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In vitro microrhizome induction in *Acorus calamus* L., a commercially important aromatic medicinal plant

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Abstract

Acorus calamus L., is a leading aromatic medicinal plant that produces pungent aromatic rhizomes that are valued worldwide as an important herbal medicine and is one of the main ingredients in several polyherbal formulations used for neurological and metabolic disorders. The present investigation aims to develop an efficient protocol for *in vitro* microrhizome induction in *A. calamus* for the large-scale production of disease-free planting material for commercial purposes. *In vitro* derived shoots were initiated on MS medium supplemented with different concentrations of sucrose alone (3–10 %) and different concentrations of sucrose (6 % and 7 %) with varying concentrations of BAP alone (0.5–2 mg/L) and combinations with IAA (0.5 and 1 mg/L) and NAA (0.5 and 1 mg/L) were carried out for the microrhizome induction experiments. In different concentrations of sucrose used, healthy and disease-free microshoots of *A. calamus* were obtained in MS media supplemented with 7 % sucrose followed by 6 % sucrose. The highest shoot length (24.79 ± 0.03 cm) and microrhizome size (4.98 ± 0.03 cm length and 399.60 ± 0.37 mg fresh weight) were obtained in MS solid medium supplemented with 1 mg/L BAP and 0.5 mg/L NAA with 7 % sucrose followed by the same hormone concentration with 6 % sucrose (shoot length-23.68 ± 0.03 cm, microrhizome length- 4.68 ± 0.03 cm and fresh weight of microrhizome-376.60 ± 0.57 mg). The developed protocol can be used for large-scale production of disease-free propagules without rooting and acclimatization and enhance the production of true-to-type planting material.

Keywords

A. calamus; microrhizome; sucrose; plant growth regulators

Introduction

Acorus calamus L., is an important aromatic medicinal monocotyledonous perennial herb belonging to the family Acoraceae. The plant is semi-aquatic, distributed in the marshy areas of temperate to sub-temperate regions and is commonly known as "Sweet Flag" or "Bach" (1-4). Naturally, the medicinal herbs are distributed in the high-altitude regions of India, predominantly in the swamps, streams and marshy areas of Himalayan and sub-Himalayan regions, Madhya Pradesh, Kerala, Andhra Pradesh, Assam, Chhattisgarh, Tamil Nadu, Karnataka, etc (2-5). The rhizome is the most commonly used part of the plant for medicinal preparations and more than 145 chemical constituents were identified from the medicinal herb. The

major chemical constituents include phenylpropanoids (α-asarone, β-asarone, eugenol, isoeugenol, etc.), sterols, triterpene glycosides, triterpenoid saponins, sesquiterpenoids, monoterpenes and alkaloids (4-7). The plant is used in the traditional system of medicine (Indian and Chinese) for the treatment of epilepsy, mental ailments, chronic diarrhoea, dysentery, bronchial catarrh, intermittent fevers, cough, throat irritations, bronchitis and tumors (8-12). The dried rhizome of the plant is widely used for the production of several ayurvedic polyherbal formulations for the treatment of neurological and metabolic disorders (4, 13, 14) and is also used in food products and alcoholic beverages as a flavoring agent (9, 15).

In India alone, 500–1000 metric tons (MT) of the dried rhizome of *A. calamus* are traded annually for the production of polyherbal formulations and Ayurvedic preparations (16). For medicinal preparations in India, bulk quantities of the rhizomes were harvested from its natural habitat and lesser quantities were gathered from cultivation areas (5). Naturally, the plant is vegetatively propagated by means of rhizome cuttings and is time-consuming for mass cultivation because of the slow growth nature of rhizome cuttings (17) and the rhizomes used for vegetative propagation are susceptible to diseases that cause tissue senescence and degeneration of the plant. So, there is a need for large-scale production of disease-free *A. calamus* planting material for commercial purposes. The present study focuses on the *in vitro* microrhizome induction in *A. calamus* under the influence of different sucrose concentrations alone and different concentrations and combinations of plant growth regulators (PGRs) with different concentrations of sucrose using MS solid media.

