

## **Asymbiotic Seed Germination of *Thunia alba*, a Variable Orchid in an Isolated Karst Hill of Xishuangbanna, Yunnan, China**

**Zi-Yu Zhou, Ju Li, Xi-Hui Peng and Sven Landrein\***

*Xishuangbanna Tropical Botanical Garden XTBG, Horticulture Department, Yunnan, Xishuangbanna, Mengla, Menglun Town, 666303 China*

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### **Abstract**

Asymbiotic seed germination of *Thunia alba* (Lindley) H.G. Reichenbach was tested on different media in order to propagate and preserve its genetic diversity. In addition different plant hormones were used in the similar germination experiments. Results from these experiments revealed that Knudson medium (KC) had the best germination ability but Mitra and modified Murashige and Skoog (MS) also showed good responses for growth and root formation in the seedling after eight weeks. Coconut water (CW) was the most efficient for promoting germination and growth, addition of 0.046  $\mu\text{M}$  and 0.46  $\mu\text{M}$  Kn also induced better germination rate and formation of leaves. The seedlings after eight months were transferred and acclimatized to grow in the garden on a medium containing bark and crushed concrete blocks. Although the species is amongst the rare orchid species that can successfully propagate asexually in the wild it is demanding for substrate and climatic conditions.

### **Introduction**

*Thunia alba* (Lindley) H. G. Reichenbach has a wide range of distribution from China, north-east India, Indonesia, Malaysia, Myanmar, Nepal, Thailand and Vietnam (Chen and Wood 2009). Seidenfaden (1986) accepted 4 species based on a preliminary study and five species are listed on the Monocot World Checklist (WCSP 2017), including *Thunia alba* var. *alba*, *T. alba* var. *bracteata* (Roxb.) N. Pearce & P. J. Cribb, *T. bensoniae* Hook. f., *T. brymeriana* Rolfe, *T. candidissima* (N.E.Br.) Rchb. f. and *T. pulchra* Rchb.f. Morphological characters to differentiate the species are based on the colour of the lip that are white or purple with yellow, red, orange or purple markings. *Thunia pulchra* differs by having 4

\*Author for correspondence: <sven@xtbg.ac.cn>.

not 8 pollinia, smaller flowers and a lip with a minute spur (Andre Shuiteman, Pers. Comm.). No recent revision of the genus exists but only *Thunia alba* is recorded for China (Chen and Wood 2009, Li et al. 2015). In Yunnan the plant is present in Dali, Lincang, Lancang, Pu'er, Wenshan and Xishuangbanna prefectures ranging from 1200 to 2300 m altitude (Gao et al. 2013). Present collection is from Southeast Xishuangbanna in Mengla county at an elevation of 1000 m, growing on a karst hill. The population in this locality is less than 15 individuals. The population is remarkable by the colour of its labellum, with a yellow centre, orange stripes on the margin and an erose margin (Fig. 1C). Although the actual knowledge in the taxonomy of the genus is insufficient to assign this taxon to any form, this represents an interesting variation for use in horticulture and *ex situ* conservation and may be linked to a particular distribution pattern within the genus.

*Thunia alba* is a lithophytic species and is amongst the few orchids that can have an asexual vegetative reproduction by producing new plantlet at the nodes of the fleshy stems (Figs 1D and 2). Node section and rooting is the easiest way to obtain new plants for *ex situ* programs and is relatively easy when provided with a suitable growing medium. However, it can only produce a limited amount of plants and does not preserve the genetic diversity obtained by using wild pollinated seeds.

Germination of orchid seeds in the wild is difficult without the symbiotic association of the mycorrhizal fungi, therefore asymbiotic seed germination is a valuable technique for *ex situ* conservation (Arditti and Ernst 1993, Arditti 1967). This represents a valuable technique for propagation and aims at preserving genetic diversity, with good re-introduction and ornamental potentials.

