

Full Length Research Paper

An efficient system for the production of the medicinally important plant: *Asparagus cochinchinensis* (Lour.) Merr.

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An *in vitro* cultivation protocol was developed for *Asparagus cochinchinensis* a species threatened by over collection due to its importance as a medicinal plant in China. Adventitious shoot induction was most successful by using hypocotyls as explants for propagation on Murashige and Skoog (Murashige et al., 1962) medium supplemented with 4.5 μM N₆-benzylaminopurine (BA) only as well as with 3.0 μM α -naphthalene acetic acid (NAA) and 4.5 μM BA. For continuous subculture, indole-3-acetic acid (IAA) and BA (μM) at a ratio of 3.0:4.5 or 3.0:9.0 had the best regeneration potential producing approximately four plantlets per nodal explants. Plantlets had 4 – 5 nodes that could be utilized for the following subculture phase to induce axillary shoots. The plantlets were placed on ½-strength MS medium, indole-3-butyric acid (IBA) was included in the media at a concentration of 2.5 or 5.0 μM . This propagation regime has the capacity for producing 1000 – 2000 plants from one shoot after 3 months long subculture cycles, making it highly attractive for implementation as an *in vitro* conservation strategy. The micro-propagated plants were easily acclimatized (80%) within a month after rooting *in vitro* and being planted *ex vitro* in a sand : soil : peat moss : vermiculite (1:2:1:1; v/v/v/v) mixture.

Key words: Medicinal herbs, *Asparagus cochinchinensis* (Lour.) Merr., micropropagation.

INTRODUCTION

Asparagus cochinchinensis (Family Liliaceae), one of about 24 *Asparagus* species that are endemic and medicinally important to China (Deli et al., 2000), is the tuberous root of *A. cochinchinensis* (Lour.) Merr. It is often used for treatment of fever, cough, hemoptysis, diabetes, constipation, swollen and throat pain. It is distributed in many provinces of China. It is reported that only a few substances such as β -sitosterol, some amino acids, monosaccharides, oligosaccharides, polysaccharides, and several furostanol saponins have been isolated until present time. Further studies on the *A. cochinchinensis*

led to the isolation of thirty compounds from the ethanolic extract of the tuberous root. The structures of the compounds were elucidated on the basis of chemical and spectral analysis (IR, MS, GC, H-NMR, C-NMR and 2D-NMR) (Tsui and Brown, 1996; Khaliq-uz-Zaman et al., 2000). Recently, the planting of indigenous China species has become very fashionable. Although *A. cochinchinensis* forms an integral part of traditional medicine in China, these Chinese endemics are likely to face increasing threats as they are heavily utilized as ethnoherbal products to treat a variety of ailments such as coughs, colds, bronchitis, sores, bacterial infections, rheumatism, arthritis, tuxicosis and tumor. Generally, limited cultivation of medicinal plants is occurring. As a consequence most of the plants with medicinal properties are gathered from the wild. Despite strict protection by laws regulating the harvesting, the tradition of indiscriminate harvesting of natural populations perpetuates. This was one of the incentives for investigating a micro-propagation strategy as a means of *in vitro* conservation, as illegal practices of plant

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BA, N₆-benzylaminopurine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, α -naphthalene acetic acid; MS, Murashige and Skoog; PGR, plant growth regulator.

harvesting continue. Other threats to the existence of these plants include: (1) The rising number of suburban developments through the country; (2) disturbance in the natural seedbank by a growing number of alien/invasive species on the ecologically fragile fynbos vegetation. Such factors are likely to impact negatively on the natural succession of these plants, leading to a dramatic decrease in plant populations from the biome.

To meet increasing commercial demands, large quantities of medical plants are desirable for the global pharmaceutical industries. *A. cochinchinensis* seeds also have the problem of low germination rate, low orderliness and long seedling time (Liu et al., 2007). The rapidness of tissue culture techniques can be advantageous for the continuous provision of a plantlet stock for cultivation and may further complement breeding programmes. We investigated the most suitable *in vitro* propagation protocol for the production of *A. cochinchinensis*. Several reports document the *in vitro* propagation of *Asparagus* species (for example: *Asparagus sehoberioides* Kunth (Qu and Yang, 1995); *Asparagus officinalis* L. (Bruno et al., 1993), but to our knowledge, there are no reports on the micro-propagation of *Asparagus cochinchinensis* (Lour.) Merr.

