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A rapid protocol for somatic embryogenesis mediated regeneration in banana (*Musa* spp.) cv. Nendran

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ABSTRACT

A simple and rapid protocol for somatic embryogenesis in banana cv. Nendran (AAB) using immature male flowers (IMF) has been developed. The IMF produced pale-white to yellow, globular embryogenic callus on MS medium supplemented with BA (0.05 – 0.50mgL^{-1}) and picloram (0.50 – 2.00mgL^{-1}) with explant response of to 30 per cent. Addition of ascorbic acid (20mgL^{-1}) and Gelrite© (0.45 per cent) to callus induction medium reduced interference from phenolic exudation. Embryogenesis was induced (33.3 to 60 per cent) on semisolid (0.30 per cent Gelrite©) MS medium supplemented with BA 2mgL^{-1} + IAA 0.5mgL^{-1}. The somatic embryos showed 60-80 per cent germination on half- strength semisolid MS medium with BA 2mgL^{-1} + IAA 0.5mgL^{-1}. Transfer of germinated embryos to semisolid MS medium supplemented with BA 2mgL^{-1} + NAA 1mgL^{-1} under 14 h light /8h dark photoperiod resulted in hundred percent conversion to plantlets. This protocol takes merely 6 months for producing plantlets from immature flower buds through somatic embryogenesis, without any intermediate liquid cultures.

Key words: Immature male flowers, somatic embryogenesis, picloram, *Musa* spp., Nendran

INTRODUCTION

Bananas and plantains belonging to the genus *Musa* of Musaceae family, are perennial herbaceous monocots. *Musa* is a major crop in the tropical and sub-tropical regions of the world, with an approximate production of 70 million tonnes per annum. India is rich in genetically diverse varieties of banana, cultivated over an area of 802.6 (000ha), with production of 297.24 lakh tonnes, contributing 33.4 per cent of the global production share (Indian Horticulture Database, 2015, National Horticulture Board, Ministry of Agriculture, Govt. of India). Owing to female sterility, parthenocarpy and polyploidy, production of new banana cultivars is difficult and time-consuming. Genetic manipulation is a promising technique for introducing desired traits in banana. A rapid and effective *in vitro* regeneration protocol is a prerequisite for any such manipulation techniques. Single-cell origin of the somatic embryos and their potential to produce non-chimeric plants makes it a desirable system for genetic manipulation. Somatic-embryogenesis-mediated regeneration is also a method for large-scale multiplication of banana.

Immature male inflorescence has been used to initiate embryogenic cultures of several banana and plantain cultivars (Cronauer and Krikorian, 1983; Escalant *et al*, 1994; Cote *et al*,1996; Navarro *et al*, 1997). Compared to soil-grown suckers, IMFs show less contamination during micropropagation, and, selection of male buds with desirable characteristics such as high number of hands and fruits per bunch is also possible (Resmi and Nair, 2007).

Nendran is a popular banana cultivar in South India where it is relished as a fruit and is used in making several value-added products. This variety is characterized by its fruits having a distinct neck with a thick, green skin, which turns buff-yellow on ripening and has a pale yellow flesh. Fruits of Nendran are larger, silky, with a firm flesh and are sweet. Bananas offer a wonderful nutritious diet due to their high protein content, are easy to digest, especially in infants. Attempts made on somatic embryogenesis in Nendran

are scarce. Most reports are on Cavendish varieties, which involve lengthy procedures (taking 18-26 months for plantlet development). This study was undertaken to develop a rapid and simple protocol for somatic-embryogenesis-mediated regeneration system in Nendran, suitable for genetic transformation experiments.

MATERIAL AND METHODS

Preparation of explant for inoculation

Male flower inflorescence was collected from field-grown *Musa* spp. cv. Nendran plants, after completion of bunch formation (i.e., 4 weeks after emergence of the inflorescence). Bracts were removed from the inflorescence to reduce its size to 7-8cm length. These explants were then surface-sterilized with 90% ethanol, inside a laminar air-flow cabinet. Immature male flower (IMF) clusters from 0-15 position from inside were separated using a sterile blade. The tiny bracts covering male

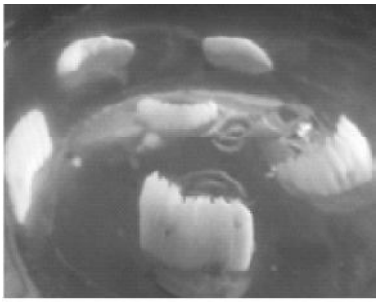

Fig. 1. Immature male flowers inoculated on callus induction medium

flowers (hands) were removed without damaging the apical dome and were inoculated onto callus induction medium (Fig.1)