There are a few reports on *in vitro* germination of seeds in the genus *Thunia* (Singh et al. 2014), and in addition such requirements are specific and depend on the orchid variety and provenance. In this study authors evaluate the effect of several media composition on the asymbiotic seed germination, protocorm and seedling growth of *Thunia alba*, from an isolated Karst population in Xishuangbanna.

## Materials and Methods

Yellowish capsules from wild pollinated plants were harvested in Xishuangbanna, Mengla on a Karst hill known for its rich orchid diversity (Gao et al. 2013). Experiments were done in the laboratory of the Horticulture Department, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science (XTBG, CAS).

The capsules were sterilized by soaking several times in 70% alcohol for 5 minutes and rinsed with distilled water. The capsules were opened, and seeds were transferred in a syringe where they could be further cleaned with a 2% sodium hypochlorite solution (NaOCl).

Four media were chosen following Singh et al. (2014), namely Knudson (KC) (1946), Vacin and Went (VW) (1949), Mitra (1976) and Murashige and Skoog (1962), to test for their effectiveness in promoting germination of seeds. MS medium was also tested using

half strength (half MS) as well as with different concentration of salts (major salts in  $\frac{1}{2}$  strength and minor salts and organic components in full strength). Three per cent sucrose and 0.8% agar were added to each medium. pH was adjusted to 5.8 by adding 0.1N NaOH or 0.1N HCl. Each medium was autoclaved at 121°C for 20 min. To study the effect of cytokinin in promoting the germination and growth of the seeds and protocorms authors also prepared MS with different concentrations of added cytokinin. Coconut water (CW) (10, 20 and 30% v/v), BAP (0.044, 0.44, 4.4 and 22.0  $\mu\text{M}$ ) and Kn (0.046, 0.46, 4.6 and 23.0  $\mu\text{M}$ ). Seeds were then transferred to the sterile media under laminar flow



Fig. 1. *Thunia alba* collected in Xishuangbanna, Mengla, 1000 m high: A. Flowering plant in July at the beginning of the rainy season. B. Fruiting plant at the end of the rainy season in October. C. Flower showing lip with yellow centre and red/purple stripes on the margin. D. Adventitious plantlet formed on an old stem's node.

and were observed and counted every week for two months. Mitra medium was problematic, because high level of charcoal content made the Petri dishes black and authors were not able to count non germinated seeds under the inverted microscope. For this reason, they only observed germination, protocorm and seedlings stages for this medium. The Petri dishes were transferred into a culture room with a 16/8 hrs photoperiod at 25°C.

Every treatment had 14 replicates and each experiment was repeated three times. The data were subjected to completely randomized design and analysed using one-way ANOVA statistics and means were compared with the LSD (Snedecor and Cochran 1989).

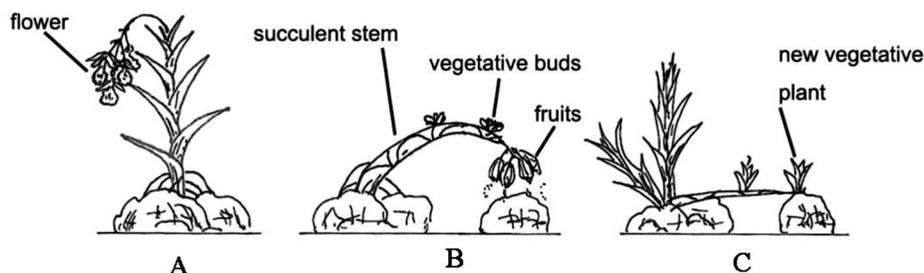


Fig. 2. Asexual reproduction of *Thunia alba*. A. Flowering plant during the rainy season. B. Fruiting plant at the end of the rainy season without leaves. C. Plantlets and roots produced on the nodes of the stem during the dry season.

