Artificial seed for short-term storage: using nodal buds in *Aquilaria malaccensis* Lam.

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Artificial seed technology is known to be the best alternative for short-term storage of plant germplasm in recalcitrant species. *Aquilaria malaccensis*, being a recalcitrant seeded tree, possesses seasonal and highly desiccation-sensitive seeds which lose viability with slight decrease in their moisture content, hence storage becomes a difficult task. The present study deals with short-term storage of *A. malaccensis* nodal buds through artificial seed technology. Nodal buds encapsulated with 2.5% sodium alginate and 100 mM calcium chloride, and polymerized for 40 min were found suitable for artificial seed preparation. Maximum regeneration rate of 83.3% and 75.0% was observed from encapsulated nodal buds stored at 4°C and 23 ± 2°C respectively, for 10 days. Storage was possible for a period of 60 days at 4°C and 50 days at 23 ± 2°C with average regeneration rate of 8.3% and 16.7% respectively.

Keywords: Artificial seeds, encapsulation, recalcitrant, storage, temperature.

GERMPLASM conservation, on a global scenario, is increasingly becoming an essential activity for safeguarding the floristic patrimony of the world due to high disappearance rate of plant species[1]. Seeds connect one generation to another in most of the plant kingdom by acting as a vehicle. Plants pass on their genetic constitution to generations through seeds and therefore the seeds are considered the most appropriate means of propagation, storage and dispersal[2]. However, all plant species do not produce seeds or bear recalcitrant seeds, thus hindering the process of propagation and stimulating depletion from their natural habitat. In such cases, application of *in vitro* techniques for conservation has been successfully reported through preservation/storage, and maintenance of gene banks of such species[3–6]. Artificial/synthetic seed technology, one of the advanced *in vitro* techniques, is usually designed for storage of seeds, zygotic embryos or any vegetative part of plants like shoot tips, axillary buds and somatic embryos[7]. It is an excellent technique for storage and propagation of rare hybrids, elite genotypes, genetically engineered plants as well as rare and endangered plants for which the seeds are either expensive or are not available[8]. Storage of artificial seed is a critical factor for conservation and exchange of germplasm, since retaining viability in terms of germination or sprouting potential even after a considerable period of storage is a desirable feature that determines the success of the technology[4,9].

Agarwood is a non-timber forest product of *Aquilaria malaccensis*, produced as a result of infection in its heartwood by endophytic fungi. It is a highly prized resin produced only by infected wood, which turns aromatic and highly valuable and has great importance in the commercial market. It is mainly valued in many cultures for its distinctive fragrance, and is used as a principal component in incense and perfumes as well as in traditional medicine[10]. In search of infected wood, its natural habitat has been severely exploited[11]. *A. malaccensis* is slow-growing and propagated mainly through seeds, but the seeds are seasonal and recalcitrant in nature[12,13]. The shelf life of the seeds is very short (5–14 days) at room temperature with higher seed germination percentage reported immediately after collection or within 4–5 days of collection[12,14]. A slight decrease in its moisture content causes a sudden fall in germination rate[12]. The rate of exploitation is higher than the germination rate leading to reduction in its population. Therefore, it is necessary to search for an alternative method to store *A. malaccensis* germplasm for longer days with no/less viability loss. So utilization of nodal buds as explants merged with artificial/synthetic seed technology and storage will provide a safer way of conserving *A. malaccensis* germplasm, since nodal buds are independent of seasonal variations and can be produced throughout the year under *in vitro* conditions. Even though there are reports on artificial seed technology and storage studies in several other species, there is none on artificial seed production and storage of *A. malaccensis* till date. Therefore, the present study explores the production of artificial seeds using *in vitro* nodal buds and examines the influence of temperature on storage.

Materials and methods

Nodal buds from two-month-old *in vitro* raised plantlets of *A. malaccensis* derived from zygotic embryos were used as explants. Various concentrations (2%, 2.5%, 3% and 4% (w/v)) of sodium alginate (encapsulation matrix)

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were prepared in aqueous MS (Murashige and Skoog) medium[15] and 100 mM calcium chloride solution was prepared in aqueous MS medium devoid of calcium chloride (Himedia, India). For preparation of artificial seeds, nodal buds (3–5 mm) were inundated in all the concentrations of sodium alginate solution separately and each bud along with the solution was suctioned with sterile pipette (tips modified by cutting with a diameter of ~0.5 cm). Nodal bud with sodium alginate solution was then released drop by drop into calcium chloride solution and maintained at different time intervals (20, 40 and 60 min) for complex formation. The artificial seeds (encapsulated nodal buds) were washed with sterile distilled water 3–4 times and inoculated in MS medium with 4 μM KN (kinetin); concentration optimized through a separate experiment.