MATERIALS AND METHODS

Establishment of *in vitro* seedlings

Seeds of *A. cochinchinensis* were collected seasonally from wild populations growing within the southwest region by Zeng Gui-Ping. Seeds were incubated in 75% (v/v) ethanol for 1 min followed by surface-decontamination in 0.1% (w/v) HgCl₂ for 10 min. Afterwards, the seeds were washed four times (5 min) with sterile distilled water prior to placing onto tissue culture medium (pH 5.8) with a Murashige and Skoog (MS) (1962) salts solidification with agar (0.7% [w/v] Agar-agar, Associated Biologic Enterprises Ding-Guo (China). After decontamination, some of the seeds were placed onto 0.8% water-agar (pH 5.8; controls). During this study, all media were autoclaved at 121 °C and 101 kPa for 20 min after adjustment of the pH to 5.8 with 1 M NaOH. Once cool, 25 ml of the medium was poured into 50 ml conical flask. The seeds were transferred to a 26 – 28 °C growth room with 24 h light illumination (200 μmol m⁻²s⁻¹ photosynthetic photon density) to induce germination. The light was provided by 'cool-white' fluorescent tubes (40W/220V×6). Seeds of *A. cochinchinensis* were placed in a lit growth room and were monitored every two days for germination for a period of 4 weeks.

Culture induction

Seeds were regarded as having germinated once the radicle (1 mm) was visible to the naked eye. However, tissue cultures were only initiated once a normal seedling with two cotyledons, and the hypocotyls and the primary root was available. Germination *in vitro* proved to be nonsynchronous because some of the seeds germinated within the month (Figure 1). Therefore, such seedlings were allowed to develop for another month until primary leaves had developed. Otherwise, hypocotyl and cotyledon explants (±1 cm) were excised from 10 to 14-day-old seedlings and compared to primary leaf sections (±1 cm) for their regeneration potential. Primary leaf tissue was obtained from seedlings that were 30 to 50-days-old. The

explants were cut and transferred under laminar conditions to shoot induction medium (20 ml MS with 0.15 g l⁻¹ myo-inositol, 30 g l⁻¹ sucrose and 1% (w/v) agar [pH 5.8]). Auxins and cytokinins were added individually to the different media. The auxins used were α-naphthalene acetic acid (NAA) (0, 2.5, 5.0 or 10.0 μM); indole-3-acetic acid (IAA) (0, 3.0, 6.0 or 9.0 μM) or 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 1.5, 3.0 or 6.0 μM), whereas N₆-benzylaminopurine (BA) (0, 2.5, 5.0 or 10.0 μM) were the cytokinins investigated for organogenesis.

The plant segments were cultured on the induction medium in conical flasks (50 ml) capped with lids that had a 10 mm vent covered with a 0.22 μm polypropylene membrane. Flasks were returned to the continuous light growth room (200 μmol m⁻²s⁻¹ photosynthetic photon density) with the temperature ranging from 26–28 °C. These culture conditions were used for all subsequent experiments. Data were collected after one month in culture and the number of explants producing shoots, roots and/or callus was recorded. When the PGR combination failed to elicit a response (callus, adventitious shoots and/or roots) at the end of the cycle, it was disregarded for use in further tissue culture steps. As all tissue culture experiments were repeated thrice, the combinations that are represented in Table 1 were the auxins and cytokinins in the culture induction experiment.

Multiplication of plantlets

In order to establish a system which could be utilized for continuous microplant production and subculturing, combinations of IAA and BA were tested for their ability to multiply and elongate *in vitro* shoots of *A. cochinchinensis*. Vegetative shoots derived from IAA and BA combinations of 3.0 and 1.5 μM, 3.0 and 4.5 μM, 3.0 and 9.0 μM, 2.0 and 8.0 μM or 0 and 2.5 μM were subcultured onto the respective PGR combinations (Table 2). One-node stem sections (±1 cm) were cut from tissue cultured shoots. These sections included a pair of axillary buds, referred to as nodal explants henceforth. Each nodal explant was placed horizontally per culture flask with the axillary buds directly in contact with the medium. Cultures were monitored regularly for shoot production. After one month, the number of shoots, length of shoots, number of roots and length of roots, if any, was recorded. Once plantlet regeneration was achieved, nodal explants were used for all subsequent subculture cycles at four-weekly intervals, with three explants being placed per glass culture vessel containing 50 ml medium. The MS medium was supplemented with 3.0 μM IAA, 9.0 μM BA, 0.1 g l⁻¹ myo-inositol, 30 g l⁻¹ sucrose and 1% (w/v) agar (pH 5.8; 1 M NaOH). This was then regarded as the maintenance medium.

