

***In Vitro* Mid-Term Conservation of *Acorus calamus* L. via Cold Storage of Encapsulated Microrhizome**

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ABSTRACT

In vitro rhizome production, encapsulation and cold storage of *Acorus calamus* were attempted for its propagation and 'true-to-type' conservation. Shoot cultures were initiated using underground rhizome buds, on 6-benzyladenine (BA) containing Murashige and Skoog (MS) medium. Maximum microrhizome production was observed in presence of 33.3 μ M BA, on modified MS medium containing 6% sucrose, 100 mg/L citric acid and 1 g/L polyvinyl pyrrolidone-40. Synthetic seeds were produced from regenerated microtubers by encapsulation in calcium alginate beads. These synthetic seeds were stored in complete darkness at 10⁰C temperature for different durations for mid-term conservation. After cold storage, synthetic seeds were re-cultured *in vitro*, 100% survival was recorded after the storage of 1, 3 or 6 months; and 80% survival was observed after the storage of 12 months. The microrhizomes were produced roots in 4.9 μ M indole-3-butyric acid containing half strength MS medium. All the regenerated plantlets were successfully transferred to field after acclimatization. It is the first report on successful one year *in vitro* cold storage of *A. calamus* synthetic seeds.

Key words: Aromatic plant, Encapsulation, Sweet Flag, Synthetic seeds, Plant tissue culture

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INTRODUCTION

Acorus calamus L. or Sweet Flag or Bach (family- Acoraceae) is a semi aquatic perennial herb distributed in Asia, North America and Europe. Plant may be indigenous to India, distributed in muddy places up to 1800 m height at 10 to 38°C temperature range¹. Plant becomes endangered in many Indian states- Madhya Pradesh, Kerala, Andhra Pradesh and Chhattisgarh; also reported vulnerable in Tamil Nadu², due to rigorous collections from natural habitat because of its economic and medicinal importance. It is a good source of β -asarone³. *A. calamus* is comprehensively used in the traditional medicines as sedative, anti-diarrheic, carminative, tonic, stimulant⁴. The essential oil extracted from rhizome of *A. calamus* used in perfumes and beverages⁵. Seed production is scanty in this herb; hence, rhizomes are mainly used as propagule for its traditional cultivation, which has a slow rate of propagation⁶. Therefore, *in vitro* techniques may provide faster mass propagation for production of its disease free quality planting material as well as means of germplasm conservation. Reports are there on micropropagation of *A. calamus*^{7,8,9,10}. Another technique namely, *in vitro* storage organ production has many advantages over *in vitro* plantlet regeneration; for example micro-tubers/rhizomes can be easily acclimatized, easier to handle, storage and exchange of germplasm, etc. Thus, selected clones can be maintained safely by tissue culture technique under disease and pest free environment. However, under the normal culture conditions, continuous growth of cultures is there induced by exogenous plant growth regulators and cultures are also subjected to frequent subcultures for long durations; may induce off types via somaclonal variations^{11,12,13}. Therefore, growth of *in vitro* cultures must be checked for storage of germplasm to overcome the risk of off type production and it is economic too in comparison to maintain fast growing cultures¹⁴. Long term *in vitro* storage is carried out in liquid nitrogen that is a costly and highly technical method. *In vitro* cold storage technique is used for mid-term conservation has many plus points i.e., simple, economic, disease free with lower requirement of manpower and space. It is also suitable for commercial micropropagation units; as *in vitro* cold stored material can be multiplied according to demand. *In vitro* microrhizome production of *A. calamus* is reported by Devi et al.⁶. Encapsulation of microrhizomes (synthetic seeds) of this species and its *in vitro* conservation by synthetic seeds *via* storage in dark at low temperature is reported firstly here.

MATERIAL AND METHODS

Explant Establishment

Mother plants of *A. calamus* were collected from Chhattisgarh State Forest Departmental Nursery, Raipur (India). The underground rhizome segments with shoot buds were used as explants to start *in vitro* culture. The rhizomes were washed thoroughly in running tap water to remove soil and then divided into approximately 2 cm pieces each. Leaves and roots were also removed from explants. Then the explants were treated with 1% Tween-80 for 15 minutes. Next step was carried out under laminar airflow hood; explants were dipped in fungicide solution (0.1% Carbendazim and 0.25% Mancozeb) for 30 minutes, followed by rinsing with sterile distilled water for 3 – 4 times. Now the explants were quickly dipped in 70% ethanol and then finally were surface disinfested with 0.2% (w/v) aqueous mercuric chloride for 20 minutes and were rinsed 4 – 5 times with sterile distilled water. Again the explants were cut to final size approximately 1 cm long and then were transferred aseptically on to culture establishment medium.