For assessing efficient regeneration in different medium states, encapsulated nodal buds (with optimal encapsulation matrix) were inoculated separately in liquid and semi-solid MS medium of different strengths (full, half and quarter) supplemented with 4 μM KN. The optimum medium obtained was considered as the regeneration medium for further studies.

The regeneration efficiency of artificial seeds was investigated through pulse treatment. Excised *in vitro* nodal buds were first treated with 6 μM BAP (6-benzylaminopurine) and 4 μM KN (concentration optimized through a separate experiment) for 30 min separately, then encapsulated using the optimized concentration of sodium alginate and complexation duration and inoculated in optimized regeneration medium.

For short-term storage studies, artificial seeds were prepared using *in vitro* nodal buds following the procedure optimized above. The artificial seeds were then packed in sterilized vials (10 artificial seeds per vial) and stored at 4°C and 23 ± 2°C separately for different time intervals. Control was maintained by culturing the artificial seeds in the regeneration medium before storage. Temperatures (4°C and 23 ± 2°C) selected for storage were monitored every day to maintain the exact and accurate temperature for storage. One vial from each temperature was taken out after every 10 days for 60 days, inoculated in the regeneration medium and transferred to growth room.

MS medium was fortified with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar. pH of the medium was adjusted to 5.8 ± 0.03 using 1 N NaOH and 1 N HCl prior to addition of agar and autoclaved at 121°C and 18 psi for 15 min. All the cultures were maintained in growth room with a temperature of 23 ± 2°C, 12 h photoperiod with an irradiance of 60 μmol m^{-2} s^{-2} provided by cool white fluorescent tubes. However, cultures inoculated into liquid medium were kept on a rotary shaker at 120 rpm with similar culture conditions. Ten replicates were maintained and the experiments were repeated two times. The artificial seeds were considered to be regenerated with the emergence of shoots from the encapsulating matrix. Regeneration observation was carried out at an interval of 15 days (from inoculation) for 60 days. Data were analysed by applying Tukey's test at 5% probability level ($P < 0.05$) using Origin8 statistical software.

Results and discussion

Concentration of sodium alginate and their complexation duration affect the shape and germination of artificial seeds. The nodal buds encapsulated in 2.5% sodium alginate held for 40 min in 100 mM calcium chloride produced iso-diametric beads with perfect consistency for regeneration. A lower concentration of sodium alginate (2%) formed soft, fragile beads which were not iso-diametric in nature due to tail formation (Figure 1 *a*) and at higher concentration (3% and 4%) the beads were iso-diametric but found to be hard. When the exposure duration for complex formation was shorter (20 min), soft beads were formed (Figure 1 *e*), this could be due to lack of optimal ion exchange between Na^+ and Ca^{2+} for proper bead formation. Usually formation of iso-diametric beads, gel complexation and bead hardness depend upon the concentration of sodium alginate, and duration of exposure for optimal ion exchange between Na^+ and Ca^{2+} (refs 16 and 17). So, for determining a successful encapsulation technique, alginate concentration, viscosity and exposure time are fundamental aspects[18].

However, nodal buds encapsulated in 2% sodium alginate showed earliest response in 15 days compared to those encapsulated in 2.5% and 3% which showed their initial response after 30 days, while it took 45 days for initiation of response in 4% sodium alginate (Table 1). This might be because of early bursting of the beads in 2% sodium alginate due to its fragile nature. It has also been reported that beads formed with a lower concentration of sodium alginate are poorly coated because of low viscosity[19]. An increase in the concentration of sodium alginate makes the beads comparatively firmer and harder, therefore delaying the initiation of response in encapsulated nodal buds. These observations are in agreement with many other reports on various species such as *Dalbergia sissoo*[20], *Punica granatum*[7], *Psidium guajava*[5,6] and *Momordica dioica*[21].

