AL-SHODHANA
A Multi-Disciplinary Double Blind Peer Reviewed Research Journal
https://sadupublications.com/journals/index.php/al-shodhana
Open Access **ISSN(P): 2320-6221**

PALMYRAH PALM IN VITRO EMBRYO CULTURE AND HISTOLOGICAL ANALYSIS OF EMBRYONIC DEVELOPMENT IN CULTURE MEDIA

Dr. Jiji George*, Dr. Anitha Karun**
*Assistant Professor, St. Aloysius (Deemed to be University), Mangaluru- 575003, Karnataka, India
**Former Director in-charge, ICAR-Central Plantation Crops Research Institute, Kasaragod-671124, Kerala, India.

ARTICLE HISTORY

Received : 24-07-2024
Revised : 02-09-2024
Accepted : 24-09-2024
Published : 12-10-2023

Author Affiliation: India

Corresponding Author:
Dr. Jiji George

Keywords:
Apocolon, Borassus flabellifer, Cotyledonary tube, Embryo culture, Hypogeal germination.

Abstract

Palmyrah palm (Borassus flabellifer) propagation relies solely on seeds due to the lack of vegetative methods. Seed germination, a slow hypogeal process forming a "cotyledonary tube" (apocolon), takes 5-6 months. This study presents the first attempt at in vitro culture of Palmyrah palm embryos with a histological analysis of their development. After 150 days, plumule emergence from the apocolon was observed in a limited number (2 out of 240) of cultured embryos. Half-strength Y3 medium yielded the fastest apocolon growth (88.33%), followed by half-strength MS medium (85%). However, shoot formation (3.33%) was observed only in half-strength Y3 medium. Histological analyses revealed an absence of clear differentiation in mature embryos during initial apocolon development. Higher magnification visualized meristematic centres within the embryonic axis, where cell division formed a proembryonal cell complex. As development progressed, the embryonic axis relocated towards the apocolon's tip. Leaf primordia formation was observed within the germ tube's posterior end after 120 days of incubation. These findings demonstrate the potential of in vitro culture for Palmyrah palm propagation, although further optimization is required to improve shoot development.

Corresponding author Email: jiji_george@staloysius.edu.in
DOI: https://doi.org/10.70644/as.v12.i2.4

Introduction

Palmyrah palm (*Borassus flabellifer L.*) is believed to be originated in Africa. The genus Borassus prefer tropical wet/dry climates. It spreads from Western Africa to Eastern Indonesia.The three species of economic importance are B. *aethiopum* Mart., occurring in Africa, B. *flabellifer* L.,

found in coastal areas of India, northern Sri Lanka, and mainland south-eastern Asia and B. *sundaicus*Becc., restricted to Indonesia (Davis& Johnson, 1987). Palmyrahpalmoccurs in India as wild as well as in cultivation, ranging from an altitude up to 760 meter above sea level. In India, the cultivated populations are prevalent in Kerala, Tamil Nadu, Karnataka, Andhra Pradesh, Maharashtra, Orissa, Madhya Pradesh, Bihar and West Bengal. The palm is also seen in scattered areasin Assam, Gujarat and Uttar Pradesh (Anonymous, 1948).

These palms are financially beneficial and widely cultivated in tropical regions. It has long been one of the most important trees of Cambodia, Sri Lanka and India. The poem "Thala Vilasam" by Arunachalam praises different Ayurvedicmedicines based on palmyrah. In Cambodia the tree is a national flora symbol/emblem that is seen growing around Angkor Wat. The government of Tamil Nadu has honoured the palm during 1978 by declaring it as a 'state tree'. Promotional campaign for introduction and popularization of the health and medicinal products, palm leaf articles, fibre processing and brush making, the government of Kerala has declared 2007 as the year of palmyrah. Kerala government established Kerala State Palmyrah Products Development and Workers' Welfare Corporation Limited (KELPALM) for the welfare of palmyrah workers across the state of Kerala.

Each part of the palm holds significant economic value. The fruit and tuberous seedlings provide food, while the sap is a source of beverages and sugar. The fibres from the fruits and leaves are utilized for making brushes, cordage, weaving, and plaiting. The trunk wood is used in construction and as fuel. Additionally, the spike and roots are key ingredients in various Siddha medicines. The male flower is specifically used in medicine to treat heart diseases. Among the four sugar-yielding palms—*Borassus flabellifer (palmyrah), Phoenix dactylifera, Caryotaurens* (sago), and *Cocos nucifera* (coconut)—the palmyrah palm was the highest producer of sugar and offered a wide range of both edible and non-edible products (Sankaralingam et al., 1999).

