Embryo culture and embryo rescue studies in wild *Musa* spp. (*Musa ornata*)

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Abstract

Seed set in *Musa* spp. is known to vary greatly among seed-fertile cultivars, but germinate at an intractably low rate in soil thus making breeding of plantains and bananas difficult. Hence, there is an increased interest in *in vitro* germination of both intact seeds and excised zygotic embryos. The present work deals with the influence of maturity and hormonal factors on germination and regeneration of *Musa ornata* seeds through embryo culture and embryo rescue. Embryos extracted from seeds harvested at various maturity stages were cultured in MS media with different concentrations of plant growth regulators. Good embryo recovery was seen in seeds from 80 and 100% mature fruits. Maturity status of embryos played a key role in direct and indirect regeneration. Medium rich in auxins led to callus (M_8) formation at all maturity levels, leading to indirect regeneration. Good direct regeneration was observed from 100% mature embryos, in media supplemented with 6-benzylaminopurine (M_4). Study revealed that zygotic embryos of *M. ornata* could be rescued and regenerated through callus when harvested at 80% maturity and media augmented with Kinetin (M6) gave the best regeneration. In general, medium rich in auxins led to callus formation at all maturity levels. Therefore, *in vitro* embryo culture and embryo rescue provide a potential tool for recovery and perpetuation of wild *Musa* species.

Key words: Banana, *M. ornata*, seed germination, embryo rescue, *in vitro* germination, Rhodochlamys.

Introduction

Banana (*Musa* spp.) is one of the most important fruit crops in tropical and subtropical countries. It forms a major staple food in most African countries and a favourite fruit in rest of the world. Though area under banana is increasing in tropical countries, but the problem of pests and diseases still exists. To site an example, appearance and spread of the virulent form of Sigatoka leaf spot disease has seriously restricted the expansion of banana and plantain cultivation. Banana breeders are therefore under increasing pressure to produce disease-resistant cultivated varieties. However, banana breeding programmes are complicated by the sterile nature of cultivated *Musa* and the low seed yield of hybrid plants. The wild progenitors of the edible bananas produce seeds while most of the edible clones are seedless with a few exceptions such as 'Pisang awak' subgroup (ABB) (Simmonds, 1966). The difficulty of obtaining seeds for breeding, the unpredictable nature of germination and low germination percentage have increased interest in *in vitro* germination of both intact seeds and excised zygotic embryos (Cox, 1960; Stotzky and Cox, 1962; Stotzky *et al.*, 1962). Several reports have indicated the limited and variable seed germination exhibited by *Musa* (Simmonds, 1952 and 1959). Therefore, *in vitro* embryo culture represents a potential tool for improved recovery of hybrid germplasm (Cox, 1960; Stotzky *et al.*, 1962; Afele and De Langhe, 1991).

Musa ornata Roxb. belongs to the section Rhodochlamys of Musaceae. It is diploid, sets fertile seeds but with medium germination efficiency (30-40%). It belongs to the ornamental group of bananas, which bear attractively coloured inflorescence which make them a beautiful garden plant. They are widely distributed in north eastern states of India and Myanmar and originally described in the early 19th century by W. Roxburgh (1824) from Bangladesh. It grows up to 3 meters; the stems are waxy and later develop black blotches. Plants bear lilac coloured erect flowers. Its distribution is endemic to Arakku Valley of Andhra Pradesh and of late reported to be slowly disappearing from the area of its cultivation (Uma *et al.*, 2006). This wild species is known to be tolerant for many diseases and pests and its genetic proximity to Eumusa makes *M. ornata* a potential parent in breeding programmes, hence can be used as a parent in hybridization programmes.

Hence, embryo culture and embryo rescue techniques in *M. ornata* may assist its rejuvenation and establishment. The objective of this work was to understand various seed components contributing to seed development, parameters facilitating better embryo germination, embryo regeneration and to standardise *in vitro* embryo culture and embryo rescue of *M. ornata*.

Materials and methods

Explant material: *M. ornata* plants grown in field gene bank of National Research Center for Banana (NRCB), Tiruchirapalli, India were used in the present study. Seeds were collected during December 2012 and were used as explant material. The diploid *M. ornata* was self-pollinated and the bunch was covered to avoid cross pollination. Fruits were harvested at 67, 88 and 110 days after-flowering corresponding to 60, 80 and 100% maturity, respectively as reported previously by Uma *et al.* (2011). The seeds were separated from pulp by continuous washing in tap water. Extracted seeds were transferred to a beaker containing water, allowed to settle for 15 minutes. Floating seeds are non viable and were discarded and only sunken seeds which are viable were used in the study.