Maximum regeneration rate of 83.3% ± 1.1% was observed in 2.5% sodium-alginate with 40 min complexation duration after 60 days of culture (Table 1). However, artificial seeds prepared in 4% sodium alginate showed the lowest regeneration rate (33.3% ± 1.4%) even after 60 days of culture (Table 1). The reason for low regeneration rate in higher concentration of sodium alginate might be due to retention of nodal buds inside the beads for longer duration because of hard coating, which disturbs shoots formation and results in yellowing and browning due to nutrient deficiency leading to death. However, the percentage response in 2 and 2.5 sodium alginate with

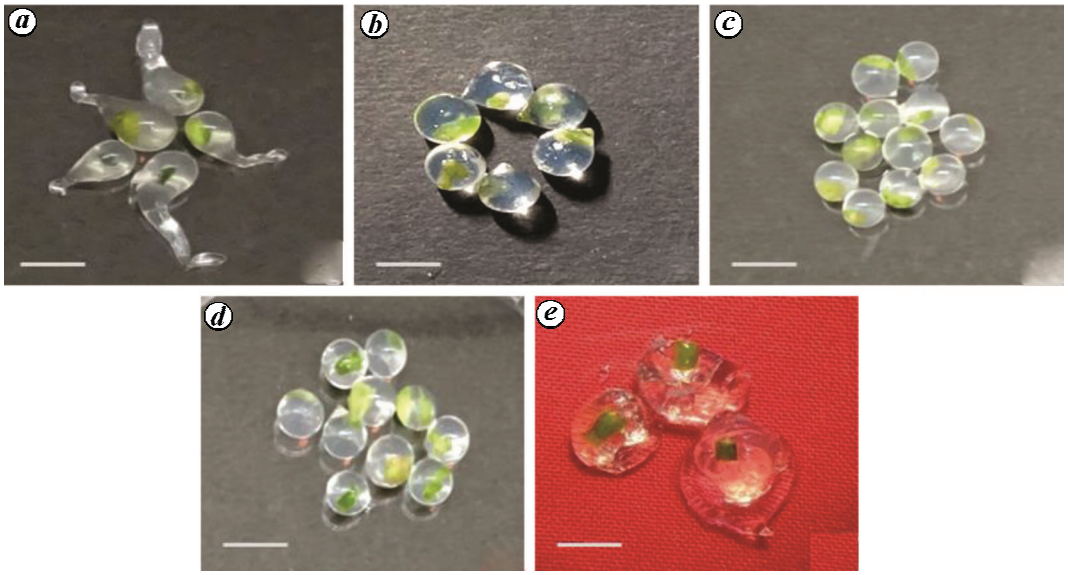

Figure 1. Nodal buds of *Aquilaria malaccensis* encapsulated in (***a***) 2%, (***b***) 2.5%, (***c***) 3% and (***d***) 4% sodium alginate and (***e***) soft and fragile beads due to inappropriate complexation duration. Bar = 0.5 cm.

Table 1. Effect of different concentrations of sodium alginate and complexation duration of calcium chloride on regeneration rate of *in vitro* nodal buds in *Aquilaria malaccensis*

Sodium-alginate (%)	Complexation duration in 100 mM $CaCl_2$ (min)	Regeneration rate (%) ± SE after			
		15 days	30 days	45 days	60 days
2	20	16.7 ± 1.1^{a}	41.7 ± 1.5^{a}	66.7 ± 1.4^{a}	75.0 ± 1.3^{b}
	40	16.7 ± 1.1^{a}	41.7 ± 1.5^{a}	66.7 ± 1.4^{a}	75.0 ± 1.3^{b}
2.5	20	0^{b}	41.7 ± 1.5^{a}	66.7 ± 1.4^{a}	75.0 ± 1.3^{b}
	40	0^{b}	33.3 ± 1.4^{b}	66.7 ± 1.4^{a}	83.3 ± 1.1^{a}
3	20	0^{b}	16.7 ± 1.1^{c}	58.3 ± 1.5^{b}	58.3 ± 1.5^{c}
	40	0^{b}	16.7 ± 1.1^{c}	58.3 ± 1.5^{b}	50.0 ± 1.5^{d}
4	20	0^{b}	0^{d}	25.0 ± 1.3^{c}	33.3 ± 1.4^{e}
	40	0^{b}	0^{d}	25.0 ± 1.3^{c}	33.3 ± 1.4^{e}

Same letters in the column signify that the values are not significantly different from each other at $P \leq 0.05$ according to Tukey's test.

