Plant Archives Vol. 20, No. 1, 2020 pp. 1175-1178 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

STANDARDIZATION OF PROTOCOL FOR *IN VITRO* SEED GERMINATION OF *CITRUS MACROPTERA* MONT.

Shabaree Y. A. Sangma[1], Lolly S. Pereira[1*] and J.C.Dang[2]

[1*,2] Department of RDAP, North-Eastern Hill University, Tura Campus, Tura, 794002, West Garo Hills, Meghalaya.
[3]Department of Botany, Don Bosco College, Tura, 794002, West Garo Hills, Meghalaya.

Abstract

Citrus macroptera Mont. is a fruit tree growing in semi-wild conditions in West Garo Hills and South Garo Hills districts of Meghalaya, locally known as 'Chambil'. The embryos of *Citrus macroptera* are mostly under developed, thereby resulting in rare and poor natural germination. Moreover, the seeds are recalcitrant in nature and cannot be preserved for long. A complete protocol for *in vitro* seed germination could be an alternative for natural seed germination, thus facilitating the conservation and utilization of this species. An experiment was conducted to standardize a protocol for *in vitro* seed germination of *Citrus macroptera* by using different concentrations and combinations of auxins and cytokinins in full strength and half strength Murashige and Skoog's (MS) medium. It was observed that full strength MS medium supplemented with BAP 0.5 mg/l showed earlier shoot initiation (4.66 days) with 88.11% seed germination, highest shoot length of 3.36 cm and highest root length of 2.60 cm.

Key words : *Citrus macroptera*, *in vitro* seed germination, protocol, conservation, Garo Hills, Meghalaya.

Introduction

The North- Eastern region of India, being the integral part of the biodiversity hot spots of the globe, has varied climatic conditions that influence a variety of plants and richness of diversity. A variety of Citrus species are found in the North -eastern region in general and a significant number of species of Citrus grow in wild natural conditions particularly in Nokrek Biosphere Reserve of Meghalaya. Citrus macroptera Mont. locally known as 'Chambil' in Garo language grows in semi-wild conditions in West Garo Hills and South Garo Hills districts of Meghalaya. It is also found in the vicinity of Shella and Dawki areas near Cherrapunji. The thick rind as well as juice of the fruit is used in preparing appetizing Garo cuisines and for making pickles. The fruit has great potential for use as commercial refreshing drink. *Citrus macroptera* Mont. is one among the seven endangered Citrus species from India as reported by Singh and Singh (2003). *Citrus macroptera* is in need of special and immediate attention for conservation due to its endemism and high degree of threat perception (Malik, *et al.*, 2006). The slow natural regeneration of this species and increasing human intervention around the Biosphere Reserve by clearing of forests for jhuming and human habitation, have become a severe threat to this species. The recalcitrant nature of seeds makes it difficult to store for longer duration. Natural seed germination of *Citrus macroptera* is a rare phenomenon owing to rudimentary embryos. *In-vitro* seed germination has been carried out successfully in many rare and endangered species as an alternative for natural seed germination. Considering the above facts, an experiment was conducted to standardize a protocol for in vitro seed germination of *Citrus macroptera*.

***Author for correspondence :** E-mail : drlollysp@gmail.com

Materials and Methods

Fruits of *Citrus macroptera* were collected from nearby villages of Tura in West Garo Hills district of Meghalaya. The seeds were separated from fruits and washed with Teepol and rinsed with tap water by keeping under running water for about half an hour. Seeds were then washed with double distilled water five times. Healthy seeds were isolated from the lot and surface sterilized with 70% absolute alcohol for 2 minutes. Seeds were

subsequently rinsed with double distilled water six times. Later seeds were surface sterilized under laminar flow chamber with 10% sodium hypochlorite for 15 minutes and 0.1% mercuric chloride for 5 minutes. Seeds were then washed thoroughly with double distilled water six times. Sterilized seeds were given longitudinal incision on both sides of the seed coat and inoculated on full strength and half strength MS (Murashige and Skoog,1962) medium supplemented with different growth regulators (auxins and cytokinins) either singly or in combination at different concentrations ranging from 0.5 to 2.0 mg/l. The cytokinins used in the experiment were kinetin (Kn) and 6-benzylaminopurine (BAP); and auxins used were Indole 3-butyric acid (IBA), Naphthalene 3-acetic acid (NAA) and Indole acetic acid (IAA). The prepared medium was adjusted to pH of 5.8 and sterilized by autoclaving at 121ºC at 15 Psi pressure for 15 minutes. Temperature of 25 ± 2ºC, photoperiod of 10 hours, light intensity of 1000 lux and 80 % relative humidity was maintained in the culture room (Sangma *et al.*, 2018).

