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Optimization of Phytohormone Concentrations for Callus Induction in Mature Embryos of Finger Millet (*Eleucine coracana)*

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ABSTRACT

Finger millet (Eleusine coracana (L.) Gaertn.) is the primary food source for millions of people in tropical dry land regions of the world. Compared to other millet, finger millet grains also have a relatively higher content of minerals such as calcium, phosphorus, iron and manganese. It has nutritional qualities superior to that of rice and is on par with that of wheat. Finger millet is vulnerable to biotic stresses like fungal blast and abiotic stresses like drought and salinity stress.

Development of efficient tissue regeneration system is an essential prerequisite for successful production of transgenic plants. For callus induction and plant regeneration in finger millet GE-3885, mature embryos were cultured onto Murashige and Skoog (MS) medium supplemented with different concentrations of 2, 4-D alone and in combination with BAP NAA and Kinetin. Callus induction was observed in all 42 different combination of phytohormone. Callus initiation was observed in 16 different combinations. Results showed that the highest callus was induced observed in MS medium containing 1.5 mg/lNAA.

Key words: *2,4-D (2,4- Di Chloro Phenoxy Acetic Acid). BAP (Benzyl Amino Purine), NAA (Nepthelic Acetic Acid), M.S Media, Callus Induction.*

INTRODUCTION

Millets are the minor cereals of the grass family, *Poaceae*. Millets are better adapted to dry and infertile soils than most of other cultivated crops and are therefore often cultivated under extremely harsh conditions - for example, high temperatures, low and erratic precipitation, short growing seasons and acidic and infertile soils with poor water-holding capacity. Although millet production is only about 2% of the world cereal production but it is an important staple food crop in semi-arid regions. Asia and Africa account for about 95% of the total millet production in the world. Asia accounts for 40% of millet production, mainly contributed by India and China[10].

India is the world's largest producer of millets. In the 1970s, all of the millet crops harvested in India were used as a food staple.

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By the 2000, the annual millet production had increased in India, yet per capita consumption of millet had dropped by 50% to 75% in different regions of the Country. As of 2005, most millet produced in India is being used for alternative applications such as livestock fodder and alcohol production. Most millets have strong, deep rooting systems, short life cycles and can grow rapidly when moisture are available in soil. The state of Karnataka is the leading producer of finger millet, known as Ragi in the region, accounting for 58% of India's Ragi production[1].

Research and improvement efforts are needed to explore the potential of finger millet to increase agricultural production, crop diversification and a better nutritional environment. Finger millet is also more vulnerable to fungal blast, which is a major constraint in finger millet production due to high seed yield loss (>50%); this demands genetic improvement of finger millet. Genetic engineering provides a new technology for generation of superior plants for different agriculturally important traits such as resistance to biotic and abiotic stresses, nutritional quality and processing, attributes as per need of farmers, consumer and processer.

Production of transgenic plants with desired qualities is possible by genetic transformation of the desired genes in to the selected plants through the methodology of tissue culture. Efficient callus formation and regeneration is an important requisite to perform *Agrobacterium*-mediated transformations for producing transgenics. Different explants have been used for raising regenerable cultures in millets. Immature embryos with scutellum at milk stage provide the best starting material. But mature embryos, whole seeds, immature inflorescence, seedling leaf bases and roots have all been used for initiating cultures. There are two principal pathways of plant regeneration in tissue cultures: organogenesis and somatic embryogenesis. Somatic embryogenesis is the most common method of plant regeneration in all the major species of cereals and grasses. In some cases such as cultured embryos of *Eleusine coracana*, first an enlarged apical dome forms and then shoot buds get differentiated on the entire surface of the dome[5].

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants[13]. Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions in vitro. The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt[4] in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells.