20 and 40 min complexation duration was not significantly different statistically, but beads obtained from 2.5% sodium alginate with 40 min complexation duration were iso-diametric with proper consistency. Therefore, further studies on storage were carried out by preparing artificial seeds in 2.5% sodium alginate with 40 min complexation duration. Earlier workers have reported that 3% sodium alginate with 30 min exposure time is the best in *Saintpaulia ionantha*[22], and 3% with 15 min in *Catharanthus roseus*[18]. However, the concentration of complexation matrix and duration of complex formation vary with different propagules and plant species[17].

Different medium states and strengths showed differences on regeneration of encapsulated nodal buds. Full-strength MS medium was the best for regeneration of encapsulated nodal buds in comparison with half and quarter strength MS medium (Figure 2). Between the two medium states tested, the regeneration rate (43.33%) of artificial seeds in liquid medium was found to be more effective than semi-solid medium (33.33%) in 30 days of culture (Figure 2). Similarly, in *Psidium guajava*, full-strength liquid MS medium was reported to be most favourable for regeneration of artificial seeds compared to semi-solid medium[5,6]. However, in the present study, shoots developed in liquid medium were found to be weak and flimsy in nature, while in semi-solid medium it appeared to be healthy and sturdy (Figure 3). Therefore full strength liquid MS medium can be selected for conversion of encapsulated nodal buds into shoots and regenerated plantlets can be transferred into semi-solid MS medium for further growth and development.

Pulse treatment of *in vitro* nodal buds of *A. malaccensis* before encapsulation improved the regeneration of encapsulated nodal buds. Regeneration rate was found

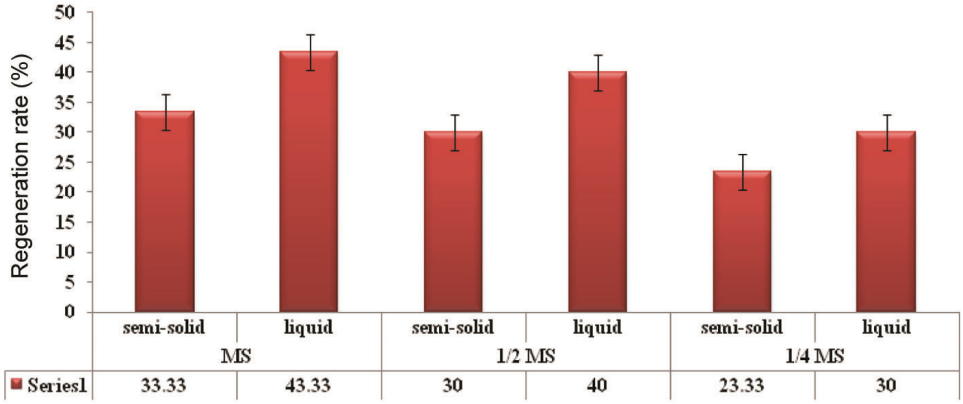

Figure 2. Effect of different medium states and strengths on regeneration of encapsulated nodal buds in *A. malaccensis* after 30 days of culture. Bars indicate standard error.

Figure 3. *A. malaccensis*: ***a***, Nodal buds encapsulated in 2.5% sodium alginate; ***b***, Plantlet regenerated from encapsulated nodal buds in semi-solid; ***c***, Liquid MS medium.

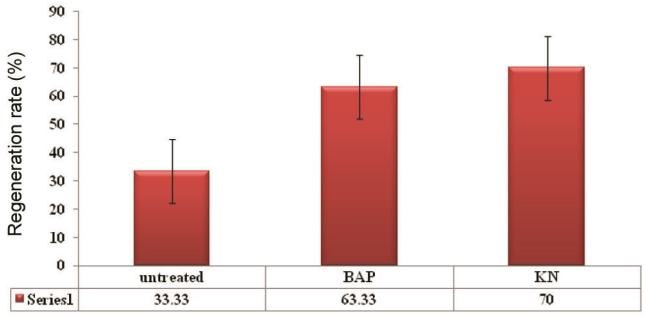

Figure 4. Effect of pulse treatment on regeneration of encapsulated nodal buds in *A. malaccensis* after 30 days of culture. Bars indicate standard error.