Table 1: Effect of different concentrations and combinations of growth regulators in full strength MS medium on *in vitro* seed germination of *Citrus macroptera.*

Treatment	Growth regulators	Conc. (mg/l)	Days for germination		Germination (%)		Length of shoot (cm)		Length of root(cm)	
T_1	Blank	0	12.77	o	50.1	m	0.53	ij	0.98	d
T_2	BAP	0.5	4.66	a	88.11	a	3.36	a	2.60	a
T_3	BAP	1	5.00	ab	80.66	abc	1.08	efghi	2.08	b
T_4	BAP	1.5	7.44	fgh	66.00	hij	3.33	a	2.01	b
T_5	BAP	2	7.33	fg	51.33	m	1.50	bcdefg	1.49	c
T_6	Kn	0.5	7.77	ghi	54.99	lm	0.52	j	0.33	ghij
T_7	Kn	1	9.66	k	65.99	hij	1.24	efgh	0.33	ghij
T_8	Kn	1.5	10.77	lmn	56.21	klm	1.98	bcd	0.66	defgh
T_9	Kn	2	11.21	n	59.88	jkl	1.03	fghij	0.17	ij
T_{10}	BAP+IBA	0.5+0.5	7.33	fg	81.55	ab	1.26	efgh	0.41	fghij
T_{11}	BAP+IBA	1.0+0.5	5.66	bc	84.77	a	2.03	bc	0.58	defghi
T_{12}	BAP+IBA	1.5+0.5	4.77	a	77.00	bcd	1.40	cdefgh	0.64	defgh
T_{13}	BAP+IBA	2.0+0.5	6.33	cd	76.66	bcde	1.36	defgh	0.33	ghij
T_{14}	BAP+NAA	0.5+0.5	7.44	fgh	70.88	defgh	1.02	fghij	0.80	def
T_{15}	BAP+NAA	1+0.5	8.22	hij	74.55	bcdef	1.06	fghij	0.95	de
T_{16}	BAP+NAA	1.5+0.5	8.33	ij	70.88	defgh	1.72	bcde	0.96	de
T_{17}	BAP+NAA	2.0+0.5	6.22	cdi	69.66	defgh	1.05	fghij	0.99	d
T_{18}	BAP+IAA	0.5+0.5	6.10	cd	67.22	fghij	0.96	ghij	0.41	fghij
T_{19}	BAP+IAA	1+0.5	8.66	j	68.44	fghi	0.97	ghij	0.59	defghi
T_{20}	BAP+IAA	1.5+0.5	11.21	n	64.77	hij	0.86	ghij	0.33	ghij
T_{21}	Kn+IBA	0.5+0.5	6.33	cd	74.55	bcdef	0.94	ghij	0.37	fghij
T_{22}	Kn+IBA	1+0.5	6.44	cde	74.44	bcdef	1.13	efghii	0.73	defg
T_{23}	Kn+IBA	1.5+0.5	6.11	cd	73.88	cdefg	2.13	b	0.68	defg
T_{24}	Kn+ IBA	2.0+0.5	7.22	efg	66.55	ghij	2.00	bcd	0.56	defghij
T_{25}	Kn+NAA	0.5+0.5	6.22	cd	68.99	efghi	1.15	efghi	0.48	fghij
T_{26}	Kn+NAA	1+0.5	6.11	cd	63.55	hijk	1.63	bcdef	0.41	fghij
T_{27}	Kn+NAA	1.5+0.5	6.10	cd	69.66	defgh	1.37	defgh	0.52	efghij
T_{28}	Kn+NAA	2.0+0.5	6.77	def	70.88	defgh	1.04	fghij	0.34	ghij
T_{29}	Kn+IAA	0.5+0.5	10.03	kl	62.33	ijl	0.89	hijg	0.22	hij
T_{30}	Kn+IAA	1+0.5	10.33	klm	63.55	hijk	0.84	hij	0.23	hij
T_{31}	Kn+IAA	1.5+0.5	10.99	mn	59.88	jkl	0.99	fghij	0.11	j
Mean		-	7.73		68.72		1.36		0.72	
C.D. (0.01)		-	1.09		10.06		0.86		0.59	