Materials and Methods

Establishment of culture

In vitro-derived disease-free and healthy microshoots of *Acorus calamus* were maintained in the *in vitro* repository of KSCSTE-JNTBGRI. The maintained explants were transferred in hormone-free Murashige and Skoog (MS) medium containing 3.0 % (w/v) sucrose and solidified with 0.65–0.7 % (w/v) agar for microrhizome induction experiments and maintained for 1 week. After 1 week, the *in vitro*-derived healthy microshoots of *A. calamus* were used for microrhizome induction experiments.

Microrhizome induction in A. calamus

In the first experiment, *in vitro*-derived shoot explants of *A. calamus* approximately 3–5 cm in length were cultured in MS basal medium for a week. For the preliminary microrhizome induction experiments, these *in vitro* shoots were initiated in MS medium supplemented with different concentrations of sucrose (3–10 % w/v). This experiment helped in confirming the accurate sucrose concentration required for good microrhizome induction. Based on the first experiment result, MS medium containing 7 % and 6 % sucrose was supplemented with different concentrations of BAP (0.5 and 1.2 mg/L) alone and different concentrations of BAP (0.5 and 1 mg/L) combined with NAA (0.5 and 1 mg/L) and IAA (0.5 and 1 mg/L) were used to test the effect of PGRs on microrhizome induction in *A. calamus*. MS medium containing 6 % and 7 % sucrose without any PGRs included as a control.

Culture condition

Initiated cultures were grown under a 12 h photoperiod at 25 ± 2 °C. All the experiments were repeated three times and observations on the number of shoots forming microrhizomes, fresh weight and number of microrhizomes per shoot were recorded after 6 weeks of the experiment.

Anatomical and histochemical studies

Anatomical and histochemical studies were carried out to confirm the microrhizome formation and to compare rhizome development. Thin transverse sections of the microrhizomes and conventional rhizomes were taken for anatomical studies. Thin transverse sections of the rhizomes were stained with Lugol's iodine solution (Himedia, Mumbai) for 2-3 min and mounted on a glass slide for histochemical localization of starch. The amount of oil cells and starch were observed, recorded and photomicrographed using Leica DM and attached to the Leica DM 2500 trinocular microscope.

Statistical analysis

Every experiment was repeated thrice and 25 explant replicates were inoculated with one explant per culture bottle. All the collected data were statistically analyzed by Analysis of Variance (ANOVA) and the means were compared by Duncan's multiple range test ($p<0.05$) using SPSS/PC + 4.0 (SPSS Inc. Chicago, USA).

Results and Discussion

Different concentrations of sucrose (3-10 % w/v) were tested without any plant growth regulators to determine the most effective concentration in the induction of microrhizome using an MS solid medium. Microrhizome formation was started from the shoot base of *Acorus calamus* in an MS medium supplemented with different sucrose concentrations. Different sucrose concentrations in MS medium showed variable responses in terms of plant length, microrhizome size and fresh weight in *A. calamus*. High sucrose concentrations in the MS medium had a positive impact on the development and growth of the microrhizome. The explants on MS media containing 4–10 % w/v sucrose produced different sizes of microrhizomes, whereas microrhizomes were not induced on MS medium with 3 % w/v sucrose. MS medium having 7 % sucrose had the highest shoot length (14.69 ± 0.04 cm), the largest microrhizome size (3.30 ± 0.03 cm length and 3.80 ± 0.14 mm diameter) and maximum fresh weight (298.68 ± 0.32 mg) followed by 6 % sucrose with an average shoot length of 13.86 ± 0.04 cm with a microrhizome of 2.71 ± 0.02 cm length and 2.80 ± 0.23 cm diameter and 251.36 ± 0.21 g fresh weight (Table 1; Fig. 1). Shoot length, microrhizome size (length and diameter) and fresh weight of *A. calamus* gradually decreased with the further increase in sucrose concentrations (80–100 % w/v).