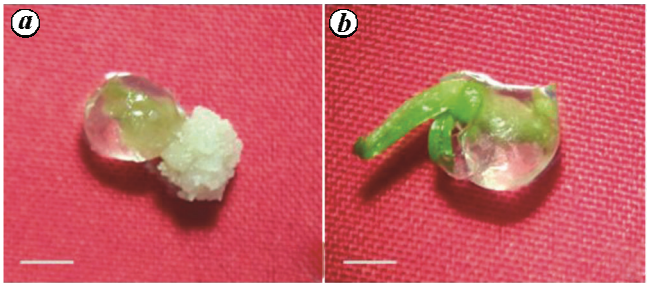

Figure 5. Regeneration from *in vitro* nodal buds of *A. malaccensis* exposed to pulse treatment for 20 min before encapsulation with (***a***) BAP and (***b***) KN after 30 days of culture.

to be higher from the nodal buds which were pulse treated with KN (70%) than BAP (63.33%) and untreated (33.33%) nodal buds in 30 days of culture (Figure 4). Development of callus was observed in BAP-treated nodal buds which slowed down the regeneration rate, whereas KN-treated nodal buds showed healthy regeneration without the intervention of callus (Figure 5).

Storage studies of encapsulated nodal buds were carried out for 60 days. As the concept and advantages of producing artificial seeds are mainly for germplasm conservation and exchange, it is therefore necessary to develop protocols which are versatile in order to fulfil and successfully achieve the general concept and advantages of producing artificial seeds. Two temperatures, i.e. 4°C and 23 ± 2°C, were chosen for storage in order to examine the regeneration capability of encapsulated nodal buds. The regeneration rate was significantly higher for artificial seeds stored at 4°C than those at 23 ± 2°C. Several workers observed similar results in other species like *Mentha arvensis*[23], *Catharanthus roseus*[18] and *Begonia*[24].

Storage temperature greatly influences regeneration rate of encapsulated nodal buds in *A. malaccensis*. Maximum regeneration rate of 83.3% ± 1.1% and 75% ± 1.3% was observed from encapsulated nodal buds stored for 10 days at 4°C and 23 ± 2°C respectively

Table 2. Regeneration rate from encapsulated nodal buds of *A. malaccensis* after storage at 4°C and 23 ± 2°C for different durations

Storage temperature (°C)	Storage duration (days)	Regeneration rate (%) ± SE after			
		15 days	30 days	45 days	60 days
4	10	0	33.3 ± 1.4^{b}	58.3 ± 1.5^{a}	83.3 ± 1.1^{a}
	20	0	25 ± 1.3^{c}	41.7 ± 1.5^{b}	66.7 ± 1.4^{b}
	30	0	16.7 ± 1.1^{d}	33.3 ± 1.4^{c}	50.0 ± 1.5^{c}
	40	0	8.3 ± 0.8^{e}	25.0 ± 1.3^{d}	50.0 ± 1.5^{c}
	50	0	8.3 ± 0.8^{e}	25.0 ± 1.3^{d}	41.6 ± 1.5^{d}
	60	0	0^{f}	0^{e}	8.3 ± 0.8^{e}
23 ± 2	10	0	41.7 ± 1.5^{a}	58.3 ± 1.5^{a}	75.0 ± 1.3^{f}
	20	0	33.3 ± 1.4^{b}	41.7 ± 1.5^{b}	58.3 ± 1.5^{g}
	30	0	16.7 ± 1.1^{d}	16.7 ± 1.1^{f}	50.0 ± 1.5^{c}
	40	0	8.3 ± 0.8^{e}	8.3 ± 0.8^{g}	41.7 ± 1.5^{d}
	50	0	0^{f}	0^{e}	16.7 ± 1.1^{h}
	60	0	0^{f}	0^{e}	0^{i}

Same letters in the column signify that the values are not significantly different from each other at $P \leq 0.05$ according to Tukey's test.

Figure 6. *A. malaccensis*: ***a***, Regeneration of shoot from encapsulated nodal buds of after storage; ***b***, ***c***, Regeneration from (***b***) yellowish and (***c***) brownish nodal buds after storage.