Tissue culture has several potential applications in crop improvement and regeneration is prerequisite in such improvement programmes. Growth and morphogenesis of plant tissues under in vitro conditions is largely governed by the composition of the culture medium. Inorganic nutrient levels used in most plant tissue culture media are based on levels established for tobacco tissue cultures[7]. These nutrient levels which were adequate for tobacco tissue culture may not necessarily be optimum for the culture of other plant species like graminaceous monocots[2]. Screening of genotypes for response to *in vitro* culture is a vital requirement for development of efficient multiplication and regeneration protocols for any given crop[8]. The present investigation was undertaken with the objectives Optimization of phyto hormones for callus induction and regeneration of finger millet (Genotype 3885)

MATERIAL AND METHOD

A high yielding variety of finger millet- GE-3885 was used for present investigation. The present studies were carried out in Plant Tissue Culture Laboratory and Teaching Laboratory in the Department of Molecular Biology and Genetic Engineering, G.B. Pant University of Agriculture and Technology, Pantnagar during 2013-2015.

Chemicals/Fine Chemicals/Phytohormones: Most of the chemicals used were plant tissue tested and analytical and molecular grades.

Glasswares, Plasticwares and Equipments: Glasswares used in the present investigation were procured from Borosil, India and Schott Duran, Germany. Plasticwares comprising of microfuge tubes, petriplates, micro tips and tip boxes were procured from Tarson, India. Micropipettes of different ranges were obtained from Eppendorf, Germany.

Preparation of Stock Solution of Murashige and Skoog Medium : A stock solution is a concentrated solution that will be diluted to some lower concentrated solution for actual use. Stock solutions were used to save preparation time, conserve materials, reduce storage, space and improve the accuracy with which working solutions were prepared. A stock solution of concentration 100X, 1000X and 10000X were prepared while dissolving the nutrients in water. One compound was added at one time to avoid precipitation. The stocks were stored at 4ºC **(Table1).**

Preparation of Hormone Stocks: Hormones were dissolved in 2.0-5.0 ml of 1M NaOH or ethyl alcohol. After that completely dissolved the solute in solvent, and then the volume was made 25 ml with double distilled water **(Table 2).**

Seed transfer in medium: Everything that we were using during inoculation should be properly sterilized. Once the seed washing is finished, the seeds were transferred to a petriplate and wiped with a sterilized filter paper. In laminar air flow, sterilized 5 to 8 seeds were inoculate in each jam bottle containing callus inducing media with different concentrations of 2 4-D, NAA , BAP and kinetin , with the help of sterilized forceps and spatula. After inoculation, the cultures were incubated under photoperiod of 16 h light and 8 h dark at 27±1 ºC temperature in culture room for 20-21 days.

Proliferation of Callus: For the proliferation of callus, the suitable callus was separated and transferred to MS media with 0.5 NAA and 1.0NAA phytohormone next for 21 days.

RESULTS

Callus induction and regeneration: Callus formation and regeneration in finger millet was reported first from mesocotyl explants[6,9] through shoot bud formation. A detailed morphogenic and histological study reported formation of apical dome like structures which, upon sub-culture produced multiple shoots. Thiru and Mohan Ram[12] reported callus formation but no regeneration from different seedling explants.

For callus induction, 2,4-D is superior to dicamba and that Picloram is also effective have been reported earlier for finger millet[3,11]. In finger millet, white nodulated calli were formed on medium with N_6 macrosalts, MS microsalts, 2,4-dichlorophenoxyacetic acid (2 mg/l), kinetin (0.4 mg/l), 1-naphthalene acetic acid (2 mg/l), and certain additives[14]. Different concentrations and combinations of various growth regulators were added to MS medium for effective callus induction. Following composition of hormones were used for callus induction from mature embryo (Table 3). Development of a genotype-independent high-frequency regeneration system is an essential prerequisite for successful production of transgenic plants.

Optimization of Phytohormone Concentrations for Callus Induction through Mature Embryos: 42 (Forty two) combinations of phytohormones with Murashige and Skoog (MS) media were used for this study. Sterilized viable seeds of finger millet were transferred to callus induction media **(Table 3)** and inoculated culture vessels were shifted in culture room at 25±1^{0}C with 16 hour light and 8 hour dark photoperiod. Every three weeks of intervals callus was excised and subcultured in same culture medium for next 15 days.

Out of 42 combinations, callus initiation was observed in 16 combinations. In MS medium supplemented with 1.5 mg/l NAA, best response (96%) for callus induction was observed (Fig 4.1). Above 90% is the Excelent response till 80%- 90% is the good growth,

between 70% - 80 % is the average growth.and below 70% is poor response. Most of the calli were white, compact in nature.

