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In Vitro Embryo Culture And Regeneration Of Seeded Banana (Musa Balbisiana Cv. Changthir)

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Abstract
Wild and seeded diploid bananas are useful resources for utilization in banana improvement programs having the traits for tolerance to various biotic and abiotic stresses. However, their regeneration, propagation and conservation face challenges due to poor germination rate of seeds and limited survivability in natural conditions. Here, we report an optimised method for the in vitro regeneration of Musa balbisiana cv. Changthir embryos using MS basal medium added with and without plant growth regulators. Zygotic embryos cultured on MS medium supplemented with 0.5 mg/L + 1.5 mg/L BAP exhibited highest percentage of germination 94.45% within 7 days follow by MS medium supplemented with 3 mg/L BAP with 84.94%. Efficient shoot induction was observed in MS 11 medium with 82.23%, while root formation was maximum in MS5 medium (0.5 mg/L NAA) with 72.22% at 21 days. Plantlet regeneration was characterised by leaf and root development, maximum in MS5 medium with average 5.67 leaves and 11.33 roots per plantlets at 48 days. These findings demonstrated a simple and reliable method of in vitro propagation of wild banana Musa balbisiana cv. Changthir, which can be applied in the conservation and banana improvement programs.
Keywords: *Changthir, Musa balbisiana, Zygote, Embryo, in vitro regeneration,*

INTRODUCTION

Wild bananas germplasms are invaluable for modern banana breeding for their capability to resist against extreme environmental stress and superior fruit qualities. *Musa balbisiana*, one of the important wild progenitors is renowned for their exceptional resistance and tolerance to water scarcity conditions, high temperatures, salinity and other abiotic stresses – originated from extremely dry, limited water and challenging habitats (1-3). In addition, recent functional studies revealed that *M. balbisiana* rapidly and effectively upregulated the transcriptional defense against pathogens – highlighting the potential of disease resistance breeding (1, 3). In contrast, another wild progenitors *Musa acuminata* contributed in food quality traits (increased size, self-life) – originated from humid, temperate forests (4, 5). Today's cultivated bananas are considered to have originated through inter/intra specific crossing over between *Musa accuminata* (AA) and *Musa balbisiana* (BB) (6). Diploid wild progenitors, are distributed in dense forests of Malaysia or Indonesia (7), and in the regions of India, Myanmar, Thailand and Philippines (8). Important agronomic traits such as fruit quality and resistance against abiotic/biotic stresses are derived from the parents with *M. acuminata* or *M. balbiasiana* or through their combinations and these are used in breeding banana programs (1,3,9).

Seeded wild bananas mainly propagates through seeds and often faces the issues of variable germination rate and seedling production rate (10-15). Scanty reports are there about the factors influencing seed germination in *Musa* and reported to be highly erratic and unproductive (16-19). Immediate sowing of freshly harvested *M. violascens* seeds could attain only 96% germination (10), while under the green house conditions, *M. balbisiana* germination rate was observed to be 90% during summer and goes down to nearly 0% during winter (20).

In vitro zygotic embryo culture technique is successfully implemented in regeneration and production of seeded bananas via rapid multiple shoot development (21). Various applications of embryo cultures techniques in the breaking seed dormancy, shortening of life cycles and recuing of immature hybrid embryos from incomparable crosses thus finding applications in various banana breeding, improvement and genetic improvement programs (22-27). A number of factors like the embryo maturity stage and culture media composition influences the banana germination rates (28). Therefore, optimization of the embryo harvesting stage and successful in vitro propagation of large number of *Musa* cultivars are gaining importance for its application in various breeding and

conservation programs (16,17,29). Several reports have also shown higher *in vitro* germination and plantlet production rate using zygotic embryos by 10 folds (30-33).

The wild seeded bananas and their relatives are abundantly found in the northeast region of India but are hugely neglected due to large number of seed contents and non-commercial value. However, the importance of the genetic resources of the crop wild relatives are gradually gaining importance with the scientific reports and findings which provides the useful information and understanding of their roles in conservation and improvement of the cultivated crops including banana. Thus, it is very pertinent to optimise an *in vitro* regeneration system of seeded banana (*Musa balbisiana* cv. Changthir) of Mizoram which will be useful for future conservation and improvement strategies.

MATERIALS AND METHODS

Explant source

Matured seeds from open pollinated banana bunches were harvested from *M. balbisiana* cv. Changthir accessions growing in the field gene bank, Department of Biotechnology, Mizoram University, Mizoram, India. Seeds were extracted from the pulp by continuous washing in tap water followed by drying at room temperature and stored at a cold and dry place for using in the experiments.

