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RESEARCH ARTICLE :

Somatic embryogenesis and plantlet regeneration from plumule explants of Finger millet [*Eleusine coracana* (L.) Gaertn.]

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SUMMARY : Plumule explants of finger millet varieties CO 9, CO 14 and TRY 1 showed better response to callus induction and plantlet regeneration compared to seed explants. High frequency of embryogenic, white, friable callus was induced from plumule explants, when cultured in MS + 2.0 mg/l 2,4-D and MS + 0.5 mg/l 2,4-D + 0.25 mg/l Kn. A concentration of 2,4-D at 0.5 mg/l with the lowest level of kinetin (0.1 mg/l) was found to be optimum for subculture and subsequent plant regeneration. Histological study of the embryogenic calli at different ages of subculture revealed the presence of somatic embryogenic pathway in plantlet regeneration and the initiation of somatic embryogenesis taking place in callus induction medium itself.

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KEY WORDS :
Finger millet, Somatic embryogenesis, Plumule culture

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BACKGROUND AND OBJECTIVES

Finger millet (*Eleusine coracana* (L.) Gaertn.) 2n = 4X = 36 is a poor man's crop originated from Ethiopia (Vavilov 1951). It is grown widely in Africa and Asia (Rangan and Vasil 1984). In India, Karnataka and Tamil Nadu are the two major states cultivating finger millet besides Andhra Pradesh, Maharastra, Uttar Pradesh and Bihar. The crop is solely utilized for human consumption, gaining popularity as relief food for diabetics. The fruit is rich in calcium and fibre (Malleshi and Desikachar 1986). The grain is malted and flour of the malted grain is used as nourishing food for infants and invalids. Plant biotechnological tools support and enhance the success of conventional breeding programmes. Nevertheless, improvement through genetic engineering or mutagenesis requires a reliable and efficient *in vitro* culture system (Fortes and Pais 2000). There is great potential of cell and tissue culture techniques in plant improvement, provided plants can be readily regenerated in large numbers (Jain *et al*. 2001).

In vitro regeneration of plants occurs either through organogenesis or somatic embryogenesis. Plant transformation studies

are revolving around a strong somatic embryogenic pathway. If the existence of somatic embryogenic pathway is unraveled, it is easy to transform the crop according to our need. In finger millet, callus formation and plant regeneration was first reported by Rangan (1976) from mesocotyl explants. Induction of somatic embryos from callus induced from different explants of finger millet have been reported by several authors (Mesocotyl: Eapen and George 1990; Immature embryos: Vishnoi and Kothari 1995; Seeds: George and Eapen 1995; Inflorescence: Jain *et al.* 2001; Shoot apex: Jain *et al.*. 2001; Latha *et al.* 2005; Sarker *et al.* 2007; Ceaser and Ignacimuthu 2008; Rao *et al.* 2009). Histological dissection of the embryogenic callus of finger millet was reported earlier (Mohanty *et al.* 1985). But, detailed description of the differentiating callus has not been carried out, so far.

RESOURCES AND METHODS

The seeds of the finger millet cultivars CO 9, CO 14 and TRY 1 released by Tamil Nadu Agricultural University, Coimbatore, India were used for the tissue culture experiments carried out during 2010. Two explants namely seed and plumule were used for *in vitro* culture studies.

The seeds were surface sterilized with 70% ethanol for 30 seconds and 0.1% $HgCl_2$ for three minutes. Plumule explants were obtained from 3-5 days old seedlings grown *in vitro* in ½ MS basal medium. The seeds as well as plumule explants were inoculated @ 4 nos/test tube in MS medium supplemented with different concentrations and combinations of 2,4-D (1.5, 2.0, 2.5 mg/l), NAA (2, 2.5, 3 mg/l), Kn and BAP (0.25, 0.50 mg/l) and the tubes were kept under dark for 3-5 days for callus initiation. Totally, 30 different growth regulator combinations were tried for callus induction.

After 15 days, the plumule derived calli were subcultured in MS medium with 9 different concentrations and combinations of 2,4-D (0.1, 0.2, 0.5 mg/l) and Kn (0.1, 0.2, 0.5 mg/l) along with previous callus induction medium used and basal medium. Callus multiplication response was recorded after 10 days and 20 days of subculture. Since, the callus induced from seed explant was poor, they were not subcultured for further studies. The embryogenic plumule callus of CO 14 induced in MS + 2.0 mg/l 2,4-D, subsequently regenerated in the subculture medium MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn was subjected to histological studies at 3rd,6th,9th,12th and 15th day of subculture as per the method adopted by Johansen (1940).