Murashige and Skoog (MS) medium¹⁵ supplemented with 3% sucrose and different concentration of 6-benzyladenine (BA) was used as culture medium. The media were adjusted to pH 5.7 with 1N NaOH and sterilized by autoclaving for 20 min at 1.05 kg/cm² pressure at 121°C. The inoculation were carried out and the cultures was incubated at 25°C ±2 under 16/8 (day/night) photoperiod with 40 µmol m⁻² s⁻¹ light intensity provided through white cool fluorescent tubes. Explant establishment and rooting experiments were carried out in 25×150 mm glass culture tubes containing 15 ml medium each, and 250 ml conical flasks containing 50 ml medium each were used for microrhizome production studies, respectively. The bud break percentage, number and length of shoots per explant were recorded after the 30 days of culture.

Microrhizome Induction

The *in vitro* elongated shoots were further used for protocol standardization of microrhizome induction. Two different levels of sucrose *viz* 6 and 9% were supplemented in MS medium. Along with sucrose three different concentrations of BA were also incorporated in the media. Activated charcoal (0.1%) was also evaluated for microrhizome induction along with 6 and 9% of sucrose supplemented medium. MS medium containing 6% sucrose without any PGR serves as control for this experiment. The number and length of microrhizomes produced from each culture were recorded after 30 days of culture.

Microrhizome Production

After 30 days, the shoots elongated from the explants were excised; leaves were trimmed and then used for microrhizome production. MS medium with best responded sucrose level was used for microrhizome production. This microrhizome production medium was also contained 100 mg/L citric acid and 1 g/L polyvinyl pyrrolidone-40 (PVP-40). The different concentrations of BA were also incorporated in to this modified MS medium for study the effect of microrhizome production. The number and length of microrhizomes produced from each culture were recorded after 30 days of culture.

Encapsulation and Cold Storage

For synthetic seed production, *in vitro* produced microrhizomes were encapsulated into calcium alginate beads. The microrhizomes were suspended in sterilized 3% sodium alginate solution in ½ strength of MS medium; and were dispensed drop-wise into sterilized 3% calcium chloride solution to trap in calcium alginate gel. These encapsulated microrhizomes were washed with sterile distilled water and were sealed in petri-plates. Further, these synthetic seeds were stored at 10°C in complete darkness for different durations *i.e.*, 1, 3, 6 or 12 months. After different time periods, survival percentage of the cold stored synthetic seeds were analyzed by re-culture them on to the microrhizome production medium under the normal culture conditions.

Rooting and Acclimatization

In vitro grown microrhizomes were subjected to rooting in the presence of 4.9 µM indole-3-butyric acid (IBA) in ½ strength MS medium. For acclimatization, plantlets with well developed roots were removed from vessels, washed thoroughly in tap water to remove the nutrient medium and then dipped in fungicide solution (0.1% Carbendazim and 0.25% Mancozeb) for 30 minutes. The plantlets were then transplanted to pots containing coco-pit. These pots were placed at 30°C ±2 and 90% relative humidity (RH) for one month. For secondary hardening, RH is gradually reduced in next two months; finally transferred to the field.

Data Analysis

All the experiments were of completely randomized design; contained ten replicates in each treatment, and each experiment was repeated thrice (total thirty replicates). The data were analyzed by the analysis of variance (ANOVA), means were separated by Duncan's Multiple Range Test (DMRT), at 5% level; using SPSS-10 software¹⁶.

RESULTS AND DISCUSSIONS

Explant Establishment

Underground rhizome bud explants of *A. calamus* were exhibited 100% bud break in growth regulator free basal MS medium (Table 1). However, shoot number as well as shoot length were highly influenced by concentration of BA, as probability (*p*) of ANOVA was <0.0001. Highest shoot number and elongation were recorded in the presence of 35.52 μ M BA, as both the values were ranked 'a' by DMRT in comparison to other concentrations including control. Beneficial effect of BA on explant establishment of *A. calamus* was also observed previously⁸.