Callus induction from IMFs

The explants were cultured in 250ml culture bottles containing 40ml MS medium (Murashige and Skoog, 1962) supplemented with various combinations of 2,4-D (0.5 to 4mgL^{-1}), IAA (0.5 – 1mg L^{-1}), BA (0.5 – 4mg L^{-1}) and picloram (0.5 – 10mg L^{-1}) along with ascorbic acid 20mg L^{-1}. Different combinations of growth regulators used for callus induction (Treatments A, B and C) are shown in Table I. Gelrite© at 0.4% was used as the solidifying agent. pH of the medium was adjusted to 5.7 using 1N NaOH or HCl and was autoclaved at 121°C, 1.06 Kg cm^{-2} pressure for 20 minutes. Cultures were incubated in the dark at 25±2ºC and at 50-60 per cent relative humidity. Callus initiated was subcultured onto the same parent medium at monthly intervals for multiplication.

Induction of somatic embryos

Calli developed on different treatments were transferred to media of four different compositions: (1) MS+ 2,4-D 2mgL^{-1} + Zeatin 0.2 mgL^{-1} + Gelrite 4% (2) MS+ 2,4-D 2mgL^{-1}+ IAA 1mgL^{-1}+NAA 1mgL^{1} + Biotin 1mgL^{-1}+ Glutamine 100mgL^{-}1 (3) MS+BA 2mgL^{-1} + IAA 0.5mgL^{-1} + Gelrite 0.3%, and (4) SH + MS vitamins + Glutamine 100mgL^{-1} + picloram 1mgL^{-1}. Sucrose at 4.5% and Gelrite at 0.2% was used in all the media. Fifteen replications with three clumps each were maintained in this study. Cultures were incubated in the dark at 25±2ºC and at 50-60 per cent relative humidity.

Maturation and germination of embryos

Somatic embryos were transferred to half-strength MS (half-strength macro nutrients) supplemented with BA 2mg L^{-1} + IAA 0.5mg L^{-1}. Cultures were incubated in the dark for 3 weeks for maturation and germination of somatic embryos.

Conversion of somatic embryos into plantlets

Germinated embryos were transferred to MS medium supplemented with BA 2mg L^{-1} + NAA 1mgL^{-1} for development into plantlets. Cultures were maintained at a light intensity of 31.4µmol$m^{-2}S^{-1}$ and 14h light/8h dark photoperiod. Plantlets at 3-4 leaf stage were transferred to coir pith compost in *protrays* and hardened in a mist chamber for a month. Plantlets were then transferred to polybags with soil and cow dung (1:1) mixture.

RESULTS AND DISCUSSION

In the present study, a protocol was developed for plantlet regeneration via somatic embryogenesis in banana cultivar Nendran. Somatic embryogenesis has been reported in many banana cultivars such as Bluggoe (ABB), Grand Naine (AAA) and Rasthali (AAB) (Novak *et al*, 1989; Escalant *et al*, 1994). However, few reports are available on this route of *in vitro* culture in the variety, Nendran (AAB group). Somatic embryos are ideal targets for genetic manipulation studies owing to their single cell origin. A rapid protocol for somatic embryogenesis can reduce time taken to develop transformants and their analysis.

Immature male flowers were used as explants in this study for establishing an *in vitro* regeneration system via embryogenesis. IMFs have been used as explants for initiating cultures of several bananas and plantain cultivars (Becker *et al*, 2000; Khalil *et al*, 2002; Namanya *et al*, 2004; Houllou-Kido *et al*, 2005). As the explants were collected after formation of the complete bunch, it was possible to superior traits of mother plant assure selection of explants for the study, based on visual evaluation of bunch quality, number of hands, pest and disease resistance, etc.

Sterilization and culture establishment from IMFs

Contamination-free cultures were established with a simple surface-sterilization procedure. Dipping the flower bud in 90% ethanol for one minute, or, wiping it with ethanol soaked cotton-swab, was found to be effective. Immature flower buds are perfectly and tightly packed within the bracts of the inflorescence. This may be the reason for obtaining contamination-free cultures with this simple procedure. Endogenous bacterial contamination is very low in inflorescence meristems compared to that in the shoot meristem, favouring selection of this explant for *in vitro* culture.