Table 1. Effect of different sucrose concentration on microrhizome induction in *A. calamus.*

Sl. No..	Sucrose (g L^1)	Response (%)	Mean shoot length (cm)	Length of microrhizome	Diameter of microrhizome (mm)	Fresh weight of microrhizome (mg)
1	30	82	2.94±0.07^{h}	0^{g}	0^{e}	0^{h}
2	40	88	5.01±0.03^{g}	0.40±0.01^{f}	1.52±0.10^{d}	126.60±0.25^{g}
3	50	86	9.95±0.02^{d}	0.83±0.01^{e}	1.92±0.09^{d}	183.68±0.30^{f}
4	60	88	13.86±0.04^{b}	2.71±0.02^{d}	2.80±0.23^{c}	251.36±0.21^{c}
5	70	82	14.69±0.04^{a}	3.30±0.03^{a}	3.80±0.14^{a}	299.68±0.32^{a}
6	80	80	10.25±0.03^{c}	3.26±0.02^{a}	3.40±0.20^{ab}	291.72±0.36^{b}
7	90	76	6.94±0.03^{e}	3.05±0.02^{b}	3.24±0.19^{bc}	246.36±0.23^{d}
8	100	76	5.34±0.02^{f}	2.92±0.02^{c}	2.92±0.21^{bc}	224.40±0.25^{e}
ANOVA		Df (n-1)= 7	F = 9941.9***	F = 3459.2***	F = 56.9***	F = 142147.6***

Data for mean shoot length, length of microrhizome, diameter of microrhizome and fresh weight of microrhizome are mean ± standard error of the mean (SE) of 25 explant replicates. Values are expressed as means followed by same letters in each column are significantly not different (Duncan's multiple range test, $p \leq 0.05$).

The result for *in vitro* microrhizome induction by the combined effect of sucrose and PGRs such as BAP alone and BAP fortified with NAA and IAA showed significant differences in the growth parameters such as plant length, microrhizome size and fresh weight (Table 2; Fig. 1). Shoot buds cultured in MS medium supplemented with 1 mg/L BAP and 0.5 mg/L NAA with 7 % sucrose responded better in terms of most parameters recorded (shoot length - 24.79 ± 0.03 cm; rhizome length- 4.98 ± 0.03 cm and fresh weight- 399.60 ± 0.37 mg) followed by 1 mg/L BAP and 0.5 mg/L NAA with 6 % sucrose exhibiting an average shoot length of 23.68 ± 0.03 cm, size of 4.68 ± 0.03 cm length and fresh weight of 376.60 ± 0.57 mg and 1 mg/L BAP and 1 mg/L NAA with 7 % sucrose averaging to a shoot length of 22.94 ± 0.02 cm, 4.48 ± 0.03 cm length and 344.24 ± 0.26 mg of microrhizome fresh weight. MS medium supplemented with different concentrations of BAP and IAA with 6 % and 7 % sucrose also showed microrhizome formation. Among the different concentrations and combinations of BAP and IAA used, 1 mg/L BAP and 0.5 mg/L IAA with 7 % sucrose showed good microrhizome production (shoot length- 21.58 ± 0.03 cm; size- 4.36 ± 0.02 cm length and fresh weight 366.08 ± 0.32 mg) followed by 1 mg/L BAP and 1 mg/L IAA with 7 % sucrose with a shoot length of

Table 2. Effect BAP and IBA/IAA/NAA on microrhizome induction in *A. calamus* containing 6 % and 7 % sucrose.