Table 1 - Effect of BA level on shoot initiation of *Acorus calamus* nodal explants on MS medium. Observations were recorded after the 30 days of culture.

BA (μ M)	Bud Break (%)	Shoot Number (Mean \pm SE)	Shoot Length (cm) (Mean \pm SE)
0	100	1.0 \pm 0 ^c	0.75 \pm 0.04 ^b
4.44	100	1.0 \pm 0 ^c	0.75 \pm 0.03 ^b
8.88	100	1.25 \pm 0.08 ^b	0.50 \pm 0.01 ^c
17.76	100	1.40 \pm 0.09 ^b	0.58 \pm 0.03 ^c
35.52	100	1.50 \pm 0.09 ^a	1.15 \pm 0.04 ^a

Each experiment consisted of 10 replicates and each experiment was repeated three times. Similar letters followed by mean values do not differ significantly at 5% level, by DMRT.

ANOVA:	<i>df</i>	<i>F</i>	<i>p</i>
Shoot Number:	4	11.248	<0.0001
Shoot Length:	4	47.988	<0.0001

Microrhizome Induction

Microrhizome induction was more influenced by enhanced level of sucrose along with combination of PGR (Table 2). Both the sucrose level (6 and 9%) in combination with slightly increased concentration of BA was found favorable for microrhizome induction. However, 6% sucrose level induced more numbers of *in vitro* rhizomes instead of 9% level. Moreover, black leaching was observed in all the rhizome inducing cultures. Thus, for microrhizome production 6% sucrose level was opted with various concentration of BA. In previous report also, 6% sucrose level was found suitable for induction of microrhizome in the same plant⁶.

In vitro cold storage of *Acorus calamus***Table 2** - Effects of sucrose level in combination with different concentration of BA on microrhizome induction of *Acorus calamus* on MS medium. Observations were recorded after the 30 days of culture.

BA (μ M)	Sucrose (%)	Activated Charcoal (%)	Rhizome Induction (%)	Rhizome Number (Mean \pm SE)	Rhizome Length (cm) (Mean \pm SE)
0	6	-	100	1.5 \pm 0.1 ^{bc}	2.5 \pm 0.2 ^b
4.44	6	-	100	2.2 \pm 0.3 ^a	2.5 \pm 0.2 ^b
17.76	6	-	100	1.8 \pm 0.2 ^{ab}	3.2 \pm 0.2 ^{ab}
35.52	6	-	100	1.4 \pm 0.1 ^c	2.8 \pm 0.2 ^c
35.52	9	-	100	1.3 \pm 0.1 ^c	1.8 \pm 0.3 ^c
35.52	6	0.1	100	1.2 \pm 0.1 ^c	1.4 \pm 0.1 ^c
35.52	9	0.1	100	1.3 \pm 0.1 ^c	2.8 \pm 0.2 ^c

Each experiment consisted of 10 replicates and each experiment was repeated three times. Similar letters followed by mean values do not differ significantly at 5% level, by DMRT.

ANOVA:	<i>df</i>	<i>F</i>	<i>p</i>
Rhizome Number:	6	6.07	<0.0001
Rhizome Length:	6	13.8	<0.0001

Microrhizome Production

For microrhizome production, the shoots which were elongated from the explants were removed from vessels aseptically after one month of culture and each shoot was divided into nodes and cultured onto microrhizome production medium. At induction stage slight blackening and phenolic leaching were observed in cultures that may inversely affect the number of microrhizome (Table 2). To overcome this problem, citric acid (antioxidant) and PVP-40 (absorbent) were also incorporated into the production medium. The problem of blackening and leaching in *Lagerstroemia parviflora* cultures were also circumvented by the similar strategy¹⁷. *In vitro* microrhizome production (number and length) was also influenced by BA level in the medium as *p* of ANOVA was highly significant (<0.0001) (Fig. 1 and Fig. 2). Maximum microrhizomes were produced in the presence of 33.3 μ M BA, as both microrhizome number and length were ranked 'a' by DMRT. Higher level of sucrose in culture medium facilitates higher carbon energy to induce formation of storage organs *in vitro*^{18,19}. Presence of cytokinin in culture medium with high sucrose level positively influenced microrhizome formation, probably because microrhizome is a modified shoot. Therefore, the best microrhizome formation in high BA and high sucrose containing medium was also reported in *Curcuma aromatica*¹⁹, *Curcuma amada*²⁰, *Kaempferia galanga*²¹.

