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IN VITRO EMBRYO RETRIEVAL TECHNIQUE FOR ARECANUT (*ARECA CATECHU* LINN.)

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ABSTRACT

An *in vitro* germination technique for the rapid germination of arecanut embryos has been developed. Seven-month-old embryos collected from four varieties *viz.* Mangala, Sumangala, Sree Mangala and South Kanara Local were cultured *in vitro* on agar gelled hormone free EeuewensY3, + strength MS or Full strength MS medium. Embryos cultured in Y3 medium supplemented with 3% sucrose showed maximum (93.3%) germination. Varietal differences with respect to germination was also recorded and showed Sumangala and South Kanara local were most responsive. *In vitro* seedlings after 8 weeks in germination media were transferred to liquid media containing reduced (1.5%) sucrose for the development of roots and expansion of leaves. Fully developed *in vitro* seedlings are being acclimatized. Developed protocol will serve as a basis for the future *in vitro* conservation (cryopreservation) studies on arecanut and is also useful in the areas of safe and convenient germplasm movement, rare embryo rescue etc.

INTRODUCTION

Areca palm (*Areca catechu* Linn.) is an unbranched erect, medium sized, monoecious tree growing in hot and humid tropical regions of the world and center of origin considered to be South East Asia (Bavappa *et al.*, 1982). Arecanut is principally valued for its masticatory uses. Regarding the production of arecanut, India contribute giant share followed by Indonesia, China, Bangladesh, Thailand and Malaysia (Rethinam and Sivaraman, 2001). Apart for its popularity as masticatory nut, it is traditionally used in religious and social functions by indigenous communities, human and veterinary medicine, in packaging and constructions etc. In view of declining popularity as masticatory agent, now researchers emphasize on alternate uses of areca palm. Tannin extracted from tender nuts has wide range of application like dyeing cloths, rope, tanning leather, adhesive etc. Alkaloids arecoline, arecadine and guvacoline are present in arecanut and are proved to have pharmacological properties. In addition to these, arecanut husk can be used for making hard board, pulping and paperboards. Leaf sheath is used for making disposable cups and plates. Areca bole is used as building materials in Indian villages (Bavappa *et. al.*, 1982). There is an urgent need to increase productivity of the crop by doubling yield and reducing unit cost of production so that declining price can be effectively defended (Rethinam and Sivaraman, 2001).

Since the crop is essentially out breeding in nature, high levels of heterogeneity among population is expected. Cultivars are to be identified/ evaluated and conserved in order to exploit heterosis. *In vitro* germination of arecanut has the potential to be useful in the germplasm movement, breeding programmes, lowering cost, satisfying most phytosanitary requirements, overcoming storage problem and rescue of rare embryo. *In vitro* germination studies of excised mature embryos (Ganapathi *et al.*, 1997) and an attempt to induce somatic embryos (Mathew and Philip, 2000) are available. In the present report, we describe a protocol for the *in vitro* germination of seven-month-old embryos excised from released varieties of CPCRI (Mangala, Sumangala, SreeMangala) and a local cultivar South Kanara Local.

MATERIALS AND METHOD

Seven–month–old green bunches collected during July-August, 2001 from CPCRI (Regional Station), Vittal, Karnataka, S. India were used as embryo source for the experiment.

Extraction of embryo from the nut

Bunches of six, seven, eight and nine month after pollination were harvested and the embryos were extracted. In case of six-month-old nuts, the embryos were very minute and difficult to extract from the nut. Whereas, the embryos from 8, 9 months were difficult to extract due to hard endosperm. The damage of the embryos while extraction is in the tune of more than 80 per cent.

The best stage of extraction of embryos is 7 month after pollination. The damage was minimum and germination response was higher.

Nuts from four cultivars *viz.* Mangala, Sumangala, SreeMangala and South Kanara Local were split open and embryo excised (Fig. 1A) and thoroughly washed with distilled water. Embryos were surface sterilized using chlorine water (50%) for 10 minutes followed by five rinses in sterile distilled water. Surface disinfested embryos were aseptically cultured on germination media. Effect of three basal salt formulations namely Eeuwens Y3 (Eeuwens, 1978), full strength MS (Murashige and Skoog, 1962) and + strength MS supplemented with 3% sucrose, 0.6% agar and 0.1% activated charcoal was studied. Prior to autoclaving the media at 121°C and 108-kPa pressure for 20 min, pH 5.7 was adjusted.

Culture conditions

All cultures were incubated at 27±1° C in dark and embryos were transferred to fresh media for two times. Germinated embryos after 8 weeks were transferred to same basal salt formulations in liquid form containing reduced (1.5%) sucrose level. Eight-week-old *in vitro* seedlings were transferred to light provided by white cool fluorescent tubes (Philips). Upon development of thick fibrous root system, 18-week-old seedlings were transferred to plastic pots containing soil, sand and coconut pith (1:1:1). Plants were initially covered with poly bags and incubated at room temperature.