The viable seeds were soaked in sterile water for 48 hours, washed sequentially in 1.4% (v/v) sodium hypochlorite solution for 10 min followed by 0.1% mercuric chloride for 10 minutes and rinsed three times in sterile distilled water. A vertical cut was given to the seeds, carefully embryos were removed under aseptical conditions and cultured in test tubes (25 mm) with one embryo per tube. The culture medium used was Murashige and Skoog (1962) salts supplemented with different concentrations of plant growth regulators (Table.1). pH of the media was adjusted to 5.8 with 1N HCl or 1N NaOH. Phytagel (2g L^{-1}) was used as solidifying agent.15 mL medium was dispensed into each test tube before autoclaving at 121 °C for 30 min. Cultures were maintained at 16-h light with a temperature of 28±2°C.

Different concentrations of 6-benzylaminopurine (BAP) (0.1 mg L^{-1}, 0.5 mg L^{-1}) and kinetin (0.1 mg L^{-1}, 0.5 mg L^{-1}) were used to study their effect on embryo germination. The effect of full and half strength MS along with sucrose concentrations (3 and 1.5%) was also compared. Apart from these treatments, MA1 medium was used which is rich in auxins. It contains Indole-3-acetic acid (IAA) (1.0 mg L^{-1}), 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (4mg L^{-1}) and (1-naphthaleneacetic acid) (1mg L^{-1}) as plant growth regulators. This medium was used in somatic embryogenesis of banana (Ma, 1991). Embryo cultures were initially maintained in dark for 15 days, followed by 16 h light and 8h dark. For the light treatment, cultures were maintained in a 16-h photoperiod under white fluorescent lamps with a light intensity of 405µE/m^{-2}/s^{-1} and a temperature of 28 ± 2°C. Each treatment contained 25 embryos, one per culture tube. The experiment was designed using a completely randomized design with five replications per treatment. The experiment was repeated thrice and data averaged is provided.

Data was analyzed by analysis of variance (ANOVA) at 95% confidence interval, where significant differences ($P \le 0.05$) between individual treatment means were determined applying Duncan's multiple range test (DMRT). All data was analyzed by SPSS for Windows, version 11.

Results and discussion

Banana seeds are in general orthodox in nature. Orthodox seeds can tolerate desiccation and regenerate into plantlets under favourable conditions. Research is limited to ascertain this fact with *M. ornata*. In the present study, various seed components contributing to seed development and factors facilitating better embryo germination and rescue were studied.

Extraction of seeds from the fruits harvested at different maturity stages revealed that number of seeds per fruit decreased with the maturity stage. Water gravity test, could differentiate the extracted seeds as sunken/viable and floating/nonviable seeds. A total of 98.4% seeds from 60% mature fruits were sunken/viable seeds. In 80% mature fruits, 78.2% of total seeds were sunken, whereas in 100% mature fruits, it was found to be 72.7%. The sunken seeds alone were taken for embryo culture, as floating seeds were devoid of either embryo or endosperm or both. The results indicated that the per cent of sunken seeds decreased with increase in maturity. Number of sunken seeds per fruit was less in seeds extracted from fruits harvested at 100% maturity indicating the possibility of degradation or drying of endosperm during maturity within the seed. Although the number of sunken seeds decreased over maturity, the percentage of good seeds with intact embryos had increased. This implies that at 60% maturity, development of embryos was incomplete; while 80% mature fruits resulted in good embryo recovery. At 100% maturity, embryo recovery (86.6 %) was highest (Fig.1).

Fresh weight gain: After 48 hours of soaking seeds in water, difference in seed weight was observed. Although increase in seed weight was observed with progressive maturity, there was a decrease in fresh weight gain (Table 2). This could be due to the hard seed coat nature of banana seeds. Uptake of water by a mature seed is triphasic with a rapid initial uptake followed by plateau phase (Bewley, 1997). Mature banana seeds are able to imbibe water regardless of initial seed moisture content. The rate of water uptake varied between ecotypes due to the difference in width of water channels. The presence of water channel between testa and operculum is reported to play a significant role in water entry into the seed (Puteh *et al.*, 2011).