OBSERVATIONS AND ANALYSIS

The results obtained from the present study as well as discussions have been summarized under following heads :

Callus induction :

Initiation of callus from seed and plumule was noticed on 10th and 5th day of inoculation, respectively. The seeds swelled initially and then started producing callus from scutellar portion. Maximum callus induction was observed on 15th day of inoculation. Response of seed for callus induction was poor. Further the induced callus was mostly watery, yellow and dried very rapidly at all combinations of media investigated. So seed derived calli were not subcultured for further studies.

Regarding plumule explants, they swelled and elongated initially and then produced callus from the basal portion (Fig. 1B) as observed by Ceasar and Ignacimuthu (2008). In general, frequency of callus induction was increased with the increasing concentrations of 2,4-D and NAA in the medium (Table 1). The callus induced in NAA containing medium was highly friable and white, at all concentrations of NAA. With 2,4-D alone in the media, the callus was highly friable at 1.5 mg/l and 2.0 mg/l of 2,4-D, but became translucent and watery at 2.5 mg/l. This indicated the deleterious effect of higher doses of 2,4-D as reported by Mohanty *et al.* (1985); Jain *et al.* (2001); Vadivoo and Vijayalakshmi (2007); Sarker *et al.* (2007). Induction of friable, white, nodular callus occurred in media containing 2 mg/l of 2,4-D alone. This callus was highly friable and embryogenic than the callus induced in medium containing both 2,4-D and Kinetin. Addition of Kn at lower concentrations enhanced the callus induction frequency (Table 1). But, with the addition of BAP to 2,4-D, the callus became watery and yellow, irrespective of the type of explant and auxin. 2,4-D was found to be better for induction of callus and somatic embryos when compared to NAA (Yamada 1977; Ceasar and Ignacimuthu 2008; Anjaneyulu *et al.* 2011; Sweta and Cahwla 2015). Rhizogenesis was observed in the callus induction medium containing NAA from plumule derived calli (Fig. 1F).

Somatic embryogenesis :

The calli induced from plumule explants of the three varieties in the five media *viz.*, MS + 2.0 mg/l 2,4-D (CIM 1), MS + 2.0 mg/l 2,4-D + 0.25 mg/l Kn (CIM 2), MS + 2.0 mg/l 2,4-D + 0.50 mg/l Kn (CIM 3), MS + 2.5 mg/l NAA (CIM 4), MS + 3.0 mg/l NAA (CIM 5) were chosen for subculture studies, as the callus induced from these media were found to be embryogenic, friable and white. In subculture medium, 2,4-D and Kn were used alone or in combination at low levels (0.1 mg/l, 0.2 mg/l, 0.5 mg/l) along with previous callus induction medium used and MS basal medium. Response of callus after 10 and 20 days of subculture was recorded. Callus induced in CIM 3 and CIM 4 showed poor multiplication and dried early in all the subculture medium used. Hence, the response of the calli induced in CIM 1, CIM 2 and CIM 3 in different subculture medium alone are discussed (Table 2).

When 2,4-D alone was present in the subculture medium, initially there was increase in the proliferation as the concentration of 2,4-D increased, in case of the callus induced in MS + 2 mg/l 2,4-D (CIM 1) but, after 20 days the callus turned brown and dried. But, at 0.5 mg/l 2,4-D, the callus remained fresh even after 20 days. In case of the callus induced in 2 mg/l 2,4-D + 0.25 mg/l Kn (CIM 2), there was moderate proliferation initially in all the concentrations of 2,4-D used alone in the subculture medium. But after 20 days they remained fresh without any further multiplication. In case of the callus induced in MS + 3 mg/l NAA, rhizogenesis was observed

Table 1: Per cent callus induction from plumule explants of different varieties in different callus induction media