## Results and Discussion

All media showed more than 60% germination and Knudson had the highest rate of germination (79.3%, Table 1). No significant difference between Knudson, MS full and half MS were observed. Seeds elongated after the first week and the first protocorms were produced after 2 weeks on half MS and only after four weeks on Mitra. All seeds swelled to become yellow to green embryo (Fig. 3A) but later all became green coloured protocorms (Fig. 3C). Cilia and roots were initiated at the latest stage after one or two leaves were produced (Figs 3F-G) and were particularly abundant on Mitra. The addition of 20% coconut water (CW) got the highest rate of germination (Table 2). BAP 0.044  $\mu\text{M}$  as well as Kinetin (0.046 and 4.6  $\mu\text{M}$ ) also improved germination rate but higher concentration of CW was not beneficial. Kn in lower concentration was also beneficial for root formation and size of the seedlings.

Similar study indicated that the protocorms may require 10 months to attain a suitable size prior to acclimatization (Jualang et al. 2014). After ten months, seedlings were approximately 5 cm long bearing 8 - 10 leaves and more than 5 roots. Seedlings were transferred on a substrate with bark and crushed concrete to recreate the Karst condition in which the species was found growing (Fig. 3H).

Morphological and genetic variation studies within the genus *Thunia* are lacking thus the number of species is still open for debate (Sudhakar 2013). Conservation work needs good taxonomic systems to be able to preserve most of the diversity. Increasing knowledge on propagation is also a way of preserving this diversity when this basic knowledge is lacking. *Thunia alba* has a wide distribution range (Chen and Wood 2009), is a variable species and it is often found growing as a lithophytic plant on isolated and disjunct Karst hills. The labellum with a yellow centre, orange stripes on the margin and

an erose margin is different from similar plants growing in Xishuangbanna (Gao et al. 2013) and more similar to plants named *Thunia alba* var *bracteata* (Sudhakar 2013), but this has no yellow markings in the centre of the lip.