A serious problem encountered in establishing IMF cultures in cv. Nendran was presence of polyphenols. The explant turned brown in 2 to 3 days after inoculation, and decayed subsequently. In this study, 20 mgL^{-1} of ascorbic acid was added to the medium in all the treatments, thus reducing the blackening of explants due to phenolic oxidation, and preventing decay of the explants. Use of antioxidants like ascorbic acid (Namanya *et al*, 2004) and citrate (Soubir *et al*, 2006) has been shown to avoid browning and improve callus formation in banana. Ascorbic acid is a reducing agent and can prevent oxidation of phenolics in the medium and it scavenges oxygen radicals produced during wounding of tissues. In this study, Gelrite© was used in the medium as a gelling agent, replacing agar. This too could overcome problems associated with phenolic exudation (Fig. 2). Spread of exudates was very restricted when Gelrite© was used. Similar findings have been reported by Khatri *et al* (2005) in callus induction studies using various banana cultivars. They also reported a positive correlation of light with browning, which may be due to the higher physiological activities under light than in the dark. In the present study too, cultures were incubated in the dark to overcome the ill effects of phenolics.

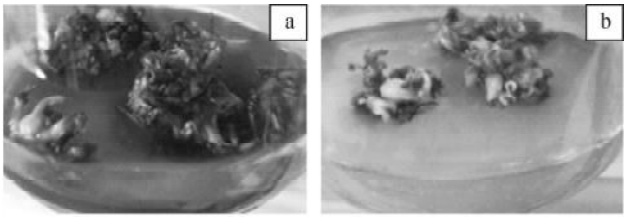

Fig. 2. Effect of solidifying agents on interference from phenolics in IMFs on callus induction medium: a) IMFs inoculated on callus induction medium solidified with agar, and b) Medium solidified with Gelrite on 10th day from inoculation.

Effect of growth regulators on callus induction in IMFs

Callus induction was observed on IMFs in 45 to 50 days after inoculation on different media tested (Table1). Explants inoculated onto MS medium supplemented with 2,4-D (0.5 - 4 mgL^{-1}) and IAA (0.5 – 1 mgL^{-1}) turned brown within a week of inoculation (A1 to A16). However, after 4 weeks of inoculation, the explants exhibited a swelling; and, after another two weeks, callus initiation was observed in treatments A1 to A14. Among treatments with a combination of 2,4-D and IAA, callus induction was highest in treatment A9 (45.80 per cent), while treatments A1 & A13 produced the least (8.30 per cent). Higher concentration of 2,4-D, above 3.5mgL^{-1} (A15, A16), did not produce any callus. Calli induced in different treatments varied in morphology too (Fig. 3a-&-b). Ganapathy *et al* (1999) were able to produce embryogenic callus from male flower buds of five banana cultivars on MS or White's medium supplemented with 2,4-D, NAA, IAA and biotin within 2 to 3 months of inoculation. According to Meenakshi *et al* (2011), IMF inoculation in 2,4-D supplemented MS medium could induce 77.70 per cent callus within a few weeks. Karintanyakit *et al* (2014) also reported the effect of 2,4-D on IMF in seven banana cultivars, and observed that callus induction was genome-specific.

IMFs inoculated on picloram (0.5-10 mgL^{-1}) supplemented medium (B1 to B11) turned brown at the base within 3 days of inoculation, but induced yellow coloured callus in treatments B4 to B11 (Fig. 3c), with the explant response ranging from 15 per cent to 60 per-cent in B4 and B8, respectively, in 45 days. Houllou-kido *et al* (2005) made a similar observation in their study where IMFs inoculated onto MS medium

Table 1. Effect of growth hormones on callus induction in IMF of *Musa spp.* cv. Nendran

