

PRODUCTION OF SYNSEED FOR HYBRID *Cymbidium* USING PROTOCORM-LIKE BODIES

Short communication

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A B S T R A C T

Synthetic seed were produced from protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light' after culture on a new medium, Teixeira *Cymbidium* (TC) medium. This new medium contained, in addition to a unique selection of macro- and micronutrients, 0.1 mg/l α -naphthaleneacetic acid and 0.1 mg/l kinetin, 2 g/l tryptone and 20 g/l sucrose, and was solidified with 8 g/l Bacto agar. Several explant types and sizes (intact PLBs, half-PLBs, PLB longitudinal thin cell layers) were tested. In addition, pretreatment of PLB-synseeds with 200 mM KNO₃ solution, the addition of activated charcoal or coconut water to synseeds, light vs dark culture, short-term (1 month) and long-term (6 and 12 months) low-temperature (4°C) storage, as well as cryostorage were also tested. All treatments resulted in less PLBs than the control treatment. Among all these treatments, only the use of TC medium or incorporation of coconut water into synseeds resulted in "germination" while low-temperature storage (1-6 months) was only possible under liquid TC. These results would allow for the short-term preservation of *Cymbidium* germplasm but not for effective cryopreservation.

Key words: PLB, synseed, Teixeira *Cymbidium* (TC) medium

Abbreviations: NAA, α -naphthaleneacetic acid; PLB, protocorm-like body; PGR, plant growth regulator; synseed, synthetic seed; TC, Teixeira *Cymbidium*; TDZ, thidiazuron (*N*-phenyl-*N*-1,2,3-thidiazuron-5'-ylurea); VW, Vacin and Went

INTRODUCTION

A synthetic seed or 'synseed' is "an encapsulated single somatic embryo", i.e., a clonal product that can be handled and used as real seed for transport, storage and sowing and that, therefore, would eventually grow either *in vitro* or *ex vitro*, into a plantlet through a process of "conversion" (Sharma et al., 2012). Synseed technology has been somewhat explored in orchid research. Synseeds were formed by the encapsulation of *Cymbidium longifolium* protocorms (Singh, 1988). *Cymbidium* protocorm-like body (PLB) synseeds embedded in a fungicide, and cocooned in chitosan resulted in a 35% germination rate when sown directly on non-sterilized substrate (Nhut et al., 2005). Protocorms 3-4 mm in size were shown to be suitable for optimal conversion frequency of encapsulated PLBs of *Cymbidium giganteum*, smaller PLBs not being able to withstand encapsulation or requiring a long time to emerge out of the capsule (Corrie and Tandon, 1993). High germination frequencies of *C. giganteum* encapsulated PLBs (Saiprasad and Polisetty, 2003) or *C. longifolium* protocorms (Chetia et al., 1998) occurred when they were stored at 4 °C. In the former study, 3% sodium alginate upon complexation with 75 mM calcium chloride for 20-30 min was the optimum combination for proper hardening of beads viz., *Dendrobium*, *Oncidium* and *Cattleya* orchids (Saiprasad and Polisetty, 2003).

Activated charcoal (AC), when incorporated into synseeds, improved

the conversion and vigour of the encapsulated propagules of tropical forest trees by not only stimulating the diffusion of gases and nutrients, but also by helping in breaking down alginate which in turn facilitates enhanced respiration of propagules, thus preventing the loss of vigour that extends the storage period significantly (Saiprasad, 2001). AC also absorbs unintended and unwanted exudates such as 5-hydroxymethylfurfural (a toxic breakdown product of sucrose formed during autoclaving) and other harmful phenolic products (Wang et al., 2007). AC also retains nutrients within the hydrogel capsule and releases them slowly, thus providing a long-term supply of nutrients to the growing tissue. AC at 1.25% (w/v) improved the conversion frequency of encapsulated somatic embryos in *Oryza sativa* (rice) (Arun Kumar et al., 2005). Natural PGRs like coconut water (CW) and tomato juice have also been successfully used for synseed conversion (Swamy et al., 2009).