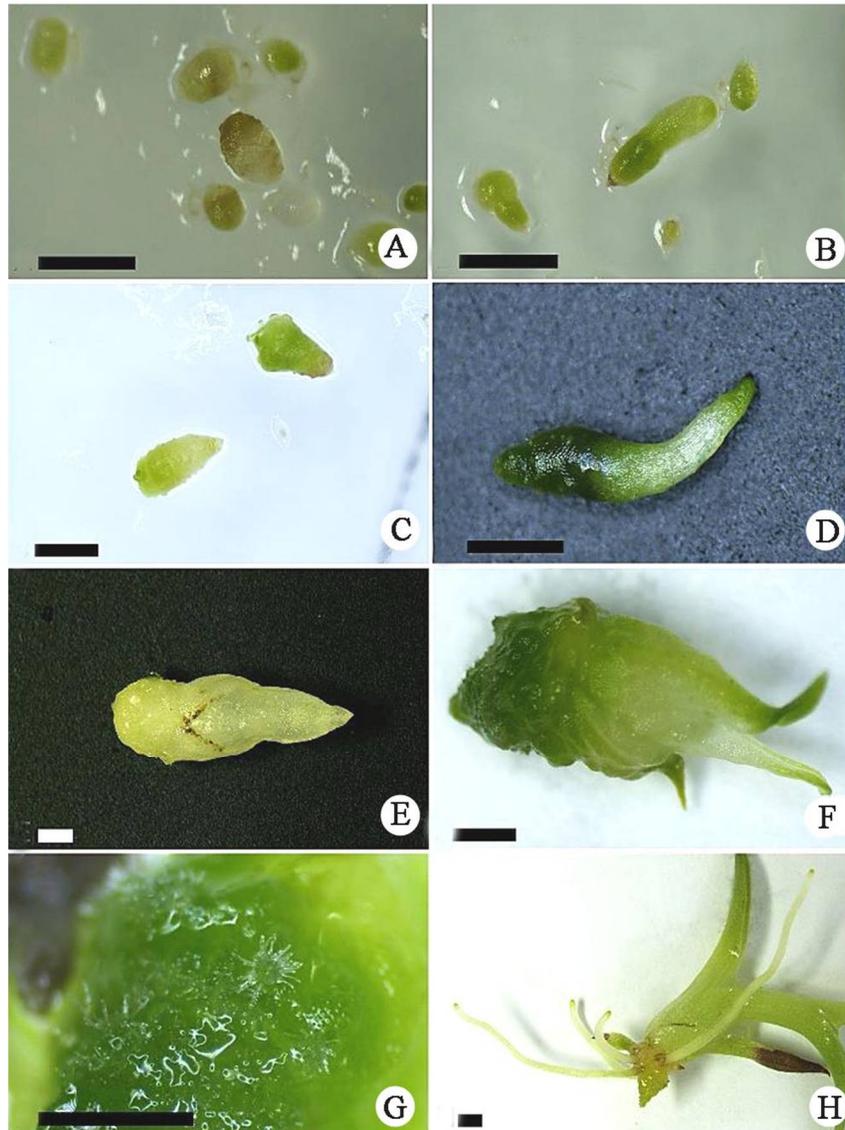


Fig. 3(A-H). Development stage of *Thunia alba* from seeds to protocorm and seedlings: A. Seeds after two weeks. B. Elongating seeds. C. Protocorm after 4 weeks. D-E. Young seedling. F. First leaves after 8 weeks. G. Papille (roots) forming after 8 weeks. H. Seedling after 4 months. Scale bar 1mm in A-H

**Table 1. Effect of different media on seed germination and seedling development of *T. alba*.**

Medium	Percentage germination after 8 weeks	Number of protocorm after 4 weeks	Number of protocorm after 8 weeks	Seedling size after 8 weeks (mm)	Roots formation (Total)	Number of leaves (if present)
Knudson (KC)	79.3 ± 13.08a	5.06 ± 4.76	117.36 ± 27.58	0.87 ± 0.27	3	1.75 ± 0.5
Vacin & Went (VW)	69.39 ± 13.04b	8.28 ± 7.26	77.01 ± 13.04	1.00 ± 0.21	0	-
Mitra	*	0 ± 0	102.90 ± 30.28	1.46 ± 0.46	20	1.71 ± 0.59
MS Full	77.3 ± 9.51ac	16.85 ± 9.16	95.61 ± 23.27	1.85 ± 0.68	4	1.79 ± 0.92
MS (1/2 strenght)	73.8 ± 16.51ab	25.58 ± 20.51	108.05 ± 39.93	1.20 ± 0.32	6	1.57 ± 0.60
MS (modified)	59.75 ± 17.60d	26.6 ± 16.96	95.71 ± 44.13	1.60 ± 0.47	17	1.97 ± 0.69

\*Indicates counting of non-germinated seeds was not possible.

**Table 2. Effect of coconut water (CW) and cytokinin on seed germination and seedling growth of *T. alba*.**

Growth regulator	Concentration	Percentage germination after 8 weeks	Number of protocorm after 4 weeks	Number of protocorm after 8 weeks	Seedling size after 8 weeks (mm)	Roots formation (Total)	Number of leaves (if present)
CW	10%	75.67±10.14ab	21.05 ± 8.25	100.31 ± 32.36	1.00 ± 0.24	0	1.12 ± 0.35
	20%	76.1 ± 15.0a	15.03 ± 6.04	90.86 ± 24.74	0.98 ± 0.32	2	1.25 ± 0.58
	30%	60.88 ± 18.14c	12.6 ± 5.37	64.61 ± 29.31	0.99 ± 0.31	1	1.15 ± 0.55
BAP	0.044 µM	70.67 ± 14.07ab	10.43 ± 4.21	70.65 ± 16.10	1.37 ± 0.42	10	1.68 ± 0.58
	0.44 µM	61.99 ± 14.34c	5.03 ± 2.87	79.08 ± 24.95	1.22 ± 0.81	2	1.20 ± 0.42
	4.4 µM	66 ± 14.36bc	5.45 ± 3.62	68.25 ± 18.24	1.49 ± 0.75	1	1.28 ± 0.47
	22 µM	57.06 ± 13.97c	3.76 ± 2.63	64.57 ± 18.60	0.83 ± 0.24	0	1.10 ± 0.32
Kn	0.046 µM	71.08 ± 9.97ab	16.06 ± 14.10	84.03 ± 14.82	1.91 ± 0.75	21	2.07 ± 0.53
	0.46 µM	69.75 ± 9.26b	27.10 ± 22.47	76.61 ± 17.84	1.62 ± 0.71	19	2.41 ± 0.50
	4.6 µM	70.38 ± 8.63ab	6.44 ± 4.87	88.03 ± 15.42	1.17 ± 0.60	12	1.81 ± 0.60
	23 µM	69.29 ± 10.02bd	5.0 ± 4.11	88.92 ± 22.45	0.85 ± 0.24	3	1.40 ± 0.51