Treatments	2,4-D (mgL^{-1})	BA (mgL^{-1})	IAA (mgL^{-1})	Picloram (mgL^{-1})	Morphology of callus	Explant response %
A_1	0.5	-	0.5	-	Pale white, hard	8.3
A_2	0.5	-	1	-	Pale white, hard	16.6
A_3	1	-	0.5	-	Pale white, hard	25.0
A_4	1	-	1	-	Pale white, hard	41.6
A_5	1.5	-	0.5	-	Pale white, hard	33.3
A_6	1.5	-	1	-	Pale white, hard	29.1
A_7	2	-	0.5	-	Pale white, hard	33.3
A_8	2	-	1	-	Pale white, hard	33.3
A_9	2.5	-	0.5	-	Pale white, hard	45.8
A_{10}	2.5	-	1	-	Pale white, hard	25.0
A_{11}	3	-	0.5	-	Pale white, watery	20.8
A_{12}	3	-	1	-	Pale white, watery	20.8
A_{13}	3.5	-	0.5	-	Pale white, watery	8.3
A_{14}	3.5	-	1	-	Pale white, watery	25.0
A_{15}	4	-	0.5	-	No response	0
A_{16}	4	-	1	-	No response	0
B_1	-	-	-	0.5	No response	0
B_2	-	-	-	1	No response	0
B_3	-	-	-	2	No response	0
B_4	-	-	-	3	Yellow, watery	15.0
B_5	-	-	-	4	Yellow, watery	35.0
B_6	-	-	-	5	Yellow, watery	35.0
B_7	-	-	-	6	Yellow, watery	50.0
B_8	-	-	-	7	Yellow, watery	60.0
B_9	-	-	-	8	Yellow, watery	40.0
B_{10}	-	-	-	9	Yellow, watery	30.0
B_{11}	-	-	-	10	Yellow, watery	40.0
C_1	-	0.05	-	0.5	Yellow, globular	8.3
C_2	-	0.05	-	1	Yellow, globular	20.8
C_3	-	0.05	-	2	Yellow, globular	16.6
C_4	-	0.1	-	0.5	Pale white, globular	16.6
C_5	-	0.1	-	1	Pale white, globular	30.0
C_6	-	0.1	-	2	Pale white, globular	25.0
C_7	-	0.2	-	0.5	Pale white, globular	8.3
C_8	-	0.2	-	1	Pale white, globular	12.5
C_9	-	0.2	-	2	Pale white, globular	8.3
C_{10}	-	0.5	-	0.5	Pale white, globular	16.6
C_{11}	-	0.5	-	1	Tiny, yellow, globular, friable	12.5
C_{12}	-	0.5	-	2	Tiny, yellow, globular, friable	8.3

Note: Callus initiation occurred in 45-50 days on all the media tested

supplemented with picloram developed necrosis in the first two weeks. However, after two months of inoculation, 80 per cent callus initiation was observed beneath the bracts. Smitha and Ashalatha (2011) reported the effect of picloram on callus induction from leaf-sheath explants. They obtained a brown, spongy callus with yellow globular structures using low concentrations of picloram in MS medium. At higher concentrations of picloram, a brown compact callus with white globular structures developed.

In treatments with BA and picloram (C1 to C12), IMFs produced small-to medium-sized, yellow, globular friable callus with a dense yellow cytoplasm in C1,C2 and C3 (Fig. 3d), whereas, treatments C4 to C10 produced pale-white, globular callus with a dense cytoplasm (Fig. 3e), and, treatments C11 and C12 (Fig. 3f) developed yellow, watery callus. Among the treatments with BA and picloram combinations in MS medium, C5 produced the maximum (30 per cent) callusing rate. Callus initiation occurred in 45-55 days from inoculation in this combination. In a study by Smitha and Ashalatha (2011), embryogenic callus was obtained from leaf-sheath explants on MS supplemented with picloram in *Musa acuminata* cv. Njalipoovan (AB). In the present study, among all the treatments tested for callus initiation, from phenolics interference was less in BA and picloram combination. Callus initiation occurred in 45-55 days of inoculation in this combination.

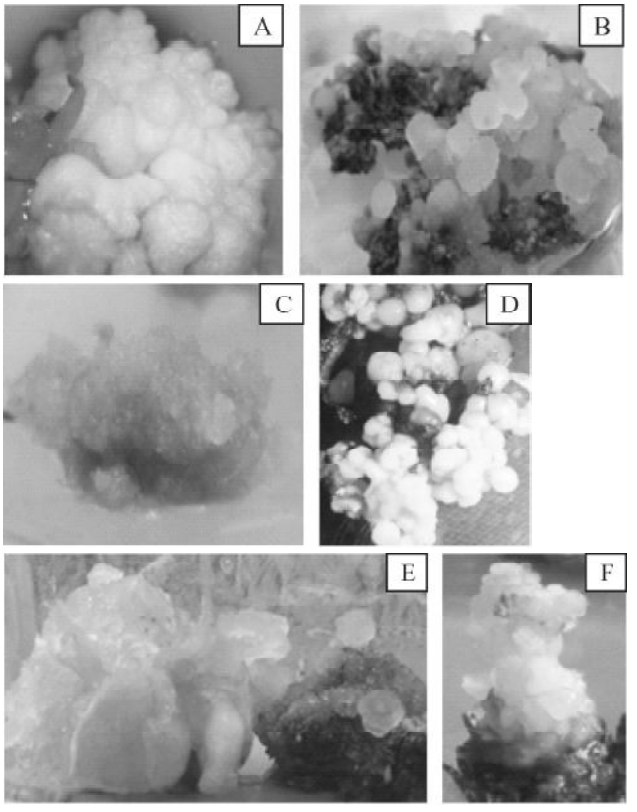

Fig. 3. Morphology of calli induced on MS medium supplemented with 2,4-D and IAA:

A) Pale-white, hard callus formed in treatments A1 to A10, i.e., 2,4-D upto 2.5mgL^{-1} and IAA (0.5 or 1mgL^{-1})

B) Pale-white watery callus formed in treatments A11 to A14 i.e. when 2, 4-D was more than 2.5 mg L^{-1}

C) Yellow coloured callus formed on MS medium with picloram (Treatments B4 to B11)

D) Yellow globular callus formed in treatments C1 to C3 (MS medium with BA 0.5mgL^{-1} and picloram 0.5 to 2mg L^{-1})

E) Pale-white globular callus formed d in treatments C4 to C10 (MS with BA 0.05 to 0.5 mg L^{-1} and picloram 0.5 to 2mg L^{-1})

F) Yellow callus formed in treatments C11 and C12 (MS with BA 0.5 and picloram 1or 2mg L^{-1})

Induction of somatic embryos from callus

Embryogenic potential of the callus induced in various treatments mentioned above was studied in four different media (Table 2). But, embryo formation was observed only on semisolid MS medium supplemented with BA 2mgL^{-1}, IAA 0.5 mgL^{-1} and 0.3 per cent Gelrite©. Only calli induced in treatments C8, C9 and C10 produced somatic embryos on this medium within 30 days of inoculation. Callus from C9 yielded 10 per cent embryo induction, which was the highest response in this study. These monocot embryos seemed elongated, with a glassy appearance (Fig. 4). Even though callus induction was high in treatments A and B, these failed to produce embryos. According to Arnold *et al* (2002), competence for somatic embryo induction may be the result of varying auxin sensitivity of cells. They observed that although callus initiation was comparatively high in treatments with 2,4-D and IAA, and, that with picloram alone, these culture failed to induce somatic embryos. Hence, it can be inferred that somatic embryogenesis may have been influenced by picloram and BA used for callus initiation. Remakanthan *et al* (2014) obtained direct

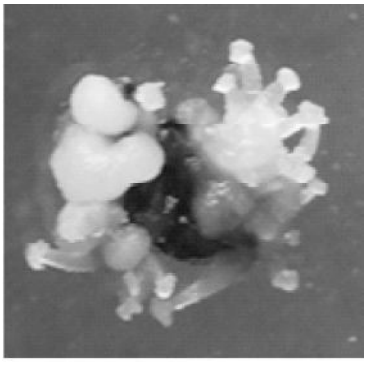

Fig. 4. Glassy, elongated monocot embryos developed on semisolid MS medium supplemented with BA 2mg L^{-1} + IAA 0.5mg L^{-1} on 0.3 per cent Gelrite

Table 2. Embryogenic response of callus initiated from various treatments

Sl. No.	Treatment for inducing somatic embryogenesis	Response of callus induced on various treatments			Per cent embryogenesis*		
		A1 to A16	B4 to B11	C1 to C12			
1	MS + 2,4-D 2 mgL^{-1}, zeatin 0.2 mgL^{-1} + Gelrite 4%	_	_	_			
2	MS+ 2,4-D 2 mgL^{-1} + IAA 1 mgL^{-1} + NAA 1 mgL^{1} biotin 1mgL^{-1} + glutamine 100 mgL^{-1} + Gelrite 0.3%	_	_	_			
3	MS + BA 2 mgL^{-1} + IAA 0.5 mgL^{-1} + Gelrite 0.3%	_	_	Embryo induction only in callus from C8, C9, C10	C8 33.33	C9 60	C10 50
4	SH+ MS vitamins + glutamine 100 mgL^{-1} + picloram 1 mgL^{-1}+ sucrose 4.5% + Gelrite 0.2%						

*Per cent embryogenesis was calculated as the number of calli showing embryogenesis out of the number of callus clumps inoculated

embryogenesis from split shoot tips of Grand Naine on MS medium supplemented with picloram and BA, used alone or in combination, obtaining the best embryogenic response when used in combination. When they used BA alone, explants produced only shoots.