Good response for callus initiation were found in MS medium supplemented with 1.0 mg/l NAA (90%), (Fig 4.2), 3.0 mg/l 2,4-D (88%), 2.0 mg/l NAA (87%), 2.5 mg/l NAA (85%) and 3.0 mg/l NAA + 1.0 mg/l Kinetin (89%) with white compact and white friable embryogenic callus (Fig 4.3). The average response were found in 2.0 mg/l 2,4-D (70%), 3.0 mg/l 2,4-D+1 mg/l Kinetin (75%), 3.5mg/l 2,4-D (79%), 2.5mg/l 2,4-D (73%), 3.0 mg/l NAA (71%). While other five combinations 0.5 mg/l 2,4-D + 0.5 mg/l BAP, 3.5 mg/l 2,4-D + 0.5 mg/l BAP, 2.5 mg/l 2,4-D + 0.5 mg/l BAP, , 0.5 mg/l NAA , 3.5 mg/l NAA resulted in significantly lower response of callus initiation from mature embryos of finger millet **(Table 4) (Fig. 1 to 6)**. Remaining 26 combinations has not shown any significant response for callus initiation.

Out of Forty-two different combinations of phytohormone best results for callus induction from mature seeds of finger millet were found in MS media supplemented with 1.5 mg/l NAA. In finger millet, high concentration of auxin (2,4-D NAA) and low concentration of cytokinin (BAP) was found better for callogenesis. The calli were subcultured in same callus induction media for proliferation. Best proliferation was found in MS media supplemented with 0.5 mg/l NAA. Most of the calli were friable and white in colour. Proliferation media was also responsive to the callus, which generated from other callus induction media.

Well proliferated calli were transferred on MS media with Fourteen different combinations of phytohormones for regeneration and the best regeneration was found in MS media supplemented with 1.5 mg/l BAP. The regenerated shoots were transferred in MS media for shoot proliferation with two different concentrations of BAP and after 21 days we got best results in MS media supplemented with 1.5 mg/l BAP.

Table 1: Composition of MS media stock solutions

Stock No.	Constituents	Stock strength	Conc. of M.S. (mg/l)	Conc. of stock solution (mg/100ml)	Volume (ml) to be taken from stock solution for 1.0 lt. medium
Stock (A)	$CaCl_2.2H_2O$	100X	440.0	4400.0	10
Stock (B)	$MgSO_4.7H_2O$	100X	370.0	3700.0	10
	KH_2PO_4		170.0	1700.0	
Stock (C)	$FeSO_4.7H_2O$	100X	27.8	278.0	10
	Na_2EDTA		37.3	373.0	
Stock (D)	H_3BO_3	1000X	6.2	620.0	1.0
	$MnSO_4.4H_2O$		22.3	2230.0	
	$ZnSO_4.7H_2O$		8.6	860.0	
	KI		0.83	83.0	
	$Na_2MoO_4.2H_2O$		0.25	25.0	
Stock (E)	$CuSO_4.5H_2O$	10000X	0.025	25.0	0.1
	$CoCl_2.2H_2O$		0.025	25.0	
Stock (F)	Nicotinic acid	1000X	0.5	50	1.0
	Pyridoxine HCl		0.5	50	
	Thiamine HCl		0.1	10	
	Glycine		2.0	200.0	

Table 2: Phytohormone stocks

S. No.	Hormones	Stock solution (mg/25ml)
1.	2,4-D (Ethanol)	25
2.	NAA (NaOH)	25
3.	BAP (NaOH)	25
4.	IBA (NaOH)	25
5	Kinetin	25

Table 3: MS medium with different concentrations of phytohormones used for callus induction