Due to their specialized biology, it is difficult to germinate orchid seeds and obtain enough viable plantlets for re-introduction in the wild. Knudson (1922) made it possible for orchid seeds to be germinated asymbiotically and to produce plants for *ex situ* conservation. Asymbiotic seed germination can be an efficient way to reproduce orchids from seeds and overcome the problem of ectomycorrhizal association in the wild. This study shows that this technique is successful when applied on *Thunia alba* and is an essential tool to preserve the population and genetic diversity of this species. Similarly, a study of *in vitro* multiplication of *Thunia marshalliana* (Singh et al. 2014) also found the genus to respond well to asymbiotic seed germination. Though they found that modified MS and addition of 10% coconut water resulted in the best germination and addition of cytokinin had no effect. Here, it was also found that addition of coconut water was the

most beneficial, but Knudson medium had a non-significant higher germination rate than modified MS. KC medium was first developed to increase germination success of orchids seeds (Curtis and Spoerl 1948) and often used for tropical epiphytic orchids (Arditti 1982). KC medium has little macro- and micro-salts and no vitamins.

Different media were chosen because their composition of mineral salts, nitrogen, organic compounds, and vitamin were different. Nitrogen concentration can influence orchid seed germination (Stewart and Kane 2006). Mitra, KC and VW contain inorganic sources of nitrogen whereas MS contains inorganic and organic sources of nitrogen (amino acid). All media showed good germination rate suggesting that both organic and inorganic source of nitrogen were used by the seeds. Nevertheless, addition of low concentration of CW increased germination success, suggesting addition of other organic compounds present in CW enhanced germination, as shown in several studies on orchid germination (Arditi and Ernst 1984).

There are some reports showing the inhibitory effects of various cytokinins on the asymbiotic germination of orchids (Stewart and Kane 2006). But other studies suggest plant growth regulators (PGR) have a positive effect on seed germination (Mathews and Rao 1980, Kishor et al. 2006). The production of auxin-like symbiotic fungus such as mycorrhizal *Rhizopogon roseolus* (Hadley and Harvais 1968) facilitates seed germination. This fact can explain the roles of exogenous plant growth regulators in asymbiotic seed germination (De Pauwet al. 1995, Lo et al. 2004). Present samples showed a better germination with 0.046  $\mu\text{M}$  and 0.46  $\mu\text{M}$  Kn but the other PGRs had no effect or were detrimental to the germination, showing that these act differently under different conditions.

In conclusion asymbiotic seed germination of *Thunia alba* can ensure a better production of large quantity of genetically diverse seedlings that can be used for *ex situ* conservation. With this experiment present authors obtained more than 100 plants in 8 months. However, the medium used for asymbiotic germination and seedling development requires more components than that for symbiotic germination, such as organic and inorganic available carbon sources as well as PGRs (Temjensangba and Deb 2005). Nitrogen as well as some cytokinin in the right concentration are essential to produce numerous and healthy seedlings.

It is hoped that using the right medium and suitable protocol for acclimatization will allow the gardeners to grow large quantity of genetically diverse *Thunia* that can be used for conservation project as well as promoting the importance of Karst in the evolution of orchids.

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