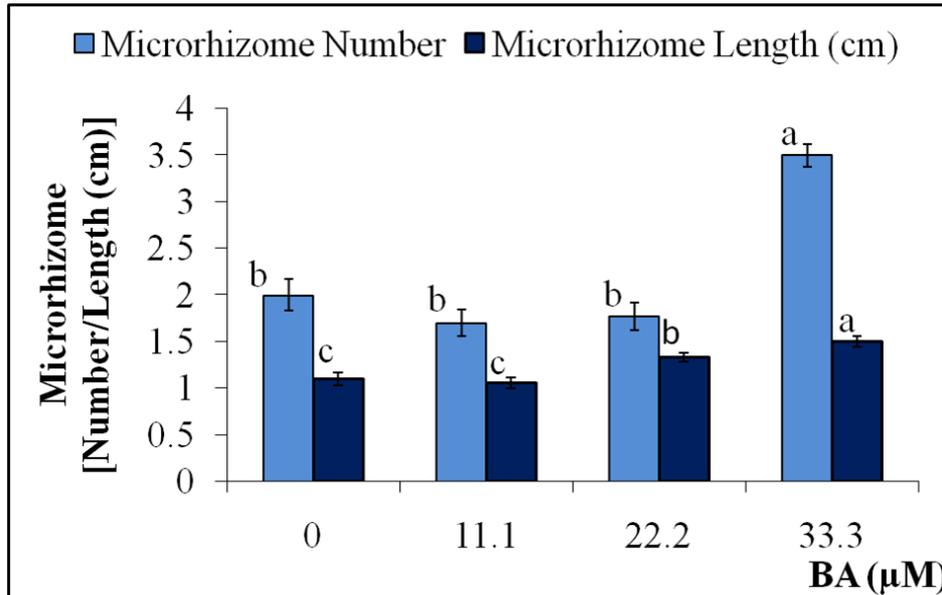


Figure 1 - Effect of BA concentration on *in vitro* microrhizome formation of *Acorus calamus* in MS medium supplemented with 60 g L⁻¹ sucrose (6%), 100 mg L⁻¹ citric acid and 1 g L⁻¹ PVP-40. Observations were recorded after the 30 days of culture.

Each experiment consisted of 10 replicates and each experiment was repeated three times. Similar letters on mean values do not differ significantly at 5% level, by DMRT.

ANOVA:	df	F	p
Shoot number:	3	33.347	<0.0001
Shoot Length:	3	12.016	<0.0001



Figure 2 - *In vitro* microrhizome production of *Acorus calamus*, on MS medium supplemented with 33.3 µM BA, 60 g L⁻¹ sucrose, 100 mg L⁻¹ citric acid and 1 g L⁻¹ PVP-40.

Encapsulation and Cold Storage

The regenerated microrhizomes of *A. calamus* were encapsulated in calcium alginate gel using sodium alginate and calcium chloride solutions to produce synthetic seeds (Fig. 3A). Synthetic seeds were also produced by encapsulation of *in vitro* grown vegetative propagules in calcium alginate beads in many species i.e., *Acacia* hybrid²², *Cassia angustifolia*²³, etc. They can be used like real seeds for transport, storage and plant production. Therefore, synthetic seeds of *A. calamus* were further tested for cold storage for mid-term conservation. For the purpose, they were

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aseptically sealed in petri-dishes and were kept at 10°C in complete darkness for different durations. Up to 6 months, the cold stored synthetic seeds were showed 100% survival when re-cultured onto the microrhizome production medium (Fig. 3B and Fig. 4). After the one year of cold storage, 80% synthetic seeds were survived. Thus, encapsulated microtubers as synthetic seeds were stored for one year without any subculture in economic manner. Although the microrhizome cold storage of *A. calamus* has not been reported previously, but the cold storage of *in vitro* raised storage organs has been reported in other plant species. In *Zingiber* microrhizomes, 50% survival was recorded up to 14 months under 16 h and 24 h light in normal culture condition; and only 20% survival under dark incubation²⁴. Potato microtubers were stored in dark at 4°C for 12 to 15 months²⁵. Nonetheless, *in vitro* culture storage provides conservation and safe exchanges of germplasm that can be bulked up again whenever required²⁶.