Seed sample preparation and inoculation

Seeds were disinfected by treating for 15 min with 1% sodium hypochlorite, then 15 min with 0.1% mercuric chloride under aseptic condition. Then this is followed by 3 times rinsing with sterilized distilled then drying on a petri plate layered with a sterilized filter paper.

By making a fine longitudinal fissure around the micropylar plug of the seeds the embryos were carefully extracted using sterilized blade and forceps. The extracted embryos were were inoculated in the germination medium consisting of Murashige and Skoog salts (34), supplementing with different concentrations of 6-benzyl adenine purine (BAP), kinetin (KIN), and 1-naphthalene acetic acid (NAA) individually or in combinations (Table 1). The inoculated embryos were maintained in a culture room at 25 ± 1° C with a 16/8 h of photoperiod timings under the white florescence light with an intensity of 55 $\mu molem^{-2}sec^{-1}$ and humidity at 60% to 80%. The response of embryos was observed and routinely sub-cultured after every 20 days.

Statistical analysis

Three replicates were used in all the experiments conducted consisting of 20 explants per treatment and repeated at least thrice. Arcsine transformation were carried out for the percentage data represented in the tables then analysed for significance using ANOVA (analysis of variance, $P \leq 0.05$) following Duncan's new multiple range test. SPSS software package version 16.0 was used for the analysis.

RESULTS

Initiation of embryo germination

The excised embryos of *M. balbisiana* cv. Chanthir inoculated on MS media containing different concentration of PGRs began responding within 3 to 8 days. Details of the *in vitro* responses of the embryos cultured are provided in Table 1. Initial germination signs of enlargement of embryos leading to the formation of globular shape accompanied by the change of colors from whitish to yellowish tones were observed (Fig. 1). Among the treatments, maximum percentage of germination was observed in MS11 medium (0.5 mg/L NAA + 1.5 mg/L BAP) with a mean germination rate of 94.45. This was followed by MS9 medium (3mg/L BAP) with mean germination percentage of 84.92, MS10 medium (2 mg/L KIN) with mean percentage of 81.00, MS8 medium (2 mg/L BAP) with mean percentage of 80.00, while the minimum germination percentage was observed in the medium MS3 (0.5 mg/L KIN) medium and MS1 (without PGR) medium with a mean germination percentage of 60.00 and 62.22 respectively.

In vitro regeneration

Shoot induction had initiated in the germinated embryos, maximum percentage of shoot induction was observed in MS11 medium (0.5 mg/L NAA + 1.5 mg/L BAP) with mean percentage of 82.23, followed by MS5 (0.5 mg/L NAA) with mean percentage of 76.67, MS8 medium (2 mg/L BAP) with mean percentage of 66.67, MS9 medium (3 mg/L BAP) with mean percentage of 66.03 respectively at 21 days.

Roots formations were initiated at 21 days in the MS medium, highest percentage of root formation was observed in MS5 (0.5 mg/L NAA) at 72.22%, followed by MS2 medium (0.1 mg/L BAP) at 61.27%, MS3 medium (0.5 mg/L KIN) and MS6 medium (0.5 mg/L NAA + 0.5 mg/L BAP) with both 56.67%.
Plantlets formation was characterised by the development of leaves and roots, maximum numbers of leaves and roots were observed in MS5 medium (0.5 mg/L NAA) with mean numbers of 5.67 and 11.33 by day 48, were followed by MS2 medium (0.1 mg/L BAP) with mean number of 5.00 leaves and 10.67 roots, and MS6 medium (0.5 mg/L NAA + 0.5 mg/L BAP) with mean number of 5.00 leaves and 5.25 roots. Even the MS media without any hormone supported the formation of leaves an average of 4.60 and 6.00 roots per plantlets at 48 days.