Currently, the palm is propagated solely through seeds, with no vegetative propagation methods available. Seed germination takes 5-6 months and follows a tubular type of process. During germination, the plumular portion of the developing embryo is pushed away from the seed by the elongation of the proximal part of the cotyledon, forming astructure commonly known as the "cotyledonary tube" or "apocolon."The first successful culture of embryos outside the seed was conducted with crucifers by Hanning (1904). Since then, embryo culture had been recognized for its several potential applications in plant research. It was particularly useful for rescuing embryos that failed to develop naturally within the fruit or seed due to defective endosperms (Johnston & Stern, 1957). Additionally, embryo culture mitigated lengthy dormancy periods caused by

physical and/or chemical inhibitors present in the fruit or seed, as excised embryos cultured in vitro, free from these inhibitors, typically germinated immediately (Hoded, 1977). Furthermore, the culture of isolated embryo segments was valuable for studying the development of primary meristems, organogenesis, and the interactions between different organs (Rabéchault& Cas, 1974).

This research paper presents the first attempt to culture palmyrah palm (*Borassus flabellifer*) embryos and provides a detailed histological analysis of their development in vitro using synthetic culture media. The study offers important insights into the stages of palmyrah embryogenesis, which are crucial for selecting the best tissues for plumule culture and somatic embryogenesis. Additionally, this research aims to establish a standardized protocol for palmyrah embryo culture, presenting a viable alternative to using seeds and seedlings for germplasm conservation. By allowing embryos to be transported and cultured in vitro, this approach can overcome the logistical challenges and biosafety risks associated with transporting seeds and seedlings, thereby enhancing the efficiency and effectiveness of germplasm conservation efforts.

Materials and Methods

Plant Material Collection

Mature fruits, approximately four months old, were harvested from the Central Plantation Crops Research Institute (CPCRI) premises in Kasaragod, Kerala. Figure 1a,b depicted a female palm bearing abundant fruits and a male palm with a cluster of male inflorescences, respectively. A total of 187 nuts were collected. Observations indicated that 18.65% of these nuts were single-seeded, 38% were double-seeded, 43.3% were triple-seeded, and 0.05% were quadruple-seeded (Figure 1c). Notably, in some triple-seeded fruits, two seeds were separated by a thin carpel (Figure 1d).