The experiment was followed the design of RBD with 12 treatments and the entire experiments were replicated thrice. Each replication consisted of 10 embryos. Embryos concomitantly produced plumule and radicle. Plumule measured >5 mm length considered as germinated. Second week onwards, data on percentage germination with respect to cultivars and media were recorded. Seedling growth parameters *viz.*, shoot height, root number and percentage of survival were recorded.

RESULTS AND DISCUSSION

Zygotic embryos cultured *in vitro* on different germination media enlarged rapidly. First sign of embryo germination was noticed after five days of inoculation. Signs of germination including the appearance of haustorial, plumular and radicular nodules occurred within one week. Embryo browning and subsequent explant death was effectively prevented by adding 0.1% activated charcoal in the medium. Activated charcoal is an adsorbing agent routinely used in *in vitro* systems, more specifically in *in vitro* culture of palms (Tisserart, 1979). First germination recorded after one week of inoculation. Germination of arecanut in natural condition takes 40 days (Nagwekar *et al.*, 1987). Excised embryo however, germinated rapidly and maximum germination recorded after 2nd and 3rd week of culture. Process of germination continued till 6th week of culture. Germination response of embryo in different basal salt media varied significantly (P=0.01). Maximum germination was recorded in Y3 medium (Table 1), followed by half MS. Least number of embryos germinated in MS medium. Suitability of Y3 medium for *in vitro* embryo germination of arecanut is further supported by rapid germination achieved in this medium. Eeuwens Y3 salt formulation is basically different from MS as macronutrients supplied in the form of halides in it as against nitrate form in MS. Y3 media has been proved to be superior than other formulations in embryo culture systems of other palms like coconut (Rillo and Paloma, 1990; Karun *et al.*, 1999). Germinated embryos produced roots and shoots and grew in to 4.22 cm size within 8 weeks (Fig. 1B). Seedling growth parameters – shoot length (Fig. 2) and root numbers (Fig. 3) were recorded. Basal media had a significant (p=0.01) role on root formation and seedling growing Y3 medium produced maximum roots. Medium however, did not significantly influences shoot growth (Table 2).

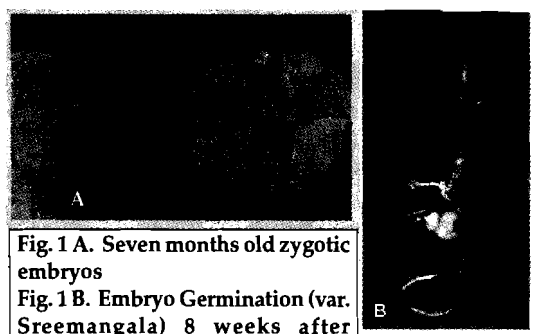


Fig. 1 A. Seven months old zygotic embryos
Fig. 1 B. Embryo Germination (var. Sreemangala) 8 weeks after inoculation

Table 1. Germination response of 7-month-old arecanut embryos cultured in different basal media

Germination media tested	%Germination				Treatment Mean
	Mangala	Sumangala	Sreemangala	South Kanara Local	
+MS	36.6	56.6	73.3	73.3	59.9
MS	33.0	50.0	63.3	66.6	53.2
Y3	53.3	63.3	93.3	93.3	75.8

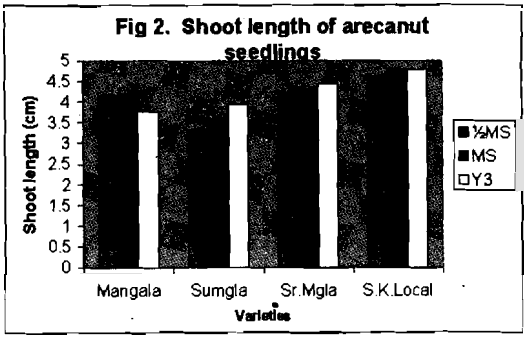
LSD (p=0.05) for treatment mean 5.972

ANOVA Summary Table

Source	df (n-1)	MS	F
Variety	3	2773.14	55.74*
Media	2	1602.77	32.22*
Variety X Media	6	50.92	1.02^{NS}

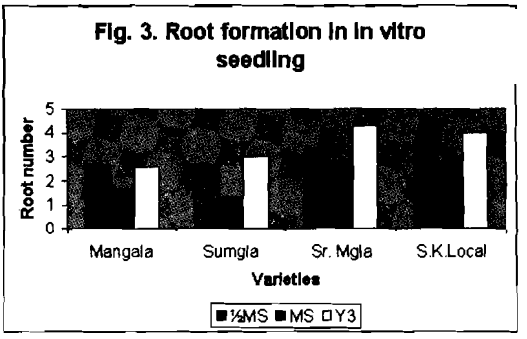
*Significant at 1% level

Survival of plant raised in different basal media varied significantly (Figure 4). Eighteen-weeks-old *in vitro* seedling produced 2-3 well-expanded leaves and thick fibrous roots (Fig. 5). They were transferred to pots for further acclimatization. Most *in vitro* seedlings raised in Y3 medium were established and survived (Figure 4). Better survival of plants raised in Y3 medium is further substantiated by maximum root development in this medium (Fig. 3).