Rooting

Plantlets on the maintenance medium (3.0:4.5; IAA: BA (μM, respectively)), were rooted after continuous culture (3 – 5 cycles). The plantlets (5 – 10 cm) were placed on ½-strength MS medium, termed ½MS henceforth, containing also: 30 g l⁻¹ sucrose, 0.1 g l⁻¹ myo-inositol. Plantlets were rooted over a four-week period. As a stimulus for rooting, indole-3-butyric acid (IBA) was included in the media at a concentration of 2.5 or 5 μM. Controls lacked this rooting additive. For preliminary experiments, plantlets were left to root on IBA for a month. However, this treatment did not appear to be for rooting. Thus rooting was achieved by growing plantlets on IBA-supplemented media for two weeks prior to transfer to ½ MS medium. Both rooted and non-rooted plants were left in the medium with activated charcoal for a further two weeks prior to acclimatization. Activated charcoal is a common additive in plant tissue culture media and its positive on organogenesis are well-documented. The root-promoting effects are comprehensively reviewed by Pan and van Staden (Pan et al., 1998) and these include an increase in

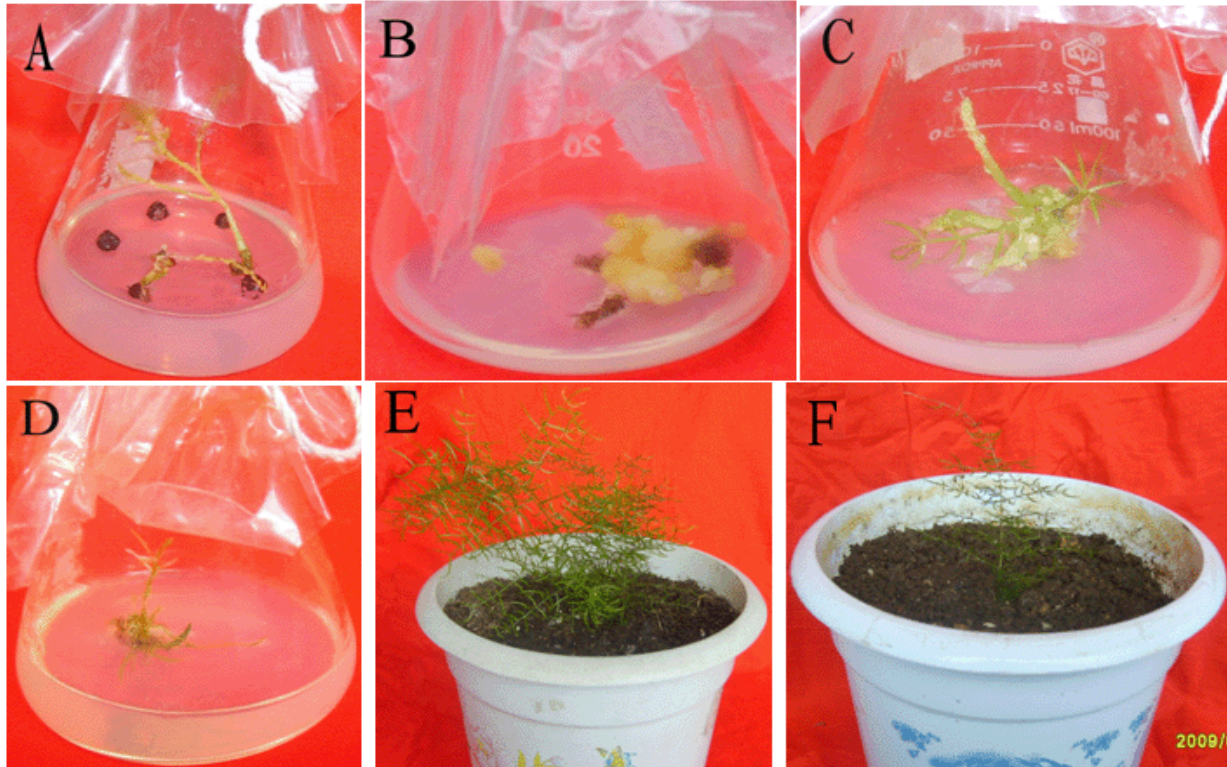


Figure 1. *In vitro* propagation of *Asparagus cochinchinensis*. **A**) A germinated seedling (24 days in culture). Hypocotyl, cotyledon and primary leaf explants were tested for regeneration; **B**) prolific callus formation from explants. Callus induction on 6.0 μM IAA and 9.0 μM BA medium from a hypocotyls; **C**) Shoot organogenesis induced by medium with 6.0 μM IAA and 4.5 μM BA; **D**) Root organogenesis induced by medium with 2.5 μM IBA; **E**) An individual regenerant acclimatized on a soil mixture with sand: soil: peat moss: vermiculite mix (1:2:1:1; v/v) in a glass culture vessel; and **F**) Acclimatized plantlet on the vermiculite substrate.

the rooting rate, the number and length of roots. When plants had rooted, extraction of plants was done with care in order not to break the roots. Controls were kept on $\frac{1}{2}$ MS without charcoal throughout the entire experiment.

Acclimatization

In the spring of 2008, all plantlets from the rooting treatments were prepared for acclimatization by gently washing-off the culture medium thoroughly using sterilized distilled water prior to transfer to transplantation substrate. Two different transplantation substrates were tested. Only rooted plantlets were transferred to culture vessels containing either vermiculite or a mixture of sand: soil: peat moss: vermiculite (1:2:1:1; v/v/v/v). Both transplantation substrates were autoclaved