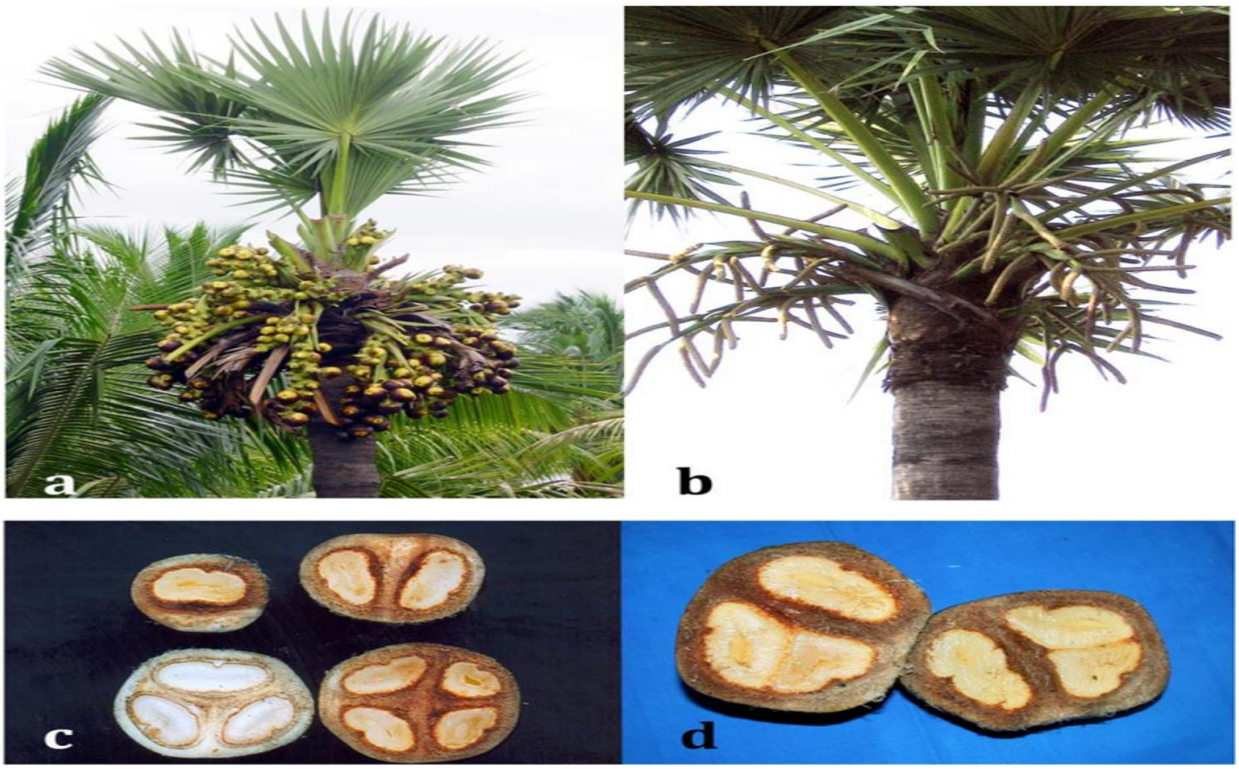

Figure 1. Palmyrah palm and C.S. of its different types of fruits showing carpel distribution. a, Female palm with profuse fruits. b, Male palm. c, C. S. of one, two, three and four seeded fruits. d, C. S. of three seeded fruit in which two seeds were separated by thin carpel.

Excision of Embryos and Surface Sterilization

The fruits were bisected using a saw, and the embryos were carefully excised with a knife. The embryos, located at the distal end of the fruit, were relatively large, measuring 0.9-1.1 cm in length and 0.7-0.8 cm in width, with a weight ranging from 170 to 190 mg. The excised embryos were then surface sterilized with a 20% sodium hypochlorite solution for 20 minutes, followed by three rinses with sterile water to remove any residual chlorine.

The embryos were inoculated into four treatment combinations using different culture media: full-strength Y3 (Eeuwens, 1978), full-strength MS (Murashige & Skoog, 1962), half-strength Y3, and half-strength MS. Each medium was supplemented with sucrose (35 g/L), agar (6 g/L), and activated charcoal (1 g/L). A total of 240 embryos were inoculated across the four treatments, with 20 embryos per treatment. The entire experiment was replicated three times. Immediately following inoculation, the cultures were incubated in darkness at 27 ± 2°C and 80-85% relative humidity to promote germination. Observations were recorded at two-week intervals. The survival of the embryos was assessed based on growth responses, such as the enlargement or elongation of the apocolon. The embryos were sub cultured monthly onto the same media

combinations. Embryos with emerging plumules were incubated under a photoperiod of 17 µmol m^{-2} s^{-1}. Data collected after 20 weeks of embryo culture were subjected to statistical analysis.

Histological Studies of Zygotic Embryogenesis

Embryos from palmyrah palm fruits at two- and four-months post-fertilization were used for histological studies. Mature embryos were fixed after incubation in culture medium for 0, 1, 2, 3, and 4 months, while immature embryos were fixed immediately after excision. Samples were fixed with Carnoy's 'B' fluid (chloroform: 30 ml, absolute alcohol: 60 ml, glacial acetic acid: 10 ml) for 24 hours, with fixation time adjusted based on tissue thickness and fixative penetration.

Dehydration, Infiltration, and Embedding

Fixed embryos were dehydrated with alcohol and butanol to remove water gradually. A mixture of paraffin wax and bee's wax (melting point 58-60°C) was used for infiltration and embedding. Dehydrated material was treated with wax in a 60°C oven, with fresh wax replacing molten wax over 3-4 days. Carmine powder was added to locate specimens before embedding in paraffin blocks.

Microtoming and Staining of Sections

The paraffin blocks were trimmed, fixed on wooden blocks, and uniform sections of 10 µm were cut using a Leica RM 2145 rotary microtome. The sections were affixed to pre-cleaned slides and applied with Gelatin (3%) as adhesive. The Slides were warmed to 45°C to flatten ribbons, then dried for 48 hours at room temperature. The Slideswere deparaffinized with xylene and dehydrated with butanol and alcohol. For water- based stains, slides were hydrated by dipping in water after alcohol treatment. The slides were stained with Periodic Schiff's reagent and Toluidine blue to localize cellular compounds. Stained sections were dehydrated, mounted in DPX, and observed under microscopes. Photographs were taken with a Leica DMLS microscope and analysed using Qwin software.

Result and Discussion

Characteristics of Embryo Development in Vitro

Four-month-old mature zygotic embryos (Figure 2a) were inoculated into four different media combinations and incubated under dark conditions. After two weeks, the embryos exhibited swelling, and the embryonic axis extended toward the germ tube. With prolonged culture, the germ tube or apocolon elongated (Figure 2b-d). The maximum survival of the embryos and apocolon growth was observed in half-strength Y3 medium (88.33%) (Figure 2e) and half-

strength MS medium (85%), both supplemented with 35 g/L sucrose, 6 g/L agar, and 1 g/L activated charcoal (Table 1). Shoot formation (3.33%) was observed only in half-strength Y3 medium (Figure 3).The embryo cultured in full-strength MS medium was subjected to severe browning, which resulted in retarded growth of the apocolon (Figure 3).