Seed components: Study of seed components with progressive seed maturity indicated that seeds from 60% mature fruits showed maximum contribution from seed coat compared to other maturity levels. Per cent contribution of embryo and endosperm was low, indicating incomplete development of these components (Table 3). Uma *et al.* (2011) reported that no proper embryo formation occurs until 70% maturity, when the endosperm was transparent fluid. In other two treatments (80 and 100 %), contribution from seed coat was reduced while contribution from embryo and endosperm had increased. There was a decrease in endosperm contribution from 80 to 100 % maturity. This could have contributed to the decrease of per cent sunken seeds in fully matured fruits.

Effect of various media on embryo culture and embryo rescue: The study was carried out to understand the initial response of embryos at various maturity levels in media with eight different compositions. Dissected embryos were initiated in these media. Signs of germination started within 3-4 days and germination
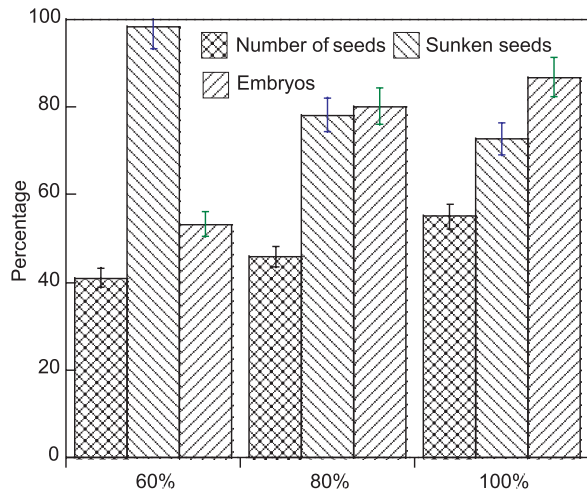
Table 1. Composition of media used in study

Culture components	Media							
	M1	M2	M3	M4	M5	M6	M7	M8
Macroelements	MS[a]	MS[a]	MS[a]	MS[a]	MS[a]	MS[a]	1/2MS[a]	MS[a]
Microelements	MS[a]	MS[a]	MS[a]	MS[a]	MS[a]	MS[a]	1/2MS[a]	MS[a]
Sucrose (g/ L)	30	15	30	30	30	30	15	30
Vitamins	Morel[b]	Morel[b]	Morel[b]	Morel[b]	Morel[b]	Morel[b]	1/2Morel[b]	MA1
6-BA (mg/ L)	-	-	0.1	0.5	-	-	-	-
Kinetin (mg/ L)	-	-	-	-	0.1	0.5	-	-
IAA (mg/ L)	-	-	-	-	-	-	-	1.0
2,4-D (mg/ L)	-	-	-	-	-	-	-	4.0
NAA (mg/ L)	-	-	-	-	-	-	-	1.0

[a] Murashige and Skoog (1962); [b] Morel (1950)

Fig. 1. Seed characterstics of *M. ornata* at various stages of fruit maturity

Table 2. Fresh weight gain of *Musa ornata* seeds

Maturity (%)	Initial weight (mg)	Final weight (mg)	Difference (mg)
60	34	52.9	18.9
80	52	61.5	9.5
100	70	74.2	4.2

Table 3. Proportion of seed components at different maturity levels

Maturity (%)	Weight of single seed (mg)	Contribution of the seed coat (%)	Contribution of the endosperm (%)	Contribution of the embryo (%)
60	33.8±0.61[c]	69.60±0.51[b]	30.01±0.57[a]	0.63±0.27[a]
80	51.8±0.61[b]	64.08±0.49[a]	34.36±0.50[c]	0.92±0.48[b]
100	70.08±0.49[a]	64.60±0.74[a]	33.47±0.64[b]	1.5±0.46[c]

Means in the same columns followed by different letters are significantly different ($P \leq 0.05$) using the student's t-test.

percentage was calculated based on the embryo response in respective medium after 10 days of initiation.