before use and one plantlet was placed per culture vessel (200 × 90 mm). To maintain the same composition of macro- and micro-nutrients prior to acclimatization, liquid $\frac{1}{2}$ MS (lacking sucrose and myo-inositol) was used to wet the acclimatization substrate. The culture vessels containing the plantlets were all sealed with lids that had a 10 mm vent covered with a 0.22 μm polypropylene membrane.

These were transferred to a thermostatically controlled glasshouse with natural sunlight and the culture vessels containing the plantlets were exposed to a PPFD range of 800 – 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (mid-day irradiance). The glasshouse thermostat was set to regulate the minimum temperature at 18°C and the maximum temperature at 28°C. The acclimatization period started on August

20 and from then onwards the glasshouse conditions were kept the same. On September 10, plantlets were transplanted from the culture vessels to pots and the number of acclimatized plantlets was recorded on October 10. During this time-period the natural day-length increased from 10 to 12 h with outdoor temperatures ranging from 14 to 24°C. The lids of the culture vessels were loosened over the acclimatization period until they were eventually completely removed (August 20 to September 10). This reduced the relative humidity from 90% to about 70%. For each transplantation substrate, fifteen micro-plants were transferred from the rooting medium to soil and the acclimatization experiments were repeated thrice.

Ex vitro growth

Plantlets were allowed to grow for one week without lids prior to transfer to pots (10 cm) filled with vermiculite or the sand : soil : peat moss : vermiculite mixture (1:2:1:1; v/v/v/v) on September 10. On this day, the number of potted plants was recorded. Those plantlets acclimatized in vermiculite inside culture vessels were transplanted to pots with the same transplantation substrate. Once in pots, plants were watered by hand two to three times a week. When a white powdery fungus was noted on the shoots, they were sprayed with broad-spectrum fungicides which also act against powdery mildews. Plants were monitored regularly. The tally of successfully acclimatized plants and the length of the propagules were recorded one month after being transferred to pots on October 10.

Table 1. Regeneration of callus, shoots and/or roots from cotyledon, hypocotyl and primary leaf explants of *Asparagus cochinchinensis* on MS media supplemented with different auxin/cytokinin combinations for a 30 day period.