In encapsulation technology, there may be physical hindrance of shoot or root emergence caused by the gel capsule. This can be overcome by adopting self-breaking alginate gel bead technology in which synseeds are pretreated with potassium nitrate (KNO_3) in which, during the pretreatment with KNO_3 , the K^+ ions replace the Ca^{2+} ions of the calcium alginate capsule thus allowing the synseeds to soften and allow the subsequent conversion to plantlets (Onishi et al., 1994). This self-breaking synseed technology was

successfully applied to *Feijoa sellowiana* (goiabeira serrana), *Oryza sativa* (hybrid rice) and *Stevia rebaudiana* (stevia) (Guerra et al., 2001; Arun Kumar et al., 2005; Ali et al., 2012). A self-breaking treatment was applied to *O. sativa* synseeds with a synthetic endosperm by immersing them in 200 mM KNO₃ solution for 60 min and rinsing in sterile tap water for 40 min or more until the beads became swollen (Arun Kumar, 2005) while *F. sellowiana* and *S. rebaudiana* synseeds were dipped in 100 mM and 200 mM KNO₃ solution for 20 and 5 min, respectively (Guerra et al., 2001; Ali et al., 2012). In *F. sellowiana*, 81.2% of capsules opened while 0% in the treatment with water opened (Guerra et al., 2001). In *O. sativa*, a 47% conversion frequency relative to normal synseeds (without a self-breaking coat) was possible (Arun Kumar et al., 2005).

When explants are incubated in the dark, they induce more root primordia followed by the addition of PGRs in the gel matrix for higher conversion from encapsulated beads (Piccioni, 1997). Pretreatment of explants with cytokinin(s) and auxin(s) has enhanced the conversion frequency of synseeds into plantlets for the following: mulberry (*Malus pumilla*) (Pattnaik et al., 1995); pineapple (*Ananas comosus*) (Soneji et al., 2002); *Dalbergia sissoo* (Chand and Singh, 2004); *Carrizo citrange* (Germanà et al., 2011); *Corymbia* spp. and African mahogany (Hung and Trueman, 2012). Moreover, when PGRs are added to the gel matrix, the efficiency of synthetic endosperm

around the vegetative propagules improves and provides a simpler method for the successful recovery of complete plantlets. When indole-3-acetic acid (IAA) was added to the gel matrix (consisting of modified MS), 100% of encapsulated nodal segments of *Dendranthema grandiflora* formed roots (Pinker and Abdel-Rahman, 2005). When silver nitrate (AgNO₃) was joined with IBA, the conversion frequency of *Chonemorpha grandiflora* synseeds improved (Nishitha et al., 2006).

Generally, 4 °C has been found to be most suitable for synseed storage (Saiprasad and Polisetty, 2003; Ikhlaiq et al., 2010) although there is a wealth of literature on the choice of optimum temperature and light conditions (reviewed in Sharma et al., 2102).

This study focuses on single layer hydrogel encapsulation of a hybrid *Cymbidium* PLBs under several conditions and variables and subsequent germination on Teixeira *Cymbidium* (TC) medium (Teixeira da Silva, 2012) to establish the efficiency of a mid- to long-term storage choice for this ornamental crop. PLBs (apolar explant) pose a risk to survival during germination unlike polar explants such as somatic embryos or unipolar explants such as shoot tips (Sharma et al., 2012), although the risk of somaclonal variation is considerably reduced (Standardi and Piccioni, 1998).

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were of the highest analytical grade avail-

able and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) originally developed spontaneously from shoot-tip culture on Vacin and Went (VW; Vacin and Went, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium or VW_{PLB}) every two months on Teixeira *Cymbidium* (TC) medium (Teixeira da Silva, 2012) supplemented with 0.1 mg/l α -naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (KIN), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA), according to procedures and advice outlined by Teixeira da Silva et al. (2005) and Teixeira da Silva and Tanaka (2006). All media were adjusted to pH 5.3 with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100 ml Erlenmeyer flasks, double-capped with aluminium foil, at 25 °C, under a 16-h photoperiod with a light intensity of 45 $\mu\text{mol}/\text{m}^2/\text{s}$ provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally bisected PLB (3-4 mm in diameter) segments, 10 per flask, were used as explants for PLB induction and proliferation and for all experiments. Culture con-

ditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva et al., 2005), biotic (Teixeira da Silva et al., 2006b) and abiotic factors (Teixeira da Silva et al., 2006a) for PLB induction, formation and proliferation.