DISCUSSION

The present study demonstrated about the successful of *in vitro* regeneration protocols for the germination and regeneration of M. *balbisiana* cv. Changthir embryos using MS medium supplemented with optimum PGRs. The combination of auxin and cytokinin in MS11 medium (0.5 mg/L NAA + 1.5 mg/L BAP) resulted maximum embryos germination rate (94.45%) and the shoot induction (82.23%). Meanwhile, MS5 medium (0.5 mg/L NAA) responded optimum root formation with complete plantlet formation, producing 72.22% rooting and mean numbers of 5.67 leaves and 11.33 roots in an individual plantlet. Similarly, the crucial roles of auxin and cytokinin in the redirection of organogenic responses in banana were discussed (1). The response of embryos in control hormone treatment in the efficient somatic embryogenesis was also similarly reported by Handayani et al. in the studies of immature embryos of M. *acuminata* ssp. *Malaccensis* (35). From the histological analysis determined that cytokinin dominated in the early cell differentiation that enhanced embryo germination and shoot induction (36).
In our study zygotic embryos directly regenerated into plantlets without callus formation, directly from embryo germination followed by shoot induction, root formation and plantlet development. Similar report of direct organogenesis with development of mature plantlet from the mature seeds was reported in M. *acuminata* (Pisang Jajee) (37). Singh et al., 2021 demonstrated about the direct regeneration of mature banana plantlets from the zygotic embryo of M. *balbisiana* (27). In our experiment the percentage of zygotic embryo germination was observed highest in MS11 medium (0.5 mg/L NAA + 1.5 mg/L BAP) with mean 94.45% at 7 days. Similar observation was demonstrated on the studies of mature embryos of *E. ventricosum* while inoculating in the MS medium supplemented with 1 µM IAA + 2 µM BAP (38). Minimum gemination was observed on the MS3 media with the supplement 0.5 mg/L KIN with a percentage of mean 60.00 but there was no direct germination in 2,4-D supplemented media, 2,4-D is popular known of using for the induction of callus.
In our study the shoot and root formations were observed at 21 days, but at different rate, highest percentage of both shoot and root with mean percentage of 77.67 and 72.22 was observed in MS5 medium (0.5 mg/L). But only shoot induction was observed maximum in MS11 medium (0.5 mg/L NAA + 1.5 mg/L BAP) with 82.23%. Similar observation of highest plantlet regeneration at 25 days was demonstrated on embryo culture of Pisang Jajee (AA) in MS media containing 4.4 µM BAP + 2.8 µM IAA (37). Maximum percentage of root induction was observed in MS5 medium supplemented with 0.5 mg/L NAA. Cytokinin (BAP) influenced in the development of shoot 82.23% with 4.67 leaf in MS11 medium 0.5 mg/L NAA + 1.5 mg/L BAP at 48 days. This result was supported by Bakry, 2008 (39) and Uma et al., 2011(37) where it was shown that either BAP alone or in combination with IAA influenced the germination of hybrid banana embryos from various crosses (37,39). It was also observed that basal MS without PGRs was found to be efficient for direct regeneration of plantlets from the cultured embryos.
Embryos with more than 90% maturity cultured on basal MS with no PGRs has been successfully used in many other crop species (40). The observations from the present study also indicates the successful induction of direct shoot leading to plantlet regeneration from the *in vitro* culture of M. *balbisiana* cv. Changthir mature embryos. Use of MS medium supplemented with low IAA concentrations of IAA breaks the inhibition of root formation and growth (41). In this study, it was observed that root induction was observed by the second with in the basal MS medium without PGR and higher concentration of cytokinin reduces the number of root formation. The optimised protocol for the *in vitro* culture and regeneration of M. *balbisiana* cv. Changthir M. *balbisiana* matured embryos has been achieved and it can be utilized for various conservation and banana improvement programs.

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Table.1. The response of *Musa balbisiana* cv. Changthir embryos cultured on MS media supplemented with different plant growth regulators.

Concentration of PGRs (mg/L) in MS medium						Germination (day)	Mean percentage of morphological changes (mean ± SD)				
Sl. No.	NAA	2,4-D	IAA	BAP	KIN		Germination as recorded during 1st week	Shoot initiation as recorded during 2nd week	Root initiation as recorded during 3rd week	No. of leaves as recorded during 6th week	No. of roots as recorded during 6th week
MS1	0	0	0	0	0	3.5	62.22±2.22^{a}	56.67±3.33^{a}	56.67±3.33^{a}	4.60±0.40^{a}	6.00±0.32^{a}
MS2	0	0	0	0.1	0	4.0	78.26 ± 3.54^{b}	61.27±2.82^{ab}	61.27±2.82^{a}	5.00± .00^{ab}	10.67±0.67^{b}
MS3	0	0	0	0	0.5	5.7	60.00±0.00^{a}	56.67 ± 3.33^{a}	56.67±3.33^{a}	4.25 ± 0.25^{a}	5.00 ± 0.41^{a}
MS4	1.0	4.0	1.0	0	0	-*	-	-	-	-	-
MS5	0.5	0	0	0	0	4.4	79.45 ± 2.42^{bc}	76.67 ± 1.67^{c}	72.22±2.77^{b}	5.67± 0.33^{b}	11.33±0.67^{b}
MS6	0.5	0	0	0.5	0	5	81.11 ± 1.11^{bc}	56.67 ± 3.33^{a}	56.67 ± 3.33^{a}	5.00 ± 0.00^{a}	5.25 ± 0.48^{a}
MS7	0	1	0	0	0	-	-	-	-	-	-
MS8	0	0	0	2	0	5.2	80.00 ± 0.00^{bc}	66.67 ± 6.67^{b}	-	3.67±0.67^{ac}	-
MS9	0	0	0	3	0	5.2	84.92 ± 0.79^{c}	66.03 ± 3.3^{b}	-	5.50±0.29^{b}	-
MS10	0	0	0	0	2	6.3	81.00 ± 1.00^{b}	53.33 ± 3.33^{ab}	43.33 ± 3.33^{c}	4.50 ± 0.29^{a}	5.25 ± 0.25^{a}
MS11	0.5	0	0	1.5	0	4.5	94.45 ± 5.55^{d}	82.23 ± 1.11^{c}	0.00	4.67 ± 0.33^{a}	-