Sl. No.	Sucrose (g L^{-1})	Plant growth regulators			Percentage of response (%)	Mean shoot length (cm)	Length of microrhizome (cm)	Fresh weight of microrhizome (mg)
		BAP	IAA	NAA				
1	60				84	13.88±0.05^{t}	2.76±0.03^{n}	251.44±0.34^{t}
2	70				82	14.70±0.04^{s}	3.30±0.04^{k}	300.64±0.23^{n}
3	60	0.5			86	16.49±0.03^{p}	2.94±0.02^{m}	272.88±0.27^{s}
4	60	1.0			84	18.70±0.03^{m}	2.93±0.02^{m}	293.80±0.27^{o}
5	60	2.0			86	15.59±0.04^{q}	2.58±0.02^{o}	232.80±0.29^{u}
6	70	0.5			80	17.60±0.04^{o}	3.77±0.02^{i}	328.56±0.23^{i}
7	70	1.0			84	19.68±0.03^{k}	3.89±0.02^{h}	350.12±0.31^{e}
8	70	2.0			86	15.47±0.02^{r}	3.48±0.03^{j}	313.76±0.44^{i}
9	60	0.5	0.5		88	18.37±0.03^{n}	3.11±0.03^{l}	283.04±0.33^{r}
10	60	0.5	1.0		88	19.50±0.02^{l}	3.45±0.02^{j}	290.80±0.31^{p}
11	60	1.0	0.5		86	21.15±0.03^{f}	3.76±0.02^{i}	299.72±0.32^{n}
12	60	1.0	1.0		88	19.46±0.02^{l}	3.22±0.02^{k}	289.20±0.34^{q}
13	70	0.5	0.5		80	18.63±0.02^{m}	3.90±0.02^{h}	343.60±0.36^{fg}
14	70	0.5	1.0		78	19.66±0.02^{k}	4.11±0.03^{g}	357.44±0.37^{d}
15	70	1.0	0.5		78	21.58±0.03^{e}	4.36±0.02^{de}	366.08±0.32^{c}
16	70	1.0	1.0		80	20.32±0.04^{i}	4.25±0.02^{e}	342.68±0.30^{g}
17	60	0.5		0.5	86	19.88±0.03^{j}	3.47±0.02^{j}	305.80±0.41^{m}
18	60	0.5		1.0	84	20.47±0.02^{h}	3.96±0.03^{h}	323.28±0.35^{k}
19	60	1.0		0.5	88	23.68±0.03^{b}	4.68±0.03^{b}	374.60±0.57^{b}
20	60	1.0		1.0	86	21.18±0.04^{f}	4.30±0.03^{ef}	324.72±0.47^{j}
21	70	0.5		0.5	82	20.90±0.03^{g}	3.90±0.03^{h}	314.32±0.34^{j}
22	70	0.5		1.0	82	22.77±0.02^{d}	4.40±0.03^{cd}	336.08±0.37^{h}
23	70	1.0		0.5	80	24.79±0.03^{a}	4.98±0.03^{a}	399.60±0.37^{a}
24	70	1.0		1.0	78	22.94±0.02^{c}	4.48±0.03^{c}	344.24±0.26^{f}

Main effect	Df (n-1)= 23	-	-	-
	F value	6130.7***	434.6***	12255.2***
Hormone type	Df (n-1)= 2	-	-	-
	F value	79.4***	44.6***	44.6***
Concentration	Df (n-1)= 5	-	-	-
	F value	1086.0***	437.8***	437.8***
Hormone type × Concentration	Df (n-1)= 17	-	-	-
	F value	509.3***	286.4***	286.4***

Data for mean shoot length, length of microrhizome, diameter of microrhizome and fresh weight of microrhizome are mean ± standard error of the mean (SE) of 25 explant replicates. Values are expressed as means followed by same letters in each column are significantly not different (Duncan's multiple range test, $p \leq 0.05$).