Encapsulation, bead preparation and parameters tested

Based on a wide literature source across many plants, the following protocol was implemented. Freshly prepared half-PLBs from which the terminal bud and base were removed (hereafter PLBs), and derived from 3-month-old PLBs donor PLBs grown on TC medium, were encapsulated as follows: each PLB was immersed with sterilized forceps in a 3.5% (w/v) sodium alginate solution. Initial trials with 3, 3.5 and 4% sodium alginate indicated that 3.5% was the most responsive concentration, 3% being too soft while 4% was too hard. Using wide-mouth glass pipettes, this solution was fed into a complexing solution made of 100 mM CaCl_2 for 45 min, drop by drop, each drop containing a single PLB. Since constant stirring resulted in poorly developed synseeds, the complexing solution was stirred gently with a sterilized metal spoon once every 10 min to prevent the synseeds from adhering to each other and fusing. After hardening, the synseeds was rinsed for 10 min in sterile distilled water to wash away CaCl_2 residue. Encapsulated PLBs (synseeds; Fig. 1A) were replated on TC basal medium under the same conditions as PLB initiation

and proliferation indicated above to assess germination. Once this optimized basic protocol was found (PLB-synseed_{Basal}), the following variables were tested (= treatments; Tab. 1). In all cases, except for d), half-PLBs were used.

- a) Full PLBs with shoot tip intact.
- b) Pretreatment of PLB-synseeds with 200 mM KNO₃ solution for 60 min and then a rinse in sterile distilled water for 1 h or longer until the beads became swollen.
- c) Half-PLBs left on TC medium for 2 weeks.
- d) PLB longitudinal thin cell layers (ITCLs) instead of half-PLBs. ITCLs were prepared as per Teixeira da Silva and Tanaka (2006). More specifically, 5-6 ITCLs were prepared from the surface of 3-month-old PLBs. ITCLs consisted of thin epidermal sections 2 mm² in area and approximately 1 mm thick.
- e) Synseeds cultured on TC containing 4% maltose for 48 h.
- f) AC at 0.5% (w/v) into the sodium alginate solution.
- g) CW at 10% (v/v) into the sodium alginate solution.
- h) Culture constantly in the dark under same temperature conditions as for light culture.
- i) Storage at 4 °C for 1 month, 6 months and 1 year under dry conditions (Petri dishes sealed with Parafilm[®]).
- j) Storage at 4 °C for 1 month, 6 months and 1 year in liquid TC.
- k) Cryostorage at -196 °C for 1 month, 6 months and 1 year. In this case, freshly prepared PLB-synseeds were plunged directly into a 50-l

liquid nitrogen tank and stored in the dark.

In all 11 treatments, the initial weight of all explants was averaged. On average, half-PLBs weigh approximately 40-45 mg (initial weight) although the fresh weights reported in Table 1 reflect additional fresh weight exceeding the initial fresh weight.

The number of encapsulated PLBs (synseeds) that germinated was observed at 2, 4 and 6 weeks, and scored at 6 weeks while the number of *neo*-PLBs or shoots that formed after plating on TC medium and their fresh weight (mg) were scored after 90 days (12 weeks). The occurrence of hyperhydricity, scored with the naked eye, was also noted at 90 days.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment (i.e., each medium). All experiments were repeated in triplicate (n = 90, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation ($p \leq 0.05$) by Duncan's New Multiple Range test (DNMRT) using SAS[®] ver. 6.12 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Only two treatments were comparable (in terms of the four performance indices) to the control (i.e., regular *in vitro* culture of PLBs without the formation of synseed):