S. No.	Concentration of phytohormone (mg/l)			
	2,4-D	**BAP**	**NAA**	**Kinetin**
1.	0.5	-	-	-
2.	1.0	-	-	-
3.	1.5	-	-	-
4.	2.0	-	-	-
5.	2.5	-		-
6.	3.0	-		-
7.	3.5	-		-
8.	0.5	0.5	-	-
9.	1.0	0.5	-	-
10.	1.5	0.5	-	-
11.	2.0	0.5	-	-
12.	2.5	0.5	-	-
13.	3.0	0.5	-	-
14.	3.5	0.5	-	-
15.	0.5	-	-	1.0
16.	1.0	-	-	1.0
17.	1.5	-	-	1.0
18.	2.0	-	-	1.0
19.	2.5	-	-	1.0
20.	3.0	-		1.0
21.	-	-	0.5	-
22.	-	-	1.0	-
23.	-	-	1.5	-
24.	-	-	2.0	-
25.	-	-	2.5	-
26.	-	-	3.0	-
27.	-	-	3.5	-
28.	-	0.5	0.5	-
29.		1.0	0.5	-
30.	-	1.5	0.5	-
31.	-	2.0	0.5	-
32.	-	2.5	0.5	-
33.	-	3.0	0.5	-
34.	-	0.5	3.0	-
35.	-	1.0	3.0	-
36.	-	1.5	3.0	-
37.	-	2.0	3.0	-
38	-	2.5	3.0	-
39.	-	3.0	3.0	-
40.	-	-	3.0	1.0
41.	-	-	3.0	1.5
42.		-	3.0	2.0

Table 4: Degree of callus initiation from mature embryos of *E. coracana* in MS medium supplemented with different concentrations of 2,4-D, BAP, Kinetin and NAA. Callus initiation was observed after three weeks of inoculation. Data was recorded from 10 replicates with two independent experiments

S. No.	Combination of phytohormones (mg/l)	% of callus initiation	Colour	Texture	Embryonic
1.	2.0 2,4-D	70.1 ± 1.57	Cream	Compact	-
2.	2.5 2,4-D	73.0 ± 0.57	Brownish	Friable	-
3.	3.0 2,4-D	88.6 ± 0.85	White	Compact	Yes
4	3.5 2,4-D	79.7 ± 0.83	Brownish	Friable	Yes
5.	0.5 2,4-D+0.5 BAP	64.0 ± 0.57	Cream	Compact	-
6.	3.5 2,4-D+0.5 BAP	63.5 ± 1.33	Greenish	Compact	-
7.	2.5 2,4-D+0.5 BAP	63.6 ± 1.43	Brownish	Compact	-
8.	3.0 2,4-D+1.0Kinetin .	75.6 ± 1.20	Brownish	Friable	Yes
9.	3.0 NAA+1.0 Kinetin	89.0 ± 1.37	White	Friable	-
10.	0.5 NAA	64.0 ± 1.42	White	Friable	Yes
11.	1.0 NAA	90.0 ± 0.32	White	Friable	Yes
12.	1.5 NAA	96.6 ± 0.33	White	Compact	Yes
13.	2.0 NAA	87.6 ± 1.33	White	Friable	-
14	2.5NAA	85.0 ± 0.78	White	Compact	-
15.	3.0 NAA	71.0 ± 1.27	Brownish	Friable	-
16.	3.5 NAA	62.6 ± 1.53	Greenish	Friable	-
CD = 7.23 **SE(m) = 2.77**					