In vitro response embryo tested from different varieties varied significantly. Arecanut is essentially out breeding in nature. High heterogeneity among varieties attributed significantly to different germination behavior in areca palm. Embryos of South Kanara Local and Sree Mangala germinated maximum in Y3 medium (Table 1).

These varieties are selections from South Indian areca populations. *In vitro* shoot growth of seedling indicated variations among varieties tested. Maximum shoot growth was recorded in South Kanara Local variety. Root formation among three varieties recorded and maximum was in South Kanara Local.



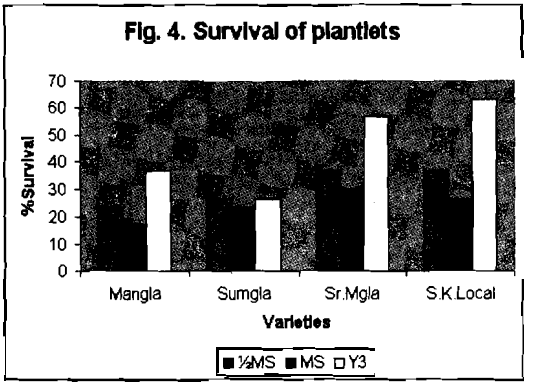


Table 2. Growth parameters of arecanut seedling raised *in vitro*

Germination media tested	Shoot length (cm)					Root number				
	Mangala	Sumangala	Sreemangala	South Kanara Local	Treatment Mean	Mangala	Sumangala	Sreemangala	South Kanara Local	Treatment Mean
+MS	4.13	3.33	4.20	4.60	4.06	2.66	2.66	3.33	3.66	3.07
MS	4.16	3.56	4.20	4.76	4.18	1.66	1.33	2.66	2.66	2.07
Y3	3.76	3.93	4.43	4.76	4.22	2.66	3.00	4.33	4.00	3.50
Variety mean		4.01	3.60	4.27	4.7		2.32	2.33	2.44	3.44

LSD (p=0.05) for variety means 0..302; LSD (p=0.05) for media 0.525

ANOVA Summary Table

Source	df (n-1)	Shoot length		Root Number	
		MS	F	MS	F
Variety	3	1.930	20.25*	3.700	9.65*
Media	2	0.083	0.88^{NS}	6.360	16.57*
Variety X Media	6	0.136	1.43^{NS}	0.175	0.46^{NS}

Table 3. Survival of seedlings raised in different basal media

Germination media tested	%Surviaval				Treatment Mean
	Mangala	Sumangala	Sreemangala	South Kanara Local	
+MS	23.3	26.6	36.6	36.6	30.77
MS	16.6	23.3	30.0	26.6	24.12
Y3	36.6	26.6	56.6	63.3	45.77
Variety Mean	25.5	25.5	41.0	42.2	

LSD (p=0.05) for variety mean 7.595
LSD (p=0.05) for treatment mean 6.570

ANOVA Summary Table

Source	df (n-1)	MS	F
Variety	3	780.55	12.93*
Media	2	1477.77	24.49**
Variety X Media	6	166.66	2.76*

*Significant at 1%

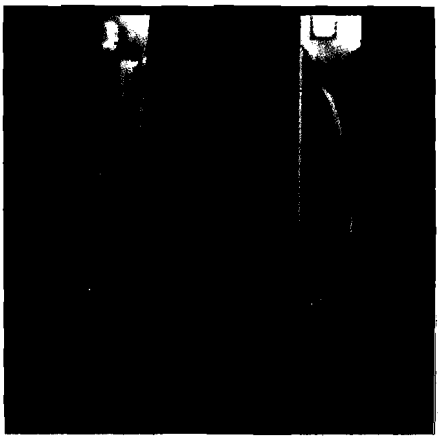


Fig. 5. *In vitro* retrieved plantlets ready to transfer to pots, Var. Sumangala

In vitro retrieval technique developed in the present study is efficient (63.3%). It can be used in the embryo rescue of rare hybrids and *in vitro* germplasm conservation etc.

CONCLUSION

A technique for the *in vitro* germination of arecanut zygotic embryos has been developed. Among different basal salt formulations tested in the present study, Y3 medium was found most suitable. Germination did not require any intervention of growth regulators. Germination response varied with varieties tested and South Kanara Local and Sree Mangala varieties gave maximum germination. The *in vitro* germination protocol developed in the present study will serve as a basis for the future in *vitro* studies of arecanut. *In vitro* germination protocol is a primary requisite for the *in vitro* conservation of arecanut germplasm.

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