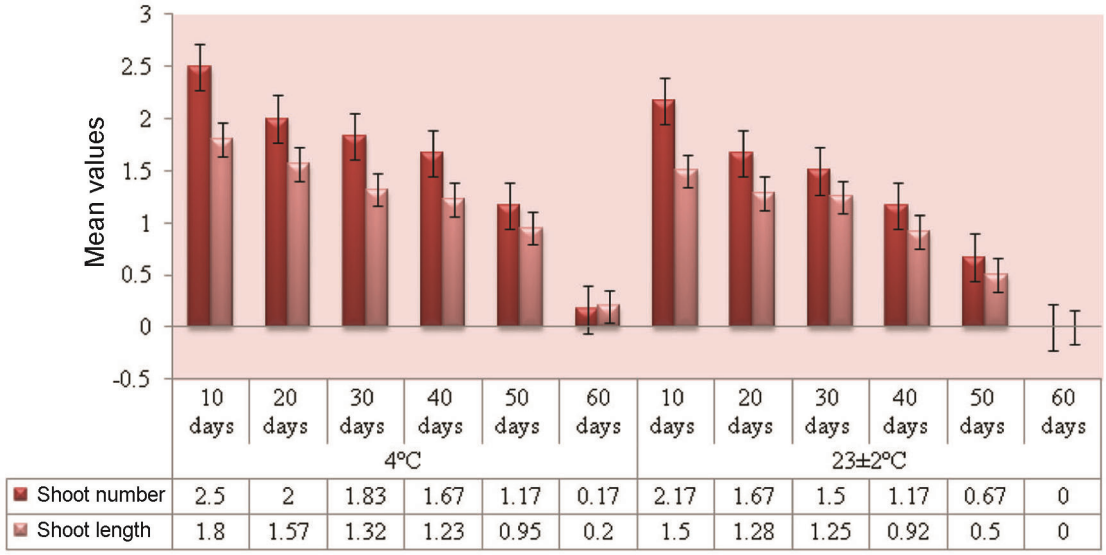

Figure 7. Shoot number and shoot length of plantlets developed from stored encapsulated nodal buds in *A. malaccensis* after 60 days of culture.

(Table 2). With the increase in storage duration, the regeneration rate of encapsulated nodal buds decreases (Table 2). Such results are supported by earlier reports on storage studies in certain plant species[5,6,24,25]. The decline in plant recovery from stored encapsulated propagules may be due to oxygen deficiency in the calcium alginate beads[26].

Most of the encapsulated nodal buds stored at both the temperatures regenerated after 30 days of culture and the regeneration rate subsequently increased with increase duration in the culture medium (Table 2). Regeneration of the encapsulated nodal buds stored for 60 days at 4°C and 50 days at 23 ± 2°C was recorded only after 45 days of culture, with a lower rate of 8.3% ± 0.8% and 16.7% ± 1.1% respectively (Table 2). However, no regeneration was recorded from encapsulated nodal buds stored for 60 days at 23 ± 2°C (Table 2). There are reports on rupture of encapsulation matrix during storage that hindered further storage[25,27]. However, in the present study rupture of encapsulation matrix was not observed; instead the nodal buds turned yellowish or brownish with increase in storage duration resulting a decline in regeneration (Figure 6). It has been reported that decline in the regeneration rate observed among encapsulated explants may be due to inhibition of respiration in the plant tissues because of alginate covers[28].

Shoot development from the encapsulated nodal buds stored at 4°C was found to be better than that at 23 ± 2°C in all the storage durations studied. The encapsulated nodal buds developed maximum shoot number (2.50 and 2.17) and shoot length (1.80 and 1.50 cm) at 4°C and 23 ± 2°C respectively, after 60 days of culture in 10 days of storage (Figure 7). A decrease in shoot number and shoot length was observed with increase in storage duration in the present study (Figure 7). This might be due to decrease in the rate of metabolic activities in the nodal buds during storage.

Conclusion

Short-term storage of *A. malaccensis* germplasm has been achieved in this study through artificial seed technology. As *A. malaccensis* produced recalcitrant seeds of seasonal nature, there were complications in storage under normal conditions. *In vitro* nodal buds can be produced throughout the year through plant tissue culture techniques to expediently integrate tissue culture manipulations with artificial seed technology for germplasm storage and conservation. As *A. malaccensis* thrives well in tropical climates, its regeneration capability at temperatures (4°C and 23 ± 2°C) other than its native habitat was studied. Encapsulated nodal buds could be stored for longer durations, i.e. 60 and 50 days in both the temperatures studied, than that reported earlier[12] for seed storage of *A. malaccensis* under normal condition. Therefore, the protocol developed here on storage of encapsulated nodal buds in *A. malaccensis* can be utilized for germplasm conservation and exchange.

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