The emergence of plumules from the cotyledonary tube was observed in 2 out of 240 embryos cultured in vitro after 150 days (Figure 2f). In vitro germination followed the same pattern as natural seed germination. As the cotyledon elongated, the posterior extremity became distinct, with a smaller anterior segment diameter and a larger posterior segment diameter. The plumule emerged through a longitudinal rift at the beginning of the posterior segment. The plumule comprised a sheath or prophyll enclosing the shoot apex, covered like a cylindrical cap, and was preceded by an eophyll (a sheath, a short petiole, and a well-developed lamina), which emerged after 150 days in culture. Masilamani et al. (2020) reported that the number of leaves emerging from palmyrah seeds during germination could be improved by soaking them in 1% CaOCl2. However, there were no reports on breaking the dormancy of palmyrah nuts during germination.

Embryo culture, initially conducted with crucifers by Hanning (1904), involved aseptically excising an embryo from the seed and culturing it in a sterile nutrient medium. This technique effectively reduced dormancy periods caused by physical or chemical inhibitors present in the fruit or seed (Hoded, 1977). Additionally, it facilitated the rescueof embryos that failed to develop naturally and supported the growth of embryos resulting from interspecific hybridization, where defective endosperms were frequently encountered (Johnston & Stern, 1957). Mature embryos, once excised and free from these inhibitors, typically germinated immediately in vitro. Douglas (1987) investigated embryo culture in H. amaricaulis, a species closely related to H. lagenicaulis, and reported that only one embryo out of 51 seeds successfully germinated to produce a true leaf.

Embryo culture proved to be a valuable technique for the collection, exchange, and conservation of germplasm, particularly for palmyrah seed nuts, which were large and heavy. No protocols for embryo culture in palmyrah existed in the literature, although well-established protocols for coconut embryo culture were available (Karunaratne et al., 1991; Rillo & Paloma, 1992; Karun et al., 1993). The palmyrah embryo culture yielded essential insights into the developmental stages of embryos. These insights are crucial for understanding and selecting appropriate tissues from the culture tube, facilitating the induction of plumule culture and somatic embryogenesis.The culture of isolated embryo segments was instrumental in studying the development of primary meristems, organogenesis, and the interactions between different organs (Rabéchault& Cas, 1974).

Among the four-culture media tested for palmyrah embryo culture, apocolon formation and elongation as a germ tube were fastest in half-strength Y3 (88.33%) and half-strength MS (85%). However, shoot formation (3.33%) occurred only in half-strength Y3.

Similar results were reported by Karun et al. (1993) in coconut embryo culture, where maximum germination was achieved in full-strength Y3 medium supplemented with 30g/L sucrose and 1 g/L charcoal. In palmyrah embryo culture, shoot emergence from the apocolon was observed in 2 out of 240 embryos cultured in vitro after 150 days (Figure 3). Similarly, Douglas (1987) investigated embryo culture in H. amaricaulis, a species closely related to H. lagenicaulis, where only one embryo out of 51 seeds germinated to produce a true leaf.

The in vitro germination of palmyrah embryos closely mirrored the natural germination pattern of seed nuts. Specifically, the seed germination of palmyrah exhibited cotyledon elongation, which carried the embryonal axis away from the seed and formed a structure known as the “apocolon” (Sankaralingam et al., 1999). Tomlinson (1960) reported hypogeal germination in certain palms, where the embryo was pushed out of the seed through the elongation of the cotyledon’s proximal portion, forming a “cotyledonary axis.” The distal portion of the cotyledon inside the seed functioned as a haustorium. Mahabale and Kulkarni (1975) referred to this as the “cotyledonary tube.”