In almost all the protocols reported for somatic embryogenesis from callus, an intermediary liquid medium is involved. Maintenance of suspension cultures is a somewhat sophisticated process, even though rate of multiplication realized is high. In our study, we could replace the same with a simple semisolid medium.

Germination of somatic embryos and plantlet formation

Somatic embryos induced on semisolid MS with BA 2mgL^{-1} + IAA 0.5mgL^{-1} failed to germinate on the same medium; But, when half-strength MS with the same combination of growth regulators was used, 60-80% germination was obtained in 3 weeks of inoculation in dark (Table 3). At this stage, shoots were pale-white to pale- green in colour (Fig. 5a).When germinated embryos were transferred onto MS medium with BA 2mgL^{-1} + NAA 1mgL^{-1} and incubated at a light intensity of 31.4$\mu mol^{-2}S^{-1}$ and 14 h light/8h dark photoperiod, these turned green and developed into complete plantlets (Fig. 5b). Meenakshi *et al* (2011) used BA and IAA to supplement MS medium with 0.2 per cent Gelrite© for conversion of somatic embryos into plantlets. Okole and Schulz (1996) and Lee *et al* (1997) reported that high BA concentrations induced only shoot formation. But Khalil *et al* (2002) reported lower rate of shoot formation at BA >5 mgL^{-1}. In the present study, BA concentration maintained at 2mgL^{-1} on half-strength MS medium resulted in regeneration of somatic embryos. Ganapathi *et al* (1999) also observed that development of embryos into plantlets was good in IAA and BA combination. Better

Table 3. Germination of somatic embryos on semisolid half MS with BA 2mg L^{-1}+IAA 0.5 mg L^{-1}

Treatment for callus induction	No. of embryo clusters inoculated	No. of clusters showing germination	Per cent germination
C_8	10	6	60.00
C_9	18	13	72.22
C_{10}	15	11	80.00

development of plantlets was observed when IAA was substituted with NAA 1mgL^{-1}. MS medium supplemented with NAA and 0.2 per cent Gelrite© was used by Meenakshi *et al* (2011) for root development. The plantlets, after primary hardening in coir-pith

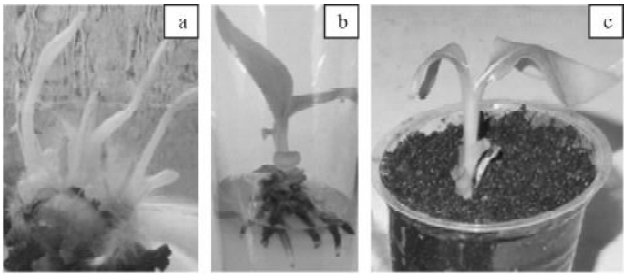

Fig 5. Germination of embryo and plantlet development

A) Germinating embryos in semisolid 1/2MS with BA 2 mgL^{-1}+ IAA 0.5 mgL^{-1}
B) Plantlet conversion in MS medium with BA 2 mgL^{-1} + NAA 1 mgL^{-1}
c) Plantlet placed for primary hardening in coir-pith compost

compost (Fig. 5c) and secondary hardening for one month in soil and cow-dung (1:1) mixture in a mist chamber, showed 100% survival.

CONCLUSION

Embryogenic culture system described in this paper demonstrates the potential of immature male flowers as explants for raising highly proliferative, embryogenic cultures with simple hormone combinations. In most of the protocols reported, liquid medium is used for proliferation of the embryos. Maintenance of embryogenic cell suspensions is laborious and time-consuming. Frequent subcultures needed in the reported procedures may lead to somaclonal variation, microbial contamination and, eventually, loss of morphogenetic potential (Kulkarni and Ganapathi, 2009). The protocol described in our present study does not require any intermediate cell suspension system, making it a more convenient and very effective method for small laboratories lacking facilities for continuous agitation. According to Georget *et al* (2000), a common feature of many banana cell culture protocols is the slow development of cell clusters, making plant regeneration time-consuming (18-24 months). The current protocol requires just 6 months for producing plantlets from the explant. It is, thus, suitable for genetic transformation experiments whereby transformants can be easily regenerated and evaluated. Further refinement of the medium may improve conversion rate, thus making it suitable for mass propagation as well.

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Authors' contribution statement: Lekshmi, R.S. (lekshmirs84@gmail.com), Ph.D. student, carried out the work; Soni, K.B. is the principal investigator; Swapna, A., Deepa S. Nair, Lekha, S. and Reghunath, B.R. are Members, Advisory Committee of the student.

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