NB: BAP= 6-benzylaminopurine; Kn= Kinetin; IBA= Indole 3-butyric acid; NAA= Naphthalene 3-acetic acid; IAA= Indole acetic acid
Similar alphabets denote homogeneous mean values.

Table 2: Effect of different concentrations and combinations of growth regulators in half strength MS medium on *in vitro* seed germination of *Citrus macroptera*.

Treatment	Growth regulators	Conc. (mg/l)	Days for germination		Germination (%)		Length of shoot (cm)		Length of root(cm)	
T_1	Blank	0	12.88	o	25.92	i	0.75	ef	0.39	h
T_2	BAP	0.5	9.33	efg	55.55	abcdef	1.00	bcdef	0.97	bcde
T_3	BAP	1.0	7.66	ab	51.84	abcdefg	1.48	b	1.02	bcd
T_4	BAP	1.5	9.44	efgh	57.4	abcd	1.21	b cde	1.21	a b
T_5	BAP	2.0	10.33	ij	48.14	bc defgh	0.93	cdef	0.93	bcdefg
T_6	Kn	0.5	10.99	jk	44.44	c defghi	0.97	cdef	0.64	defgh
T_7	Kn	1.0	10.33	ij	53.70	abcdef	0.86	def	0.68	defgh
T_8	Kn	1.5	9.00	def	55.55	abcde	0.97	cdef	0.95	bcdef
T_9	Kn	2.0	9.66	fghi	48.14	bcdefgh	0.79	ef	0.79	bcdefgh
T_{10}	BAP+IBA	0.5+0.5	9.33	efg	53.70	abcdef	0.93	cdef	0.91	bcdefg
T_{11}	BAP+IBA	1.0+0.5	7.1	a	66.66	ab	2.10	a	1.65	a
T_{12}	BAP+IBA	1.5+0.5	11.55	klm	55.55	abcde	1.24	bcde	1.06	bcd
T_{13}	BAP+IBA	2.0+0.5	11.77	klmn	39.25	defghi	0.99	bcdef	1.17	a bc
T_{14}	BAP+NAA	0.5+0.5	8.22	bcd	40.73	cdefghi	0.97	cdef	0.97	bcde
T_{15}	BAP+NAA	1.0+0.5	8.11	bc	70.36	a	1.04	bcdef	1.04	bcd
T_{16}	BAP+NAA	1.5+0.5	8.66	cde	59.25	abc	1.35	bcd	0.88	bcdefg
T_{17}	BAP+NAA	2.0+0.5	8.77	cde	42.96	cdefghi	1.06	bcef	0.79	bcdefgh
T_{18}	BAP+IAA	0.5+0.5	12.53	no	37.30	efghi	0.91	cdef	0.46	gh
T_{19}	BAP+IAA	1.0+0.5	11.88	lmn	44.44	cdefghi	1.00	bcdef	0.47	fgh
T_{20}	BAP+IAA	1.5+0.5	11.33	kl	35.55	ghi	1.04	bcdef	0.5	efgh
T_{21}	Kn+IBA	0.5+0.5	9.44	efgh	38.14	defghi	0.93	cdef	0.93	bcdefg
T_{22}	Kn+IBA	1.0+0.5	9.22	ef	41.48	cdefghi	1.40	bc	1.00	bcd
T_{23}	Kn+IBA	1.5+0.5	8.99	def	48.14	bcdefg	1.06	bcdef	0.97	bcde
T_{24}	Kn+ IBA	2.0+0.5	10.22	hij	42.96	cdefghi	0.98	cdef	0.98	bcde
T_{25}	Kn+NAA	0.5+0.5	10.11	ghi	35.55	ghi	0.70	cf	0.70	cdefgh
T_{26}	Kn+NAA	1.0+0.5	9.66	fghi	39.25	cdefghi	0.79	ef	0.79	bcdefgh
T_{27}	Kn+NAA	1.5+0.5	9.77	fghi	32.22	hi	0.97	cdef	0.73	bcdefgh
T_{28}	Kn+NAA	2.0+0.5	10.33	ij	33.33	ghi	0.86	def	0.86	bcdefgh
T_{29}	Kn+IAA	0.5+0.5	12.55	no	33.33	ghi	0.68	f	0.46	gh
T_{30}	Kn+IAA	1.0+0.5	12.22	mno	29.62	hi	0.77	ef	0.48	fgh
T_{31}	Kn+IAA	1.5+0.5	12.33	mno	29.62	hi	0.84	ef	0.47	fgh
Mean		-	10.12		44.77		1.02		0.83	
C.D. (0.01)		-	1.05		25.65		0.65		0.63	