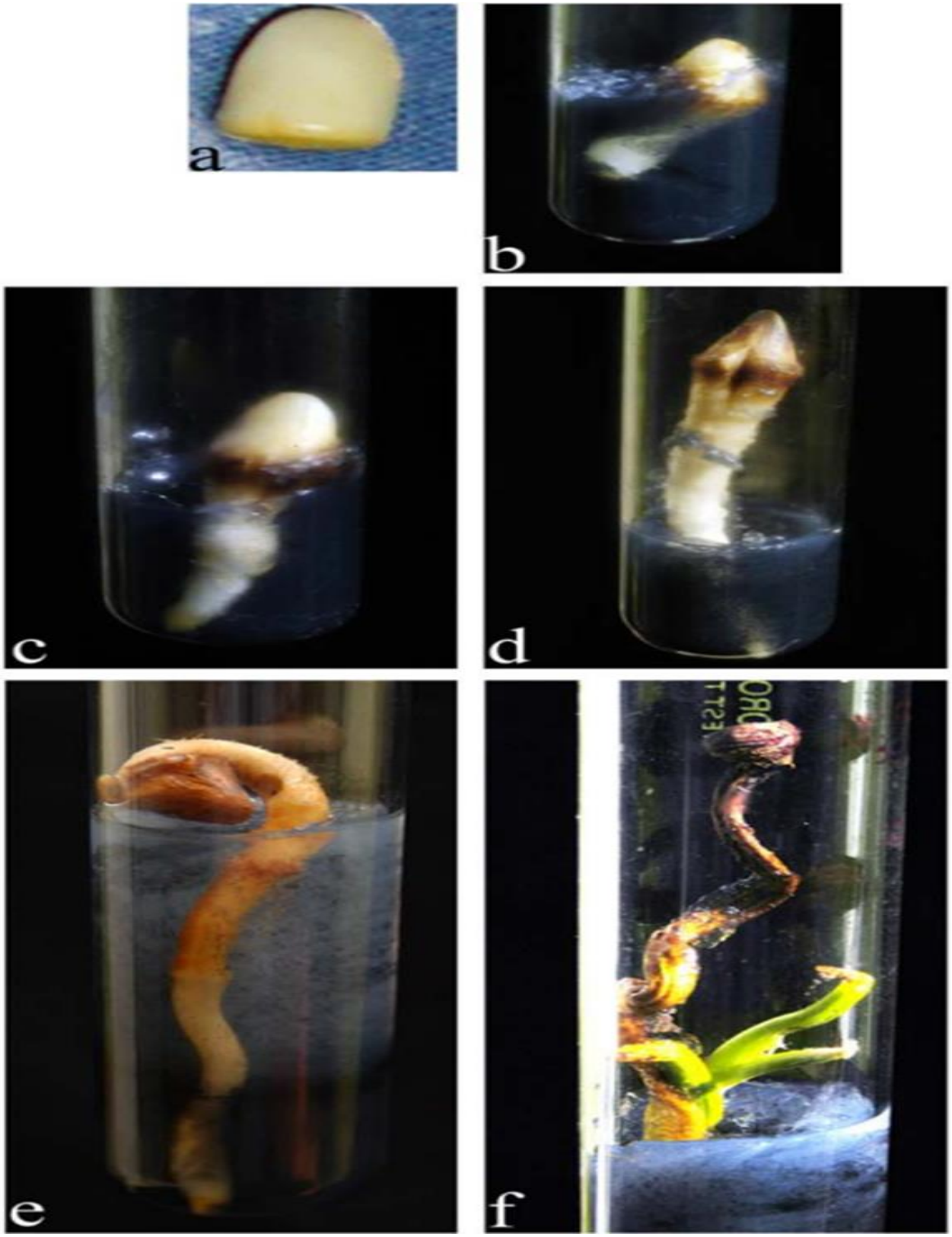

Figure 2. Developmental stages in palmyrah zygotic embryo culture. a, four months old mature palmyrah embryo. b, cultured palmyrah embryo for a period of one month in half strength Y3 medium with 35 g/l sucrose and 1 g/l charcoal. c, in vitro grown embryo after two months with elongated cotyledonary stalk. d, in vitro grown embryo after three months. e, elongated

cotyledonary stalk after four months in half strength Y3 medium. f, Plumule emerged from the posterior end of cotyledonary stalk after 150 days in embryo culture medium.

Table 1. Percentage of browning, survival, average length of apocolon and plumule emergence from embryos cultured on different media after 150 days.

Treatment	Media	Browning (%)	Survival (%)	Plumule emerged (%)	Average apocolon length (cm)
1.	Full strength Y3	3.33 [b]	60.00 [b]	0	4.31 [b]
2.	Full strength MS	100.00 [a]	45.00 [c]	0	2.35 [c]
3.	Half strength Y3	0	88.33 [a]	3.33 [a]	8.59 [a]
4.	Half strength MS	0	85.00 [a]	0	5.05 [b]
	Mean CD (p=0.05)	25.83 5.44	69.58 11.86	0.83 2.72	5.07 1.60

Figures in a column bearing same alphabets as superscript are not significantly different.