Sr. No.	Medium	CO 9	CO 14	TRY 1
1.	MS + 1.5 mg/l 2,4-D	40.00	42.50	40.00
2.	MS + 2.0 mg/l 2,4-D	90.00	85.00	77.50
3.	MS + 2.5 mg/l 2,4-D	80.00	75.00	75.00
4.	MS + 1.5 mg/l 2,4-D + 0.25 mg/l Kn	30.00	40.00	30.00
5.	MS + 1.5 mg/l 2,4-D + 0.50 mg/l Kn	52.50	42.50	35.00
6.	MS + 2.0 mg/l 2,4-D + 0.25 mg/l Kn	100.00	90.00	100.00
7.	MS + 2.0 mg/l 2,4-D + 0.50 mg/l Kn	100.00	85.00	90.00
8.	MS + 2.5 mg/l 2,4-D + 0.25 mg/l Kn	80.00	70.00	75.00
9.	MS + 2.5 mg/l 2,4-D + 0.50 mg/l Kn	100.00	100.00	87.50
10.	MS + 1.5 mg/l 2,4-D + 0.25 mg/l BAP	25.00	30.00	32.50
11.	MS + 1.5 mg/l 2,4-D + 0.50 mg/l BAP	30.00	32.50	25.00
12.	MS + 2.0 mg/l 2,4-D + 0.25 mg/l BAP	77.50	60.00	52.50
13.	MS + 2.0 mg/l 2,4-D + 0.50 mg/l BAP	70.00	65.00	55.00
14.	MS + 2.5 mg/l 2,4-D + 0.25 mg/l BAP	75.00	60.00	57.50
15.	MS + 2.5 mg/l 2,4-D + 0.50 mg/l BAP	82.50	67.50	60.00
16.	MS + 2.0 mg/l NAA	62.50	57.50	60.00
17.	MS + 2.5 mg/l NAA	75.00	70.00	67.50
18.	MS + 3.0 mg/l NAA	92.50	90.00	97.50
19.	MS + 2.0 mg/l NAA + 0.25 mg/l Kn	50.00	50.00	52.50
20.	MS + 2.0 mg/l NAA + 0.50 mg/l Kn	42.50	47.50	45.00
21.	MS + 2.5 mg/l NAA + 0.25 mg/l Kn	57.50	55.00	50.00
22.	MS + 2.5 mg/l NAA + 0.50 mg/l Kn	50.00	50.00	42.50
23.	MS + 3.0 mg/l NAA + 0.25 mg/l Kn	70.00	47.50	65.00
24.	MS + 3.0 mg/l NAA + 0.50 mg/l Kn	60.00	45.00	50.00
25.	MS + 2.0 mg/l NAA + 0.25 mg/l BAP	32.50	30.00	35.00
26.	MS + 2.0 mg/l NAA + 0.50 mg/l BAP	32.50	25.00	27.50
27.	MS + 2.5 mg/l NAA + 0.25 mg/l BAP	37.50	35.00	37.50
28.	MS + 2.5 mg/l NAA + 0.50 mg/l BAP	35.00	40.00	25.00
29.	MS + 3.0 mg/l NAA + 0.25 mg/l BAP	42.50	40.00	47.50
30.	MS + 3.0 mg/l NAA + 0.50 mg/l BAP	35.00	35.00	42.50

after 20 days in the subculture medium with moderate initial proliferation.

When kinetin alone was present in the subculture medium at 0.1 mg/l, 0.2 mg/l, there was initial moderate proliferation from the callus induced in MS + 2 mg/l 2, 4-D and the callus produced shoots and roots after 20 days which dried later. In case of the callus induced in other two callus induction media (CIM 2 and CIM 5), the proliferation was poor in subculture medium containing Kn alone. However, green tissues and embryo like structures appeared after 20 days, which did not develop into plantlets further.

In case of combinations of 2,4-D and Kn used in subculture medium, the initial proliferation was good from the callus induced in 3 types of media. Kinetin at lower concentrations found to have synergistic effect on callus proliferation, when combined with a higher concentration of 2,4-D (Vadivoo and Vijayalakshmi 2007). In case of callus induced in MS + 2mg/l 2,4-D (CIM 1), the callus produced shoots and roots in the subculture medium containing lowest level of Kn (0.1 mg/l) along with 2,4-D which later produced plantlet. This callus at different ages of subculture was used for histological studies. But, when Kn was present at 0.2 mg/l along with 2,4-D at 0.5 mg/l, although shoots and roots were produced, they did not develop into plantlets. In case of other two types of callus used for subculture, neither caulogenesis nor rhizogenesis was observed.

Fig. 1 : Tissue culture experiments (A) Plumule explants of CO 14 inoculated for callus induction in MS + 2.0 mg/l 2,4-D (B) Callus induction from plumule explants of CO 14 in MS + 2.0 mg/l 2,4-D (C) Proliferating embryogenic plumule callus in MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn (D) Regenerating plantlet from plumule callus in MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn (E) Non embryogenic plumule callus of CO 14 in MS + 2.0 mg/l 2,4- D (F) Rhizogenesis of CO 14 plumule callus in NAA medium (MS + 3.0 mg/l NAA)

Histology:

Biological organization of any life coordinated with several events, as though a craftsman was moulding it according to a plan. In this process, the individual parts do not develop independently, but all are knit together into an organised system. The phenomenon of morphogenesis leading to organogenesis or somatic embrygenesis depends on the factors like polarity, differentiation and regeneration of individual cells and the fate of a cell is a function of its position.