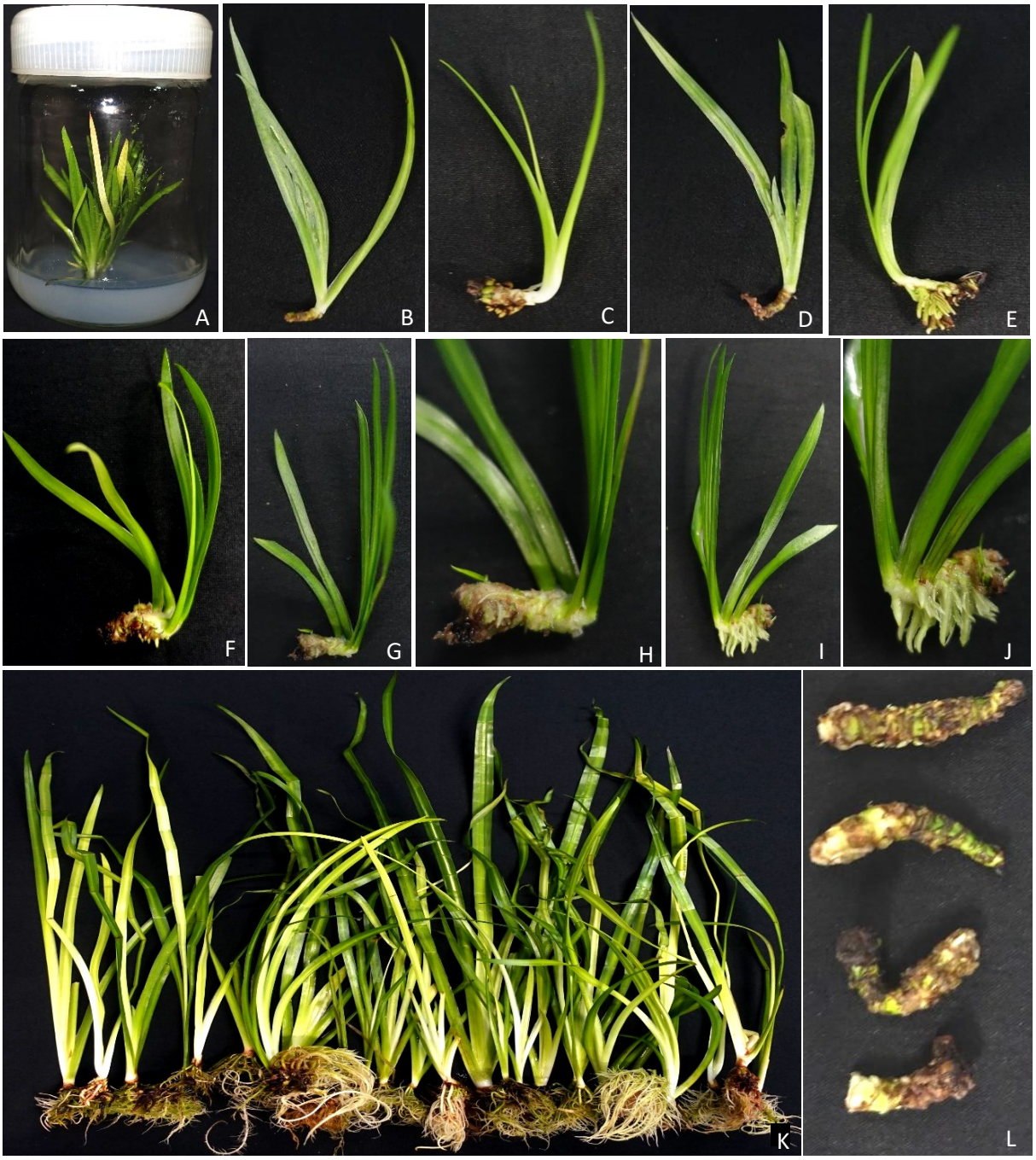

Fig. 1. Microrhizome induction in *A. calamus*. **A**: *In vitro* derived microshoots for microrhizome induction; **B** & **C**: Microrhizome formation at the base of *in vitro* derived shoots in MS medium with 6 % sucrose (**B**-after 4 weeks; **C**-after 6 weeks); **D** & **E**: Microrhizome formation at the base of *in vitro* derived shoots in MS medium with 7 % sucrose (**D**-after 4 weeks; **E**-after 6 weeks); **F**-**H**: Microrhizome formation at the base of *in vitro* derived shoots in MS medium with 1 mgl/L BAP+0.5 mgl/L NAA+7 % sucrose (**F**- after 4 weeks; **G** & **H** after 12 weeks); **I** & **J**: Microrhizome formation at the base of *in vitro* derived shoots in MS medium with 1 mgl/L BAP+0.5 mg/L IAA+7 % sucrose (**I** & **J**-after 12 weeks); **K**: Microrhizome induced rooted mericlones of *A. calamus* after 12 weeks of culture. **L**: Harvested microrhizome.

21.15 ± 0.03 cm, 4.25 ± 0.02 cm length and 357.44 ± 0.37 mg of microrhizome fresh weight (Table 2; Fig. 1 and 2). The result indicated that BAP has less effect on microrhizome induction in *A. calamus* as compared to sucrose. Microrhizome induction of *A. calamus* was further confirmed by anatomical and histochemical studies using Lugol's iodine solution. Transverse sections of *in vitro* raised microrhizomes and conventional rhizomes indicated a high amount of oil cells. The color of oil cells appeared light yellow, starch content was high throughout the cells and it appeared dark blue (Fig. 3). Transverse sections of *in vitro* raised microrhizomes did not show any variations when compared with conventional rhizomes. So, the developed protocol can be applied to the large-scale production of high-value medicinal plants.