Histology of Embryo Development in Vitro

Anatomical differentiation during palmyrah zygotic embryo development was investigated using histological techniques. Various developmental stages of embryos were utilized for this study. In two-month-old fruits, the embryo size was very small (about 3 mm) and the endosperm was established as jelly form (Figure 4a,b). In four months, old fruit the endosperm was very hard, and the embryo size ranges between 0.7 to 1 cm in length (Figure 4c). Histological sections revealed that mature embryos do not show clear differentiation during the initial stages of apocolon development (Figure 4b-d). After one month in culture medium, histological studies showed that embryos enlarged in size and started germination through the elongation of the germ tube (Figure 4e,f), with the embryonic axis moving towards the tip of the germ tube, resembling hypogeal germination. Higher magnification revealed meristematic centres at the embryonic axis, where cells divided to form a proembryonal cell complex consisting of several proembryo units (Figure 4g, h). As development progressed, the embryonic axis moved towards the extremity of the apocolon, eventually locating at its tip region (Figure 5a-d). Leaf primordia development was observed within the posterior end of the germ tube after120 days of incubation in culture medium in vitro (Figure 5e, f).

Lossi et al. (2006) documented a remote-tubular type of germination in Phoenix roebelenii, while Pinheiro (2001) observed a similar pattern in Schippia concolor Burret. Histological insights into the ontogeny of palmyrah palm zygotic embryogenesis can provide valuable information for refining tissue culture techniques, especially for plantlet regeneration through plumule culture.

Despite plumule development occurring within each apocolon during palmyrah embryo culture, only a few progresses into complete plants. Further refinement of this technique is necessary to expedite plumule emergence rather than apocolon elongation and to enhance the percentage of complete plant development. The embryo culture technique can alleviate challenges associated with seedling transportation, particularly in palms like palmyrah, where germination follows a hypogeal/tubular pattern.

Figure 3. Percentage of browning, survival, average apocolon length and plumule emergence of palmyrah embryos cultured in various media combinations.