NB: BAP= 6-benzylaminopurine; Kn= Kinetin; IBA= Indole 3-butyric acid; NAA= Naphthalene 3-acetic acid; IAA= Indole acetic acid. Similar alphabets denote homogeneous mean values.

The number of days required for germination was recorded. Percentage of germination and length of shoots and roots were recorded at 4 weeks after inoculation.

Ten replicates per treatment were taken and the experiment was repeated three times. The experiment was laid out in Completely Randomised Design. The data were subjected to statistical analysis using Fischer's one-way analysis of variance (Panse & Sukhatme, 1989) using Ag. Res. Statistical Software, (c) 1994 Pascal Intl. Software Solutions, Version 3.01 and significant differences were compared by Least Significant Differences (LSD). The level of significance employed in 'F' test was P=0.01. Critical difference was calculated for comparison wherever the 'F'test was found significant.

Results and Discussion

Among the various treatments of growth hormones used for *in vitro* seed germination of *Citrus macroptera* in full strength MS medium, it was observed that MS medium supplemented with BAP 0.5 mg/l (T_2) showed

the earliest germination in 4.66 days, followed by combination of BAP 1.5 mg/l + IBA 0.5 mg/l (T_{12}) and BAP 1.0 mg/l (T_3) which showed germination in 4.77 days and 5 days respectively. The highest percentage of germination (88.11%) was observed in BAP 0.5 mg/l (T_2) followed by BAP 1.0 mg/l + IBA 0.5 mg/l (T_{11}) and BAP 0.5 mg/l + IBA 0.5 mg/l (T_{10}) which exhibited 84.77% and 81.55% respectively. The highest shoot length of 3.36 cm was observed in MS medium supplemented with BAP 0.5mg/l (T_2) followed by BAP 1.5 mg/l (T_4) with 3.33cm and combination of Kn 1.5 mg/l + IBA 0.5 mg/l (T_{23}) with 2.13cm. The longest root (2.6 cm) was recorded in MS medium supplemented with 0.5mg/l of BAP (T_2) followed by 2.08 cm in BAP 1.0 mg/l (T_3) and 2.01 cm in BAP 1.5mg/l (T_4)(Table1). Out of the 31 treatments tried in full strength MS medium, T_2 exhibited the best results.