PGR combination (μM)	Cotyledon explants			Hypocotyl explants			Leaf explants		
	Callus Production (%)	Shoot Production (%)	Root Production (%)	Callus Production (%)	Shoot Production (%)	Root Production (%)	Callus Production (%)	Shoot Production (%)	Root Production (%)
5.5 NAA:9.0 BA	100	35	0	100	38	7	100	27	30
0.0 NAA:4.5 BA	83	25	0	45	46	0	43	29	0
3.0 NAA:4.5 BA	71	37	0	100	42	25	100	21	0
1.0 NAA:9.0 BA	100	33	0	100	33	0	100	33	0
6.0 IAA:9.0 BA	100	47	12	75	19	14	100	33	8
6.0 IAA:4.5 BA	100	33	0	100	33	0	100	33	0
4.5 2,4-D:9.0 BA	100	33	0	100	33	0	100	33	0
3.0 IBA:2.0 BA	100	22	67	100	52	0	100	26	43

Data collected from media that failed to induce a regeneration response or that resulted only in explant elongation after 30 days without evident callus, shoot and/or root formation within 30 days were omitted. Ten replicates were used per treatment and experiments were repeated thrice.

Statistical analysis

For all germination experiments, 10 seeds were placed per conical flask and five conical flasks were prepared per treatment. For culture induction, multiplication and rooting experiments, one explant was placed per flask and each flask represented a replicate. Ten replicates were used per treatment. Experiments were based on a completely randomized design and repeated three times. All quantitative data were subjected to standard analysis of variance using the General Linear Model Procedure generated by the SPSS14.0 program (Tables 2 and 3). The LSMeans and LSD values were calculated at a 5% significance level. The test controls the Type I comparison-wise error rate. Otherwise data from all other experiments were subjected to the M-L χ^2 Chi square analysis using Statistical Release 7 (Statsoft Inc. 2005), to test whether the observed differences were or not at a 5% level.

RESULTS AND DISCUSSION

Seed germination and culture induction

The seeds of *A. cochinchinensis* followed a non-

synchronous pattern; they started to germinate a few days after culture initiation whilst most of the seeds germinated two months after culture induction on all media tested (Figure 1A). 80% of the seeds had produced normal seedlings ($P = 0.0024$ for the M-L χ^2 -test) after 60 days on MS. Use of auxin/cytokinin combinations that failed to induce a regeneration response from explants within 30 days were regarded as unsuitable as this study aimed at finding a simple PGR regime that easily and rapidly induced direct organogenesis. The first signs of regeneration from the explants tested were noted within 10 days on the medium with 6.0 μM IAA and 9.0 μM BA (Table 1; $P = 0.035$). Callus production (Figure 1B) was prolific for all explants tested occurring with most PGR treatments at a frequency of 33 – 100% (Table 1). Adventitious shoot regeneration (Figure 1C) was strongly influenced by the explant type, shoot production being highest when hypocotyls were placed on medium with 4.5 μM BA only, 3.0 μM NAA and 4.5 μM BA, or 3.0 μM IBA and 2.0

μM BA contrary to the study by Santos-Gomes and Fernandes-Ferreira (Santos-Gomes et al., 2003) who considered 2,4-D superior for shoot production in *Salvia officinalis*. Indirect organogenesis increases the probability of genetic variation in culture (George and Sherrington, 1984) which may adversely affect the quality of the essential constituents produced from propagated plants.

Multiplication for continuous culture

When 3.0 μM IAA was combined with BA at 4.5 or 9.0 μM , shoot induction differed from the other combinations tested with an average of 3 shoots being produced (Table 2, $P = 0.002$).

Although these two treatments affected shoot regeneration, their effect on shoot elongation was statistically similar to the other IAA and BA combinations. This may imply that for shoot regeneration, the ratio of IAA to BA is important for successful plantlet regeneration. However, the

Table 2. Multiplication and elongation of shoots on MS medium with different combinations of IAA (0–3.0 μM) and BA (1.0–10.0 μM) for a 30 day period.