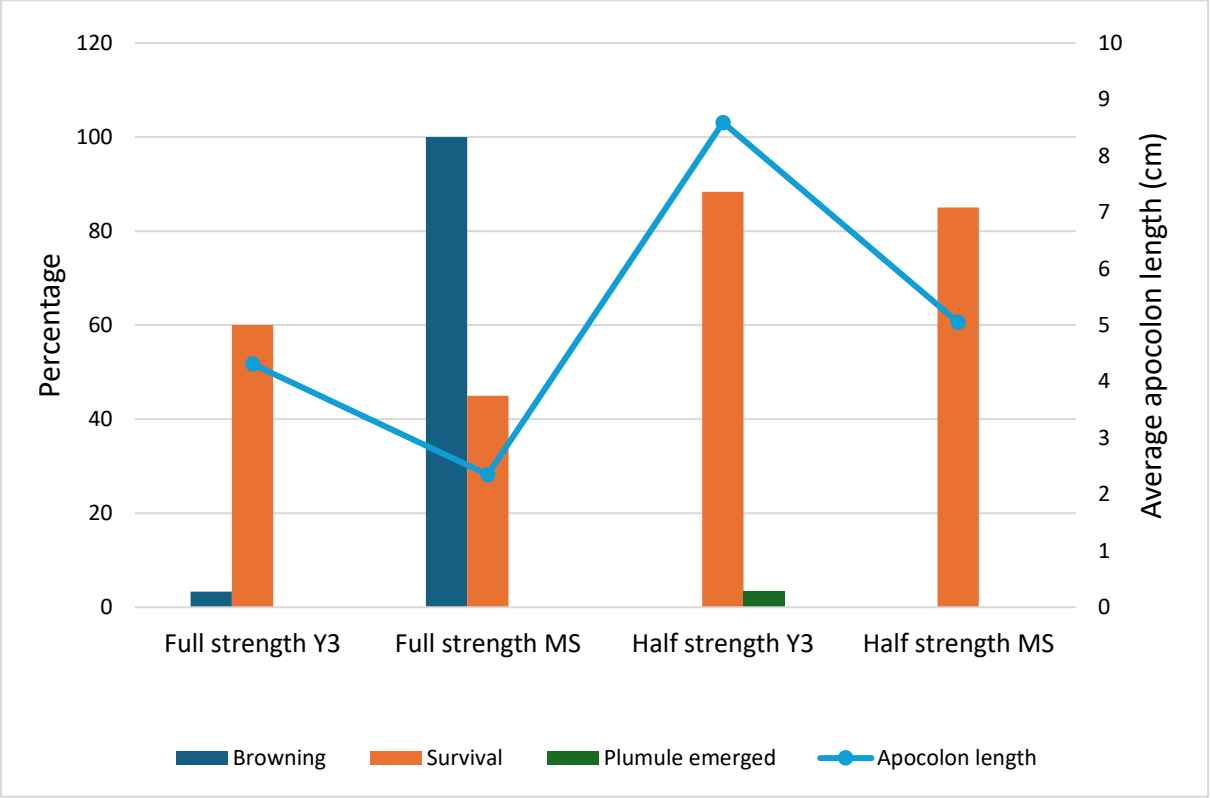

Figure 4. Histological studies on early stages of zygotic embryo development in vitro in palmyrah. a, immature embryo with endosperm (2 months after fertilization). b, developing embryonic axis in a longitudinal section of immature palmyrah embryo. c, a mature embryo of B. flabellifer (4 months old). d, L. S. of a mature palmyrah embryo showing embryonic axis. e, germinating embryo after one month incubation in embryo culture medium. f, L.S. of one month grown embryo in vitro. g, embryonic axis of one month grown embryo at higher magnification. h, centres of meristematic activity showing large, nucleated cells in the embryonic axis (arrow).

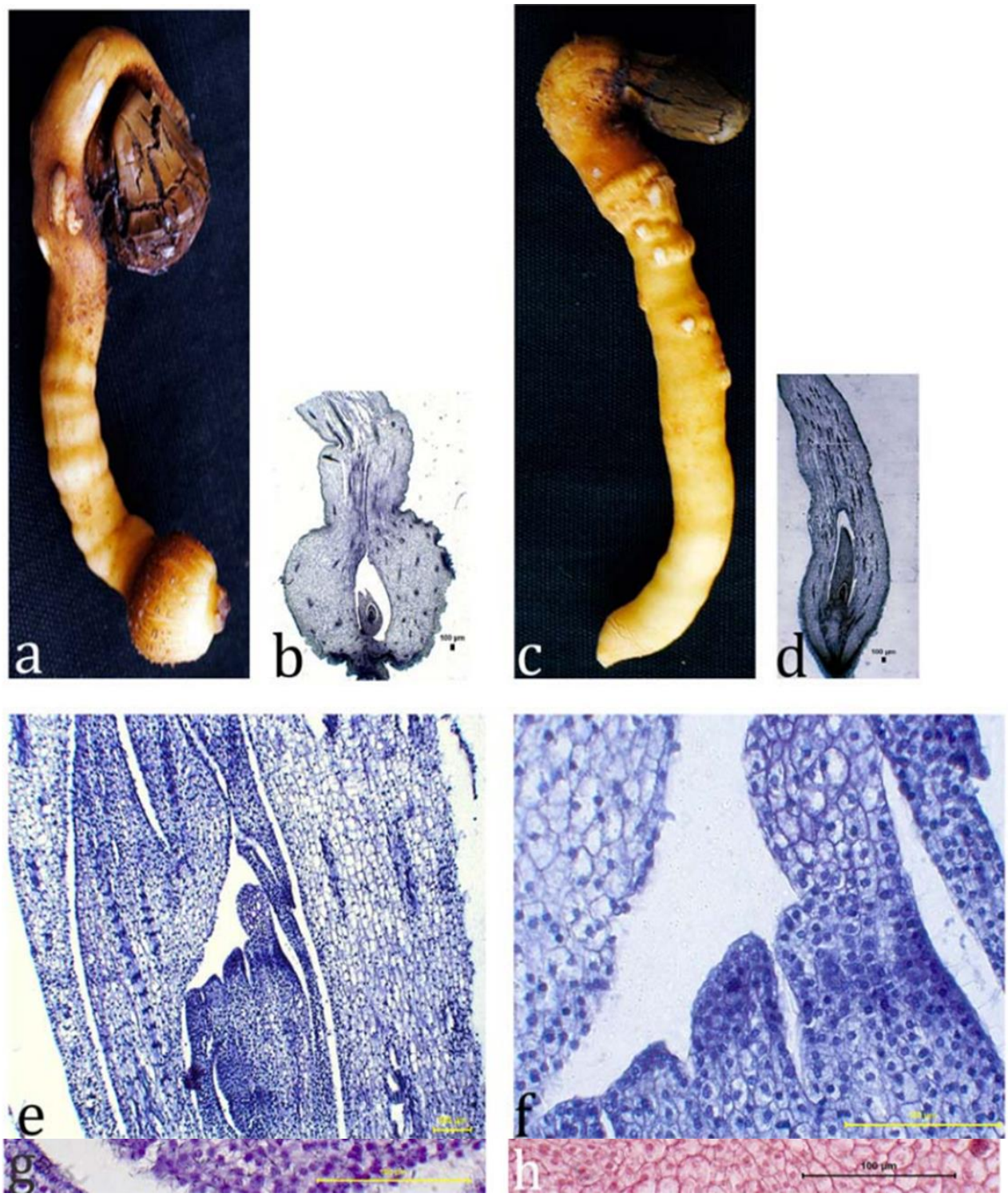

Figure 5. Histological analysis of embryo development in Borassus flabelliferin vitro after 120 days in culture medium. a, elongated cotyledonary stalk with a bulge at the posterior end. b, L.S. of the bulged cotyledonary stalk showing plumule development. c, elongated cotyledonary stalk without bulging. d, plumule development from the posterior end of cotyledonary stalk without bulging (L.S.). e & f, Magnified view of shoot meristem subtended with leaf primordia (L.S.)