Regeneration of plants via somatic embryogenesis is preferred to organogenesis because of the single cell origin of embryoids, thereby making such embryogenic cells suitable for genetic manipulation (Kumar *et al.* 2001). Street and Withers (1974) defined the *in vitro* embryogenesis as the developmental process, producing a perfect embryo from a single cell, all the derivatives from which become a part of a structure, which achieve bipolarity at an early stage as occurs during the development of zygotic embryos.

Table 2: Response of CO 14 calli induced from different callus induction media in different subculture media

Callus induction medium	Subculture medium	Response observed	
		Callus multiplication response after 10 days	Response after 20 days
1) MS + 2.0 mg/l 2,4-D	MS + 2.0 mg/l 2,4-D	Moderate, white, watery	No further multiplication, dried later
	MS Basal	Good, slight watery, white	Turned yellow, rhizogenesis
	MS + 0.1 mg/l 2,4-D	Poor, white, friable	Turned brown and dried later
	MS + 0.2 mg/l 2,4-D	Moderate, yellow, watery	Turned brown and dried later
	MS + 0.5 mg/l 2,4-D	Good, white, watery	No further multiplication, remained fresh
	MS + 0.1 mg/l Kn	Moderate, white, watery	Produced shoots and roots, dried later
	MS + 0.2 mg/l Kn	Moderate, white, friable	Produced shoots and roots, dried later
	MS + 0.5 mg/l Kn	Moderate, white, watery	Turned brown and dried later
	MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn	Good, white, watery	Produced shoots and roots, Developed into
	MS + 0.5 mg/l 2,4-D + 0.2 mg/l Kn	Good, white, friable	plantlets
	MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kn	Good, white, watery	Produced shoots and roots, dried later
			Turned brown and dried later
2) MS + 2.0 mg/l 2,4-D + 0.25 mg/l Kn	MS + 2.0 mg/l 2,4-D + 0.25 mg/l Kn	Good, yellow, friable	No further multiplication, remained fresh
	MS Basal	Good, yellowish white, watery	Rhizogenesis, turned brown
	MS + 0.1 mg/l 2,4-D	Moderate, yellowish white, watery	No further multiplication, remained fresh
	MS + 0.2 mg/l 2,4-D	Moderate, yellowish white, watery	No further multiplication, remained fresh
	MS + 0.5 mg/l 2,4-D	Moderate, yellowish white, watery	No further multiplication, remained fresh
	MS + 0.1 mg/l Kn	Poor, white, watery	Induced green tissue, later dried
	MS + 0.2 mg/l Kn	Poor, white, watery	Induced green tissue, later dried
	MS + 0.5 mg/l Kn	Poor, white, watery	Turned brown and dried later
	MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn	Good, white, watery	No further multiplication, remained fresh
	MS + 0.5 mg/l 2,4-D + 0.2 mg/l Kn	Good, white, watery	No further multiplication, remained fresh
	MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kn	Good, white, watery	Turned brown and dried later
3) MS + 3.0 mg/l NAA	MS +3.0 mg/l NAA	Good, white, friable	Turned yellowish white, Rhizogenesis
	MS Basal	Poor, induced roots	No further multiplication
	MS + 0.1 mg/l 2,4-D	Moderate, white, friable	Rhizogenesis, no further multiplication
	MS + 0.2 mg/l 2,4-D	Moderate, white, friable	Rhizogenesis, no further multiplication
	MS + 0.5 mg/l 2,4-D	Good, white, friable, rhizogenesis	Rhizogenesis, no further multiplication
	MS + 0.1 mg/l Kn	Poor, yellow, rhizogenesis	Formed embryo like structures
	MS + 0.2 mg/l Kn	Poor, yellow, rhizogenesis	Formed embryo like structures
	MS + 0.5 mg/l Kn	Poor, yellow, watery	No further multiplication, dried later
	MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn	Good, yellowish white, friable	Formed embryo like structures
	MS + 0.5 mg/l 2,4-D + 0.2 mg/l Kn	Good, yellowish white, friable	Formed embryo like structures
	MS + 0.5 mg/l 2,4-D + 0.5 mg/l Kn	Good, yellow, watery	No further multiplication, dried later