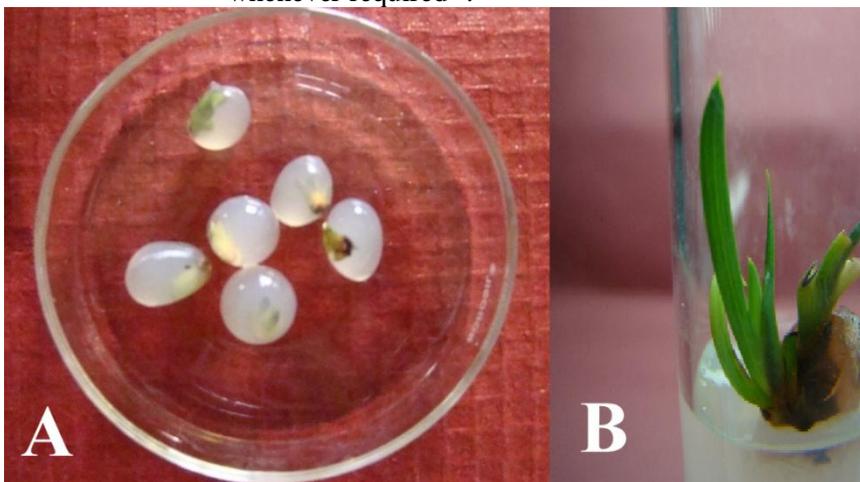


Figure 3 - *In vitro* cold storage of *Acorus calamus*. A: Synthetic seeds produced by microrhizome encapsulation in calcium alginate beads. B: Re-growth of synthetic seed after one year of storage at 10°C in dark.

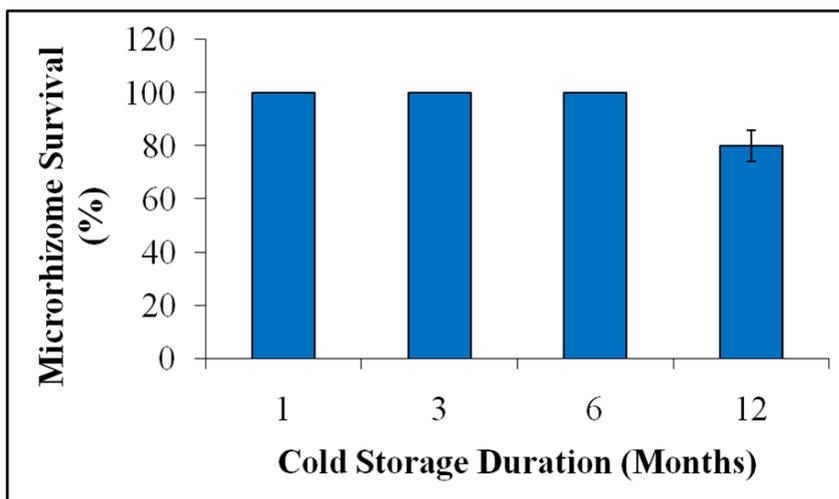


Figure 4 - Survival percentage of *in vitro* cold stored (10°C in dark) synthetic seeds of *Acorus calamus*, after the re-culture. Each experiment consisted of 10 replicates and each experiment was repeated three times.

Rooting and Acclimatization

Each microrhizome was aseptically transferred to rooting medium for complete plantlet formation. These microrhizomes were easily rooted in presence of an auxin-4.9 µM IBA in half strength MS medium. All the regenerated plantlets were

survived after one month of acclimatization in greenhouse. Similar, high rate of survival in *A. calamus* microrhizomes after acclimatization was also recorded previously⁶.

CONCLUSION

Thus, the developed protocol facilitates simple *in vitro* technique for synthetic seed production by encapsulation of microrhizomes and conservation *via* these synthetic seeds in complete dark at 10°C up to one year; for this economically important medicinal and aromatic plant- *A. calamus*.

ACKNOWLEDGEMENTS

Authors are thankful to Head of School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur (India) for providing laboratory facilities and support.

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Received: February 03, 2016;
Accepted: July 14, 2016