Among the various concentrations and combinations of hormones used in half strength MS medium for *in vitro* seed germination of *Citrus macroptera*, combination of BAP 1.0mg/l + IBA 0.5mg/l (T_{11}) showed earlier shoot initiation in 7.1days followed by BAP 1.0 mg/l (T_3) and BAP 1.0mg/l + NAA 0.5mg/l (T_{15}) which showed shoot initiation in 7.66 days and 8.11 days respectively. The highest percentage of germination (70.36%) was observed in combination of BAP1.0 mg/l + NAA 0.5mg/l (T_{15}) followed by BAP 1.0 mg/l+IBA 0.5mg/l (T_{11}) and BAP 1.5 mg/l+ NAA 0.5mg/l (T_{16}) with 66.66% and 59.25 % respectively. The highest shoot length of 2.1 cm was observed in T_{11} (BAP 1.0mg/l + IBA 0.5mg/l) followed by T_3 (BAP 1.0 mg/l) and T_{22} (Kn 1.0 mg/l + IBA 0.5 mg/l) with 1.48 cm and 1.40cm respectively. The longest root (1.65 cm) was recorded in T_{11} (BAP 1.0mg/l + IBA 0.5mg/l) followed by 1.21 cm in T_4 (BAP 1.5mg/l) and 1.17 cm in T_{13} (BAP 2.0 mg/l+ IBA 0.5 mg/l) (Table 2).Out of the treatments used in half strength MS medium, T_{11} proved to be the best.

Among the treatments used in full strength and half strength MS medium for *in vitro* seed germination of *Citrus macroptera*, it was observed that full strength MS medium supplemented with 0.5 mg/l BAP exhibited the best response. Similar experiments on *in vitro* seed germination of various Citrus species were carried out by many researchers. Hassanein and Azooz (2003) reported that MS medium supplemented with BAP 0.5mg/l showed better results for *in vitro* germination of seeds of *Citrus reticulata* than in soil. Azim *et al.*, (2011) recorded the highest shoot formation (70%) from seeds (without seed coat) of *Citrus sinensis* inoculated in MS medium with 1.0 mg/l BA (6-benzyl adenine). Kamruzzaman *et al*., (2015) observed that half strength MS medium supplemented with BAP (0.5mg/l) + NAA (2.0 mg/l) + Kn (1.0mg/l) showed highest germination response of 90% in Kinnow mandarin (*Citrus reticulata* Blanco) while half strength MS medium with BAP (1.0 mg/l) + NAA (0.5 mg/l) showed highest seed germination of 92% in Citron (*Citrus medica)*.

Conclusion

Among the treatments of growth hormones used in full strength and half strength MS medium, it was observed that full strength MS medium supplemented with 0.5 mg/l BAP exhibited the best response for *in vitro* seed germination of *Citrus macroptera* showing earliest germination (4.66 days), highest germination percentage (88.11%), longest shoot (3.36 cm) and longest root (2.6 cm).

References

Azim, F., M.M. Rahman, S.H. Prodhan, S.U.Sikdar, N. Zobayer and M. Ashrafuzzaman (2011). Development of Efficient Callus Initiation of Malta (*Citrus sinensis*) Through Tissue Culture. *Int. J. Agril. Res.Innov. & Tech.*, **1(1&2):** 64-68.

Hassanein, A.M. and M.M. Azooz (2003). Propagation of *Citrus reticulata* via *in vitro* seed germination and shoot cuttings. *Biologia Plantarum*, **47(2):**173-177.

Kamruzzaman, M., A. Akther, Md.O. Faruq, A.Pervin, S. Myti and S.H. Prodhan (2015). Establishment of an efficient callus induction method from leaf and stem in kinnow mandarin *(Citrus reticulata* Blanco) and citron (*Citrus medica* L.). *Academic Journals*, **14(15):** 1290-1296.

Malik, S.K., R. Chaudury, O.P. Dhariwal and R.K. Kalia (2006). Collection and characterisation of *Citrus indica* Tanaka and *C. macroptera* Mont.: Wild endangered species of Northeast India. *Genetic Resources and Crop Evolution*, **53:** 1485-1493.

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

Panse, V.G. and Sukhatme (1989). Statistical Methods for Agricultural Workers. Indian council of Agricultural Research. New Delhi.

Sangma, S.Y., L.S. Pereira and J.C. Dang (2018). *In vitro* Seed germination of *Citrus macroptera* Mont. - Endangered Species of Meghalaya. *Environment and Ecology*, **36(3):** 855-859.

Singh, I.P. and S. Singh (2003). Exploration, collection and mapping of *Citrus* genetic diversity in India. Technical Bulletin **No.7**, National Research Centre for Citrus, Nagpur, 230.