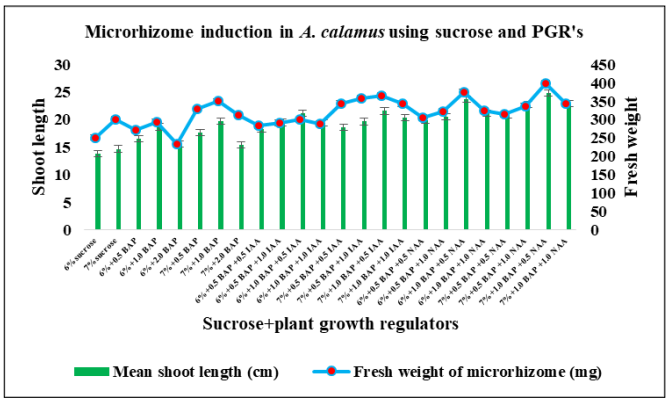

Fig. 2. Effect of BAP and IBA/IAA/NAA on microrhizome induction in *A. calamus* containing 6 % and 7 % sucrose.

elevated sucrose levels (3–10 %) and larger microrhizomes were capable of survival in the field without any direct acclimatization procedure (22-24). In addition, these microrhizome-developed plants can be easily transported as they do not require any culture medium for their survival or any other special measures to prevent contamination for a short period of time (17, 24, 25).

Higher levels of sucrose in the MS medium induce the formation of microrhizome in *A. calamus* (17-19). It was reported that the first instance of microrhizome induction in *A. calamus* using MS dual-phase media (agar solidified phase overlaid by a liquid fraction of the same medium). Different sucrose concentrations (2–10 %) were employed to determine the most effective concentration in the induction of microrhizome. The different sucrose concentrations showed a variable response in terms of size and fresh weight of the microrhizomes, and 6 % of the sucrose concentrations had the largest size (3.9 cm length and 0.47 cm diameter) and maximum fresh weight of 0.72 g. The best response was observed in the medium supplemented with 2.0 mg/L IBA and 6 % sucrose, which produced a maximum fresh weight of rhizome (0.82 g) and size (length 4.8 cm; diameter 0.55 cm) in 6 weeks. Studies reported that microrhizome production in *A. calamus* using MS media. Maximum microrhizome production was observed in the presence of 33.3 µM BA on a modified MS medium containing 6 % sucrose, 100 mg/L citric acid and 1 g/L polyvinyl pyrrolidone-40 (18). Based on the previous reports, different sucrose concentrations (2–10 %) were used for microrhizome