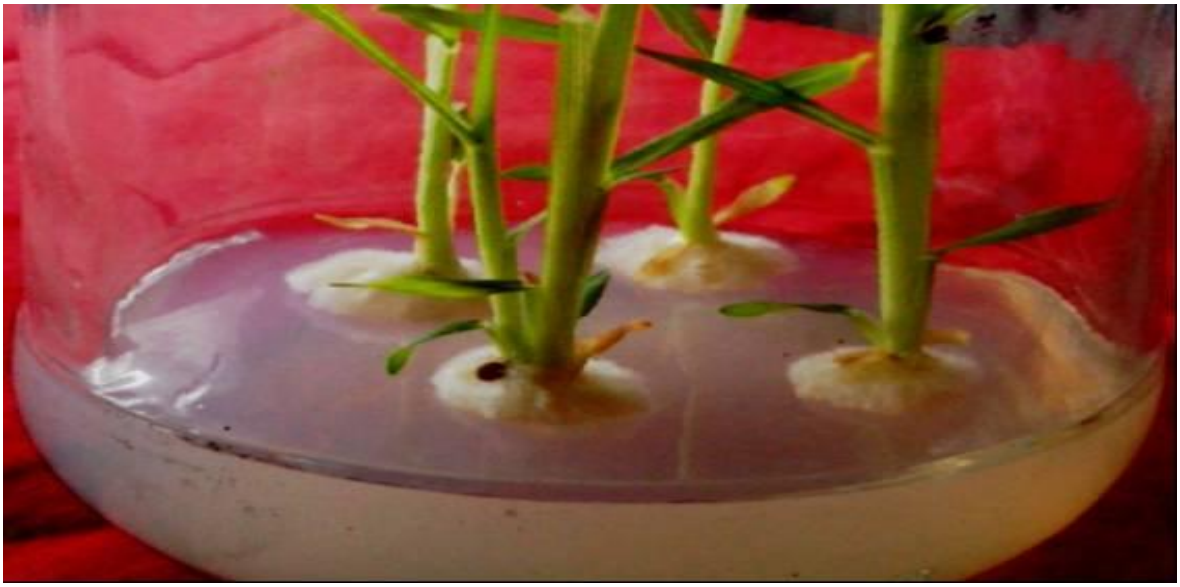

Fig. 1: Callus induction on MS media supplemented with 1.5 mg/l NAA from mature embryos of finger millet

Fig. 2: Well developed white and friable callus on MS media supplemented with 1.5 mg/l NAA

Fig. 3: Callus induction on MS media supplemented with 1.0 mg/l NAA from mature embryos of finger millet

Fig. 4: Well developed white and friable callus on MS media supplemented with 1.0 mg/l NAA

Fig. 5: Callus induction on MS media supplemented with 3.0 mg/l NAA+1.0 mg/l Kinetin from mature embryos of finger millet

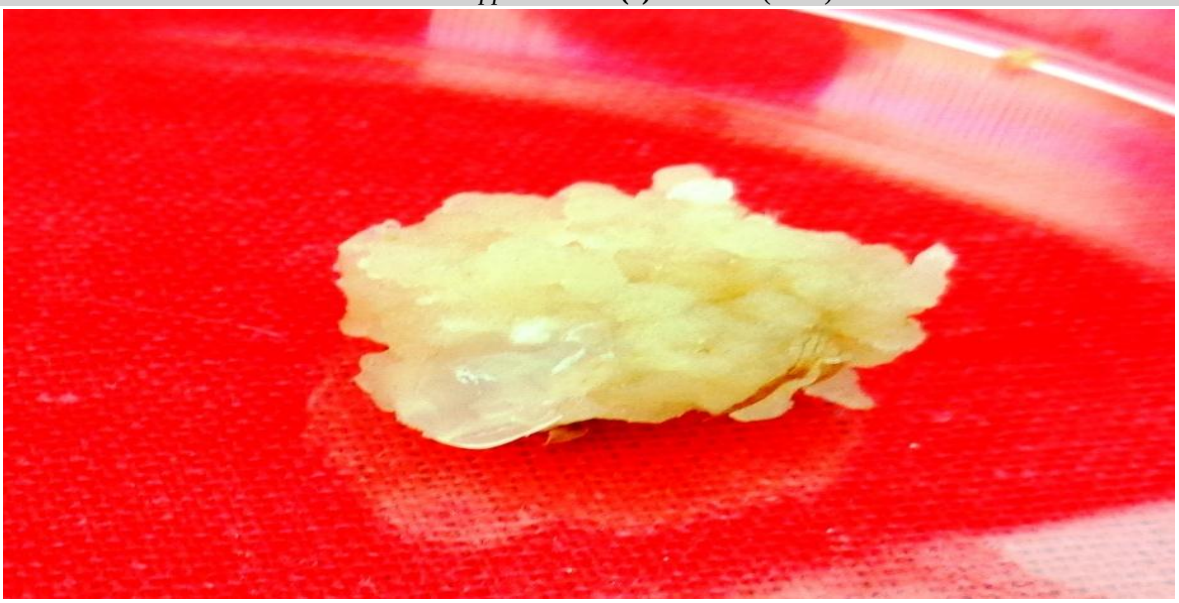

Fig. 6: Well developed white and friable callus on MS media supplemented with 3.0 mg/l NAA+1.0 mg/l Kinetin

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