Means (±) within the same column followed by different letters are significantly different ($P \leq 0.05$) using Duncan's new multiple range test, - absence of data; * callus formation.

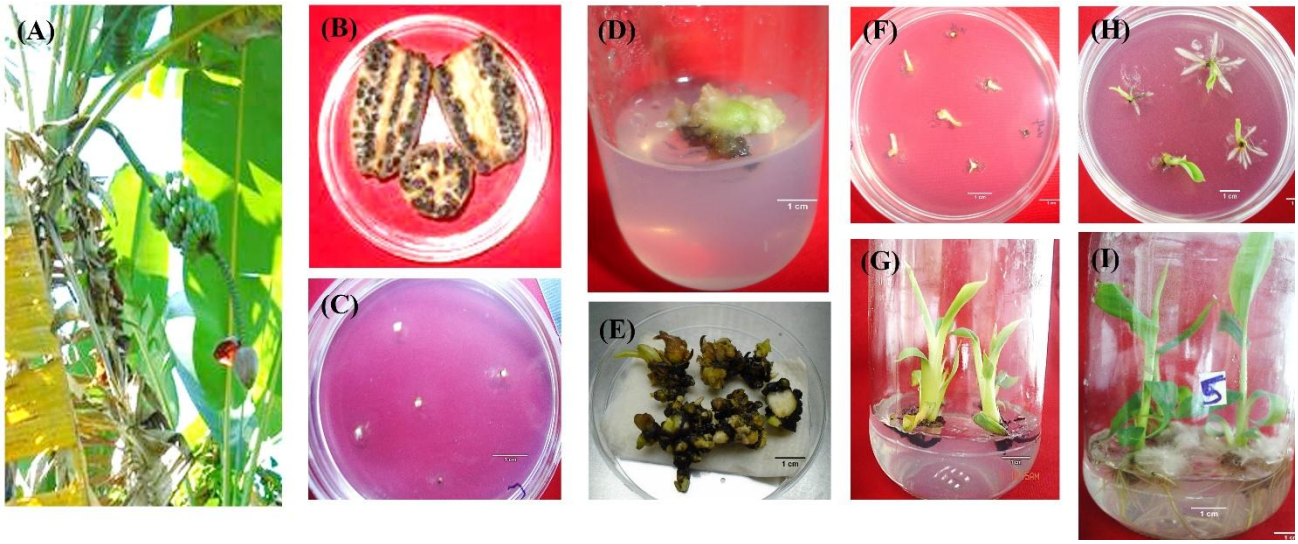

Fig.1. *In vitro* germination and regeneration of plantlets from zygotic embryos of *M. balbisiana* cv. Changthir, (A) Mother plant with mature fruit, (B) Longitudinal and transverse section of mature fruit, (C) Inoculated zygotic embryo, (D) Callus induction in MS4 medium (1 mg/L IAA + 1 mg/L NAA + 4 mg/L 2,4-D), (E) Initiation of shoot bud from callus in MS4 medium (1 mg/L IAA + 1 mg/L NAA + 4 mg/L 2,4-D), (F) Initiation of shoot bud in MS11 (0.5 mg/L NAA + 1.5 mg/L BAP) at 7 days, (G) Shoot formation in MS11 medium (0.5 mg/L NAA + 1.5 mg/L BAP) at 21 days, (H) Initiation of shoot and root induction in MS5 (0.5 mg/L NAA) media at 7 days, (I) Plantlet regeneration in MS media (0.5 mg/L NAA).