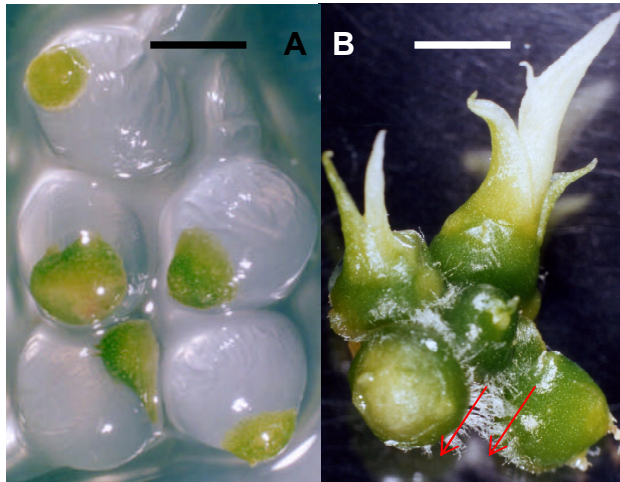


Figure 1. (A) Synseed from half-PLBs. (B) Development of neo-PLBs that emerged from PLB-synseed on Teixeira *Cymbidium* medium (Teixeira da Silva 2012). Bars = 2 mm (A), 5 mm (B). Arrows = vestiges of synseed sodium alginate coat

preculture of half-PLBs left on TC medium for 2 weeks (Fig. 1B), and the addition of CW at 10% (v/v) into the sodium alginate solution (Tab. 1). In the former, most likely the PLBs that were used had already survived any injury from explant preparation and had already initiated the development of *neo*-PLBs while in the latter, CW tends to include a large number of unknown substances, including PGRs, which may have stimulated the growth of PLBs even further, as found for other orchids (Wu et al., 2012). Even though the use of intact PLBs (treatment a) resulted in higher fresh weight of PLB-synseed + *neo*-PLBs, the actual number of PLBs/explant was low, so it was only given a mildly effective code (Tab. 1). Hyperhydricity was observed in some of the treatments (Tab. 1). Rady and Hanafy (2004)

also observed hyperhydricity in regenerating *Gypsophila paniculata* plantlets following synseed germination.

One of the possible reasons for the low level of germination (i.e., percentage of synseed forming *neo*-PLBs in Tab. 1), in which germination is not the traditional formation of a shoot, but rather the *neo*-formation of PLBs or the formation of *neo*-PLBs, may be because half-PLBs were used rather than intact PLBs, suggesting that tissue injury may have negatively impacted the regeneration capacity. The reader is reminded that PLBs are in fact considered to be somatic embryos in orchid (Teixeira da Silva and Tanaka, 2006), and would thus represent the ideal form of a dipolar propagule for synseed, although, in our case, half-PLBs were used. *Neo*-PLB formation

Table 1. The growth and developmental response of hybrid *Cymbidium* Twilight Moon 'Day Light' PLB-synseeds to several treatments

Treatment ¹	Effectiveness coding ⁴	Percentage of synseed forming <i>neo</i> -PLBs [%]	Number of PLBs per ex-plant/synseed	Fresh weight ⁵ (mg) of PLB-synseed + <i>neo</i> -PLBs	Hyperhydricity ⁶
TC ²		100 a	8.3 a	526 b	-
PLB-synseed _{Basal} ³		46 b	4.6 b	321 d	-
a		96 a	3.6 b	618 a	-
b		23 c	1.8 c	186 f	+
c		100 a	7.9 a	478 c	-
d		41 b	1.4 c ⁵	263 e	+
e		26 c	2.6 bc	312 d	+
f		11 d	0.2 d	68 g	-
g		100 a	7.6 a	581 ab	+
h		0 e	0 d	0 h	No growth
i 1 month		0 e	0 d	0 h	No growth
6 months		0 e	0 d	0 h	No growth
12 months		0 e	0 d	0 h	No growth
j 1 month		23 c	0.3 d	81 g	++
6 months		0 e	0 d	0 h	No growth
12 months		0 e	0 d	0 h	No growth
k		6 e	0 d	44 gh	No growth

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($P = 0.05$). $n = 90$.