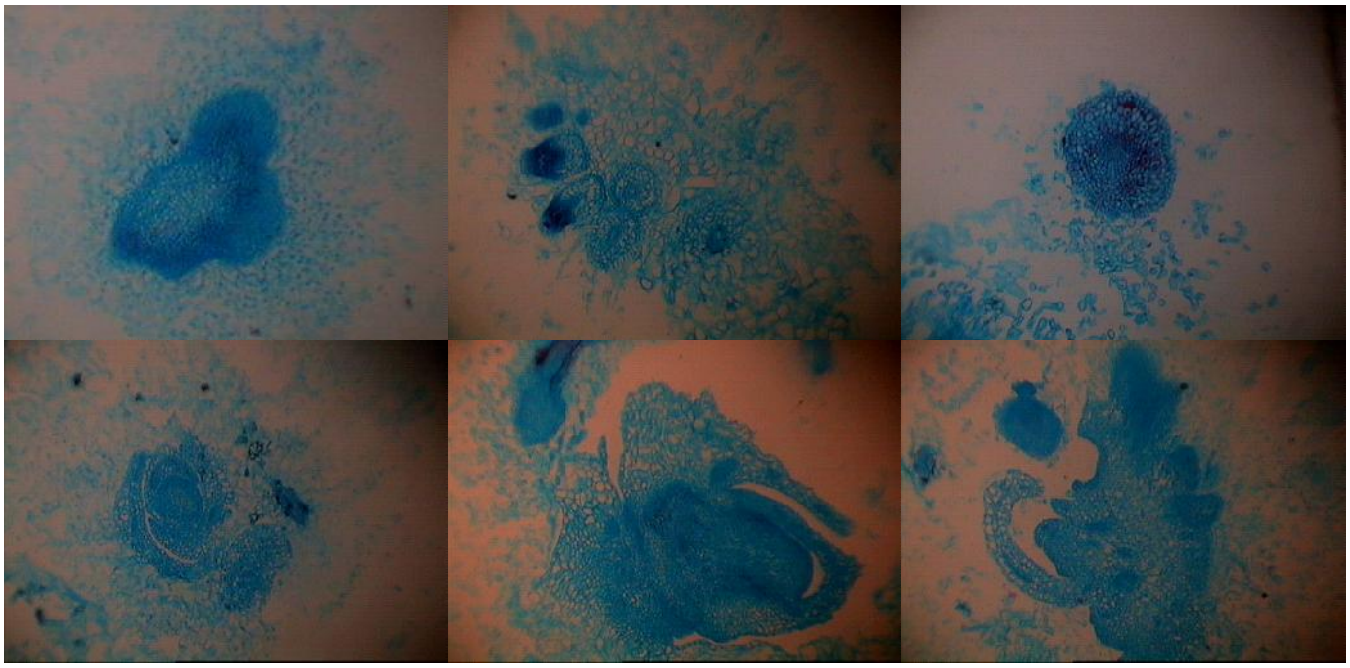

Fig. 2 : Histological analysis of the callus (A) Callus showing tendency for organised divisions (B) Embryogenic cell initials with meristematic activity (C) Proembryo with intense division (D & E) Somatic embryo showing bipolarity (F) Apical meristem in growing somatic embryo, A, B, E & F - 400 X magnification, C & D- 100X magnification

Histological analysis of the callus induced in 2 mg/l 2,4-D, subcultured in 0.5 mg/l 2,4-D + 0.1 mg/l Kn at different ages revealed different stages of somatic embryogenesis (Fig 2). Three days old subcultured callus showed clusters of unorganized cells indicating the occurrence of organization of cells in the callus induction medium itself (Fig 2A). On 6th day, meristematic regions were observed (Fig 2B), either in the periphery of the cell or found few to several layers below the epidermis of the callus. Proembryos were developed on 9th day of subculture (Fig 2C). On 12th day, the embryos further developed and showed bipolarity with distinct shoot and root initials with the absence of interconnections between the embryos (Fig 2 D & E) as defined by Street and Withers (1974). Fifteen days old culture showed apical meristems in the fully developed somatic embryo (Fig 2F). Thus, in the present investigation, the regeneration of plants was achieved through somatic embryogenesis as reported by Mohanty *et al.* (1985).

To conclude, the media suggested for callus induction and somatic embryogenesis are MS + 2 mg/l 2,4-D and MS +0.5 mg/l 2,4-D + 0.1 mg/l Kn, respectively which was confirmed through histological studies. So the above system can be used for induction of somatic embryogenesis in finger millet using plumule explants.

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