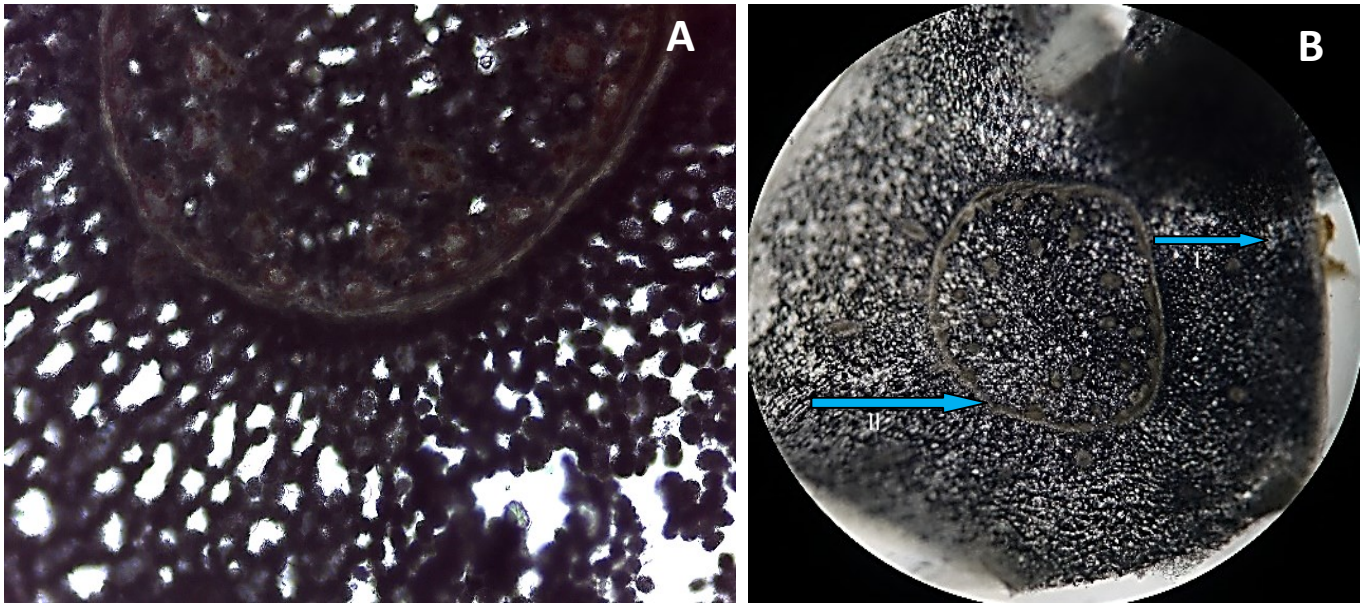

Fig. 3. Anatomical analysis of rhizome and *in vitro* derived microrhizome of *A. calamus* (**A**) Rhizome of *A. calamus* and (**B**) *In vitro* derived microrhizome of *A. calamus* (**I** -Starch cells (appeared as blue)) and **II**- Oil cells (appeared as yellow))

A higher level of sucrose in plant tissue culture medium facilitates higher carbon energy to induce the formation of storage organs (microrhizome) *in vitro* (18-24). In recent times, *in vitro,* induction of storage organs such as rhizomes has been encouraged because the developed large-scale disease-free and true-to-type shoots can be directly transferred to the field without any acclimatization or hardening process. Microrhizomes of many rhizomatous plants were found to develop *in vitro* in MS medium with induction in *A. calamus.* Among different concentrations used in the previous studies, 6 % sucrose in MS media was observed for good microrhizome induction in *A. calamus.*

The present study showed the influence of sucrose and PGRs in microrhizome induction and variable growth responses in *A. calamus.* Better microrhizome production in terms of variable growth responses such as shoot length (24.79 ± 0.03 cm), microrhizome length (4.98 ± 0.03 cm), and fresh weight of microrhizome (399.60 ± 0.37 mg), was

obtained in MS medium supplemented with 1 mg/L BAP and 0.5 mg/L NAA with 7 % sucrose. According to the aforementioned results, *A. calamus* was able to induce microrhizomes at a significantly higher rate in smaller amounts of PGRs with 7 % sucrose when no further additives or media modifications were introduced.

The promoting effect of sucrose and plant growth regulators on the formation of storage organs (microrhizome) has been reported earlier (26, 27). Several studies reported the specific concentrations of sucrose that were effective in the induction of microrhizome. 6 % sucrose concentration in *Curcuma zedoaria* (28), 6 % in *Curcuma aromatica* (29) and 6 % and 9 % in *Kaemferia rotunda* (22) and a study detailed the improved rate of *in vitro* organ formation. The increasing concentration of sucrose, which can be attributed to the presence of high carbon energy, is stored in the form of sucrose in the storage organs, which mostly store carbohydrates. The role of growth regulators and sucrose concentration in the *in vitro* induction of microrhizome in *A. calamus* can be compared to the *in vitro* formation of tubers, bulbs and corns in other plants (30). In the present study, good microrhizome induction and subsequent plant growth were observed. Compared to the previous studies, microrhizome helps bypass any hardening or acclimatization steps, thereby reducing the time span of 2 months compared to the normally raised *in vitro* plants that need these adaptation steps.

Conclusion

Production of microrhizome in *Acorus calamus* is significantly influenced by sucrose concentration and plant growth regulators. In the present study, the highest shoot length, largest size and maximum fresh weight of the microrhizome were achieved in MS medium fortified with 1 mg/L BAP and 0.5 mg/L NAA with 7 % sucrose. Increased microrhizome size enhanced establishment rate in the field, shoot height and number of shoots. The results of this work have applications in both germplasm conservation and large-scale multiplication of disease-free, true-to-type planting material that may be directly transferred to the field without acclimatization. The study discussed can help to attain numerous plants in a short span of time that can be exploited commercially in the future to meet the current market needs. Furthermore, standardizing a good potting mixture can, in turn, help in attaining efficient growth patterns.

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Authors' contributions

SF did the whole experiment, data collection, analysis and drafted the manuscript. AA participated in the experiments and manuscript preparation. RKR participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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