¹ In total there were 11 treatments:

- Full PLBs with shoot tip intact.
- Pretreatment of PLB-synseeds with 200 mM KNO₃ solution for 60 min and then a rinse in sterile distilled water for 1 h or longer until the beads became swollen.
- Half-PLBs left on TC medium for 2 weeks.
- PLB longitudinal thin cell layers (ITCLs) instead of half-PLBs. ITCLs were prepared as per Teixeira da Silva and Tanaka (2006). More specifically, 5-6 ITCLs were prepared from the surface of 3-month-old PLBs. ITCLs consisted of thin epidermal sections 2 mm² in area and approximately 1 mm thick.
- Synseeds cultured on TC containing 4% maltose for 48 h.
- AC at 0.5% (w/v) into the sodium alginate solution.
- CW at 10% (v/v) into the sodium alginate solution.
- Culture constantly in the dark under same temperature conditions as for light culture.
- Storage at 4°C for 1 month, 6 months and 1 year under dry conditions (Petri dishes sealed with Parafilm®).
- Storage at 4°C for 1 month, 6 months and 1 year in liquid TC.
- Cryostorage at -196°C for 1 month, 6 months and 1 year. In this case, freshly prepared PLB-synseeds were plunged directly into a 50-l liquid nitrogen tank and stored in the dark.

² TC = Teixeira *Cymbidium* medium (Teixeira da Silva 2012) (-ve control; no encapsulation)

³ +ve control; optimized conditions = immersion of each half-PLB in a 3.5% (w/v) sodium alginate solution, dripped into a complexing solution made of 100 mM CaCl₂ for 45 min, and final rinse in sterile distilled water for 10 min.

⁴ **Green** = most effective; **orange** = mildly effective; **red** = ineffective

⁵ Even though the number of PLBs per ITCL was low, after converting using the Growth Correction Factor (Teixeira da Silva and Dobránszki, 2011, unpublished data), the actual comparable number is higher.

⁵ In all 11 treatments, the initial weight of all explants was averaged. On average, half-PLBs weigh approximately 40-45 mg (initial weight) although the fresh weights reported in Table 1 reflect additional fresh weight exceeding the initial fresh weight.

⁶ Hyperhydricity: - = no occurrence; + = slight (< 5% of final PLBs)

in *Cymbidium* is clearly without an intermediate callus phase, or the explant dies. The two routes tend to be very clear. Undoubtedly TCLs suffer much more tissue wounding than half-PLBs or full PLBs, which could explain their lower *neo*-PLB regeneration ability.

Seed germination in orchids, although a good way to preserve rare germplasm and to induce novelty through natural variation inherent through sexual reproduction, can take several years to achieve a successful culture (Deb and Pongener, 2011; Hossain et al., 2012). Therefore, synseeds present an excellent way to store orchid material at room temperature, under cold storage, or even cryopreservation for weeks to months, or even years (Teixeira da Silva, unpublished data), while maintaining the clonal stability of the material. Synseeds in orchid biotechnology has particular relevance considering that they produce tiny, non-endospermic seeds. Corrie and Tandon (1993) encapsulated *Cymbidium giganteum* protocorms and healthy plantlets formed after synseed were transferred either to nutrient medium or directly to sterile soil. The conversion frequency was 100% *in vitro* but lower *ex vitro* (88% in sand, 64% in sand and soil mixture). PLB encapsulation is well documented in many orchids, including *Cymbidium giganteum*, *Dendrobium wardianum*, *Dendrobium densiflorum*, *Phaius tonkervillae*, *Oncidium*, *Cattleya* and *Spathoglottis plicata* (Ara et al., 2000; Vij et al., 2001; Saiprasad and