Combination of IAA and BA (μM)	Mean number of shoots per nodal explant	Shoot length (cm)	Mean number of roots per plantlet	Root length (cm)
3.0+1.0	2.0 \pm 1.73 b	6.00 \pm 1.73 a	0.0 \pm 0.72 bc	0.00 \pm 0.65 a
3.0+4.5	3.0 \pm 1.06 a	7.07 \pm 1.06 a	1.0 \pm 0.44 ac	0.49 \pm 0.40 a
3.0+9.0	3.0 \pm 0.48 a	6.58 \pm 1.15 a	0.0 \pm 0.48 bc	0.0 \pm 0.43 a
1.0+10.0	1.0 \pm 0.48 b	7.38 \pm 1.15 a	2.0 \pm 0.48 a	0.37 \pm 0.43 a
0.0+2.5	2.0 \pm 0.72 b	5.37 \pm 0.73 a	0.0 \pm 0.30 bc	0.77 \pm 0.27 a

Nodal explants used for this experiment were generated from shoots produced on the same respective media (IAA/BA combinations) as those reported in Table 1. Data indicate mean \pm standard error and treatments denoted by the same letter in a column were not different ($P \leq 0.05$) using the LSD test. Ten replicates were used per treatment and experiments were repeated thrice.

Table 3. Rooting of *Asparagus cochinchinensis* plantlets on $\frac{1}{2}$ MS medium supplemented with 5% (w/v) activated charcoal after a two-week exposure to different concentrations of IBA (0–5 μM).

IBA (μM)	Mean shoot length (cm)	Mean number of roots per plantlet	Root length (cm)
0	3.5 \pm 0.68 a	2 \pm 0.38 a	1.05 \pm 0.38 a
2.5	5.5 \pm 0.52 b	5 \pm 0.62 b	3.84 \pm 0.50 b
5	4.4 \pm 0.23 b	4 \pm 0.85 c	2.01 \pm 0.35 c

Data indicate mean \pm standard error and treatments denoted by the same letter in a column were not different ($P \leq 0.05$) using the LSD test. Ten replicates were used per treatment and experiments were repeated twice.

internodal elongation does not appear to be dependent on the auxin : cytokinin ratio as the length of those on cytokinin alone was similar to those on the combined PGRs.

There were no significant differences with respect to the multiplication and/or elongation potential when the medium contained IAA and BA at a ratio of 3.0:4.5 or 3.0:9.0 (μM). As a result, the former PGR combination is now routinely utilized in our laboratories for continuous culture of *A. cochinchinensis*. On average, four to five nodes became available from each plantlet (5.5 – 7.0 cm) at the time of subculture. With an average of three plantlets regenerating per node, after three 4-week cycles, approximately 1000 – 2000 plants may be produced from one shoot—thus validating the use of this continuous culture regime.

In vitro rooting and *ex vitro* transfer

A. cochinchinensis was able to root spontaneously on multiplication medium (1.0 μM IAA and 9.0 μM BA) with 2 roots being produced per plantlet (Table 2 and Figure 1D). Even so, root induction could be improved by incubating plantlets in medium containing 2.5 μM IBA for two weeks prior to transfer to medium with 5% activated charcoal. This treatment produced five roots per plantlet (Table 3) as opposed to two roots per plantlet using IBA-free medium (controls). The roots reached an average length of 3.84 cm (Table 3), longer than controls.

Plant performance *ex vitro* was highly dependent on the transplantation substrate as plants transplanted onto the soil mixture with sand : soil : peat moss: vermiculite had the best survival rate of 80% ($P = 0.0094$) and shoot growth (30 – 40 cm) (Figure 1E). The survival of the plants growing in vermiculite only was lower (40%; 18 – 25 cm) (Figure 1F). Plantlets were fully acclimatized at the time of transferring them to pots and no further losses were recorded thereafter. Six months later, all the *in vitro* derived plants were still alive and displayed normal development (and seed development) similar to non-cultured plants.

In conclusion, the micropropagation strategy reported here was characterized by a rapid proliferation of shoots. Such a system could be extremely useful as a model for undertaking studies of a genetic or molecular nature in order to gain insights into the processes governing vegetative propagation of plants; particularly the Laliaceae. Not only was a high multiplication rate observed but the plantlets easily acclimatized also to the external environment from the tissue culture, thereby undergoing normal physiological development. This is highly advantageous for the conservation of this species and may further aid in molecular manipulation in *A. cochinchinensis*.

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