25**: 3-22, 2004.

Hren, M., Baebler, Š., Camloh, M., Kovač, M., Ravnikar, M., Žel J.: Yew (*Taxus × media* Rehd.) cell suspension cultures as a source of taxanes. - Acta Physiol. Plant. **28**: 3-8, 2006.

Jaziri, M., Zhiri, A., Guo, Y.W., Dupon, J.P., Shimonura, K., Hamada, H., Vanhaelen, M., Homès, J.: *Taxus* sp. cell, tissue and organ cultures as alternative sources for taxoids production: a literature survey. - Plant Cell Tissue Organ Cult. **46**: 59-75, 1996.

Kadkade, P.: Cryopreservation of diverse plant cells. United States Patent Application Publication Pub. No.: US 2003/0031998 A1, 1-30, 2003.

Ketchum, R.E.B., Gibson, D.M., Croteau, R.B.: The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* folowing elicitation with methyl jasmonate. - Biotechnol. Bioeng. **62**: 97-105, 1998.

Kim, S.I., Choi, H.K., Son, J.S., Yun, J.H., Jang, M.S., Kim, H.R., Song, J.Y., Kim, J.H., Choi H.J., Hong, S.S.: Cryopreservation of *Taxus chinensis* suspension cell cultures. - Cryo-Letters **22**: 43-50, 2001.

Menges, M., Murray, J.A.H.: Cryopreservation of transformed and wild-type *Arabidopsis* and tobacco cell suspension cultures. - Plant J. **37**:635-644, 2004.

Reed, B.M.: Implementing cryopreservation for long-term germplasm preservation in vegetativly propagated species. - In: Towill, L.E., Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 50. Cryopreservation of Plant Germplasm II. Pp 22-31. Springer-Verlag, Berlin - Heidelberg 2002.

Ryynanen, L., Häggman, H.: Recovery of cryopreserved silver birch shoot tips is affected by the pre-freezing age of the cultures and ammonium substitution. - Plant Cell Rep. **20**: 354-360, 2001.

Schrijnemakers, E.W.M., Van Iren, F.: A two-step or equilibrium freezing procedure for the cryopreservation of plant cell suspensions. - In: Day, J.G., McLellan, M.R. (ed.): Cryopreservation and Freeze-drying Protocols (Methods in Molecular Biology. Vol. 38). Pp. 103-111. Humana Press, Totowa 1995.

Tabata, H.: Paclitaxel production by plant-cell-culture technology. - Adv. Biochem. Eng./Biotechnol. **87**: 1-23, 2004.

Van Iren F., Schrijnemakers, E.W.M., Reinhoud P.J., Kijne J.W.: Cryopreservation of plant cell cultures. - Biotechnologia **32**: 60-67, 1996.

Wang, Q., Gafny, R., Sahar, N., Sela, I., Mawassi, M., Tanne, E., Perl, A.: Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspension by encapsulation-dehydration and subsequent plant regeneration. - Plant Sci. **162**: 551-558, 2002.

Wildholm, J.M.: The use of fluorescein diacetate and phenosafranin for determining viability of cultured plant cells. - Stain Technol. **6**: 169-194, 1972.

Yu, L.J., Lan, W.Z., Chen, C., Yang, Y., Sun, Y.P.: Importance of glucose-6-phosphate dehydrogenase in taxol biosynthesis in *Taxus chinensis* cultures. – Biol. Plant. **49**: 265-268, 2005.

Zhong, J.J.: Plant cell culture for production of paclitaxel and other taxanes. - J. Biosci. Bioeng. **94**: 591–599, 2002.