Polisetty, 2003). Saiprasad and Polisetty (2003) encapsulated the most suitable stage of PLBs for *Dendrobium*, *Oncidium* and *Cattleya*. Fractionated PLBs at the leaf primordia stage, 13-15 days after culture, were most suitable for encapsulation. Similarly, in this study, treatment c), in which half-PLBs had been left to develop for 2 weeks prior to encapsulation, resulted in higher synseed survival (treatment c; Tab. 1). An encapsulation matrix prepared with MS medium ($\frac{3}{4}$ -strength) supplemented with BA and NAA resulted in 100% conversion of encapsulated PLBs when cultured on MS medium supplemented with BA and NAA (*Dendrobium*) or NAA alone (*Oncidium* and *Cattleya*) (Saiprasad and Polisetty, 2003). Sarmah et al. (2010) produced *Vanda coerulea* synseeds by encapsulating PLBs regenerated from the leaf base with a 94.9% conversion frequency. Nagananda et al. (2011) encapsulated the PLBs of *Flickingeria nodosa* and achieved 95% conversion after 3 months' storage at 4 °C. Cold storage was not successful for *Cymbidium* hybrid (treatments i and j, Tab. 1), nor was cryopreservation, suggesting that conditions need to be further optimized before successful mid- to long-term storage can be achieved. Gantait et al. (2012) alginate-encapsulated *Aranda* × *Vanda* PLBs with 3% sodium alginate and 75 mM calcium chloride, leading to a 96.4% conversion. PLBs of *Dendrobium candidum* (Yin and Hong, 2009) were successfully cryopreserved

using encapsulation-vitrification, offering a long-term perspective for orchid germplasm preservation.

Synseeds using half-PLBs of hybrid *Cymbidium* can be produced using alginate encapsulation. Although PLB-derived TCLs could also be encapsulated, neo-PLB formation was low, but this is a function of their lower surface area (Teixeira da Silva and Dobránszki, 2011). Pre-culture of half-PLBs for 2 weeks improved neo-PLB recovery as did inclusion of 10% CW into the synseed TC medium.

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PRODUKCJA SZTUCZNYCH NASION HYBRYDY *Cymbidium* Z UŻYCIEM TWORÓW PROTOKORMOWYCH

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S T R E S Z C Z E N I E

Otrzymano syntetyczne nasiona z tworów podobnych do protokormu (protocorm-like bodies, PLBs) hybrydy *Cymbidium* Twilight Moon 'Day Light' wyhodowanych w kulturach na nowej pożywce Teixeira *Cymbidium* (TC). Oprócz specjalnie dobranych makro- i mikroelementów ta nowa pożywka zawierała 0,1 mg/l kwasu α -naftalenoocetowego i 0,1 mg/l kinetyny, 2 g/l tryptonu i 20 g/l sacharozy, i była zestalona 8 g/l agaru Bacto. Przetestowano kilka rodzajów i rozmiarów eksplantatów (niezaruszone PLBs, pół-PLBs, podłużne cienkie warstwy komórek PLB). Ponadto zbadano wpływ wstępnego traktowania syntetycznych nasion z PLB roztworem 200 mM KNO_3 , dodatku węgla aktywowanego lub wody kokosowej do tych nasion, prowadzenia kultur w świetle i w ciemności, przechowywania krótkoterminowego (1 miesiąc) i długoterminowego (6 i 12 miesięcy) w niskiej temperaturze (4°C), jak również przechowywania kriogenicznego. Wszystkie traktowania dawały w rezultacie mniejszą ilość PLBs niż w traktowaniu kontrolnym. Spośród wszystkich zastosowanych traktowań jedynie wykorzystanie pożywki TC lub zastosowanie wody kokosowej do nasion syntetycznych spowodowało „kiełkowanie”, natomiast przechowywanie w niskiej temperaturze (1-6 miesięcy) było możliwe tylko w ciekłej pożywce TC. Wyniki te wskazują na możliwość krótkoterminowego przechowywania plazmy zarodkowej *Cymbidium*, ale nie na skuteczną krioprezerwację.

Słowa kluczowe: PLB (twory podobne do protokormu), nasiona syntetyczne, pożywka Teixeira *Cymbidium* (TC)