Conclusion

In vitro culture of palmyrah embryos effectively recapitulates the natural germination process, including the characteristic cotyledon elongation. This technique offers valuable insights into the developmental stages of palmyrah embryogenesis, facilitating the selection of appropriate tissues for efficient plumule culture and somatic embryogenesis. While embryo germination rates exceeding 80% were achieved, further refinement is necessary to optimize conversion of germinated embryos into complete plantlets.Overall, this approach has the potential to revolutionize germplasm conservation efforts, particularly for palms like palmyrah with their complex hypogeal/tubular germination. This in vitro culture technique eliminates the logistical challenges and biosafety risks associated with seed and seedling transport, thereby streamlining germplasm conservation efforts.

References

Anonymous, (1948). The Wealth of India-Borassus. *Council Sci and Industr Res*, New Delhi, Vol (1), 203-207.

Davis, T. A. & Johnson, D. V. (1987). Current utilization and further development of the palmyrah palm (Borassus flabellifer L., Arecacea) in Tamil Nadu, India. *Economic Botany*, 41(2), 247- 266.

Douglas, G. C. (1987). Embryo culture of a rare plant, HyophorbeamaricaulisMartius (Palmae). *Journal of Plant Physiology*, 130, 73-77.

Eeuwens, C. J. (1978). Effects of organic nutrients and hormones on growth and development of tissue explants from coconut (Cocos nucifera L.) and date (Phoenix dactylifera L.) palms culture in vitro. *Physiologia Plantarum*, 42, 173-178.

Hanning, E. (1904). Zur physiologuepflanwlicherEmbryonen I Ueberdieculturvon cruciferin-Embryonenausserhalb des embryosacks. *Bot Zeitg*, p 62.

Hoded, D. (1977). Notes on embryo culture of palms. *Principes* 2, 103-108.

Johnston, F. B. & Stern, H. (1957). Mass isolation of viable wheat embryos.Nature,179, 160-161.

Karun, A., S. Shivashankar, Sajini, K. K. &Saji, K. V. (1993). Field collection and in vitro germination of coconut embryos. *Journal of Plantation Crops*, 21, 291- 294.

Karunaratne, S., Gamage, C. &Kovoor, A. (1991). Leaf maturity, a critical factor in embryogenesis. *Journal of Plant Physiology*,139, 27-31.

Lossi, S. F., Moro& Sader, R. (2006). Seed anatomy and germination of Phoenix roebeleniiO'Brien (Arecaceae). *Sements*, 28 (3), 21-26.

Mahabale, T. S. & Kulkarni, K. M. (1975). Studies on palms: fruits, seeds and their germination in Livistona chinensis R. Br. *Journal of Bombay Natural History Society*, 72(3), 655–661.

Masilamani, P., Alex Albert, V. & Govindaraj, M. (2020). Effect of dormancy breaking treatments ongermination of Palmyrah (Borassus flabellifer L.). *Indian Journal of Forestry*, 43, 114-118.

Murashige, T. &Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15, 473-497.

Pinheiro, C. U. B.(2001). Germination strategies of palms: the case of Schippia concolor Burret in Belize. *Brittonia*, 53 (4), 519-527.

Rabechault, H. & Cas, S. (1974). Recherches sur la culture in vitro des embryons de palmier a huile (Elaeisguineensis Jacq var. dura Becc.). *Oleagineaux*, 29, 73- 79.

Rillo, E. P. & Paloma, M. B. F. (1992). In vitro culture of Makapuno coconut embryos. *Coconuts Today*, 9, 90-108.

Sankaralingam, A., Hemalatha, G. & Mohamed Ali. (1999). A Treatise on Palmyrah. InH. Hameed Khan (ed.), *All-India Corordinated Research Project on Palms*(pp. 1-40)., Tamil Nadu Agricultural College and Research Institute, India.

Tomlinson, P. B. (1960). Essays on the morphology of palms. In Germination and the seedling. *Principes*, 4, 56–61.