At 60% maturity level, germination response of embryos was poor. Among the treatments, M_2 (MS+ half sucrose concentration) showed highest germination percentage of 42.4%. Treatments M_1 (MS basal media), M_3 (BAP 0.1mg/L) and M_5 (Kinetin 0.1mg/L) exhibited 25% germination. Very low germination percentage was observed in M_6 (Kinetin 0.5mg/L) and M_8 (MA1). No germination was recorded in M_4 (BAP 0.5mg/L) and M_7 (half MS) media. Germination percentages of 80% mature embryos were observed in 8 different media, M_6 (kinetin 0.5mg/L) recorded highest germination (99.97%), followed by M_1, M_2, M_3 and M_7 all showing 83% germination. Lowest germination percentage was recorded in M_4 medium (50.23%). In treatments involving 100%
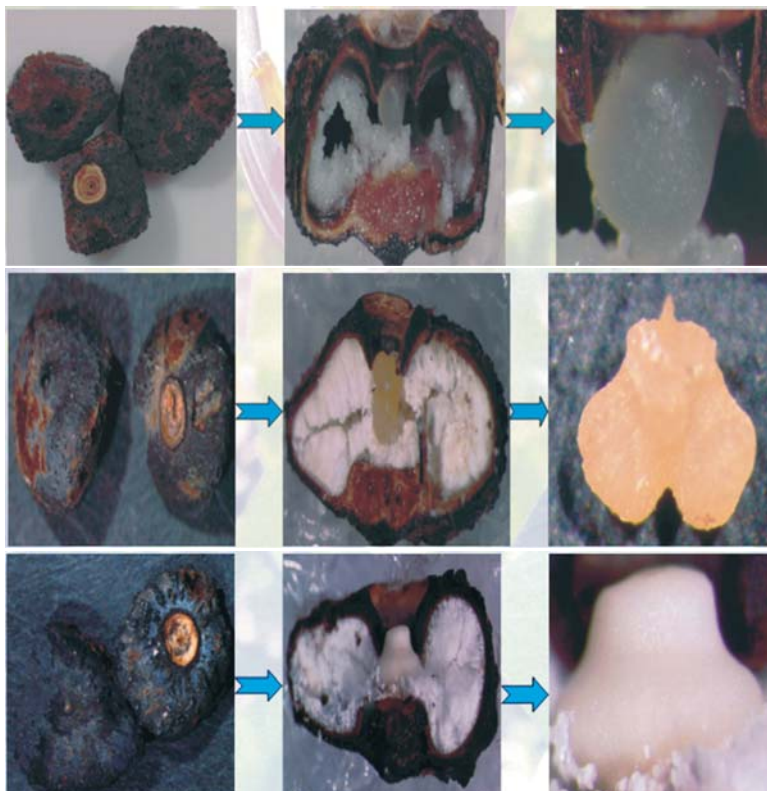
mature embryos, only M_8 (MA$_1$ medium) showed the highest percentage (90.17%) of germination. In M_4 and M_1, 72.04 and 63.1%, respectively, rest of the treatments exhibited less than 50% germination (Table 4).

These results imply that 80% mature embryos responded uniformly well in all the media tried, while 60% mature embryos gave moderate response in M_2 (42.4%) alone. 100% mature embryos showed good response in M_8, M_4 and moderate response in all other treatments. Embryos extracted at 60% maturity, showed incomplete development which would have been the reason for poor embryo germination. At 80% maturity, seeds had developed embryos which attained the physiological maturity by then, hence good germination was noticed (Fig. 3). Similar results have been obtained by Uma *et al.* (2011) who reported that less differentiated and younger tissues are more amenable to *in vitro* culture. Whereas irrespective of the media, embryos from 100% mature seeds showed moderate response. Hence, at 80% maturity, a minimum of 50.23% (M_4) and maximum of 99.97% (M_6) of embryos was noticed.

Regeneration response of zygotic embryos: Regeneration efficiency of embryos was recorded after 30 days of initiation and the results indicated significant differences among the treatments. At 60% maturity, indirect regeneration of embryos into callus was seen in M_3, M_5 and M_8. At 80% maturity, indirect regeneration was observed high in M_6 followed by M_3, M_7 and M_1. In all other treatments moderate regeneration percentage was noticed. At 100% maturity, both direct and indirect regenerations were observed. High rate of direct regeneration of embryo into plantlets was observed in M_4 followed by M_6 and M_3 while the lowest per cent of direct regeneration was observed in M_2 and M_5. The indirect regeneration in the form of callus development was noticed high in M_8 and M_1 followed by M_7 and M_5 (Fig. 4). According to Johri and Rao (1984), the regeneration of excised banana embryos was influenced primarily by two factors, embryo maturity at excision and the *in vitro* culture medium.

Both 60 and 80% mature embryos resulted in only callus formation. Maximum regeneration efficiency across all the treatments was observed with 80% mature embryos. The treatment M_6 showed highest regeneration per cent of 88.2 while all other treatments exhibited good regeneration. Embryos in M_8 (MA-1 medium) gave rise to callus at all three maturity levels. This may be due to high amount of auxins

Fig. 2. Various maturity stages of embryo (I Row -60%, II Row- 80%, III Row-100% maturity)
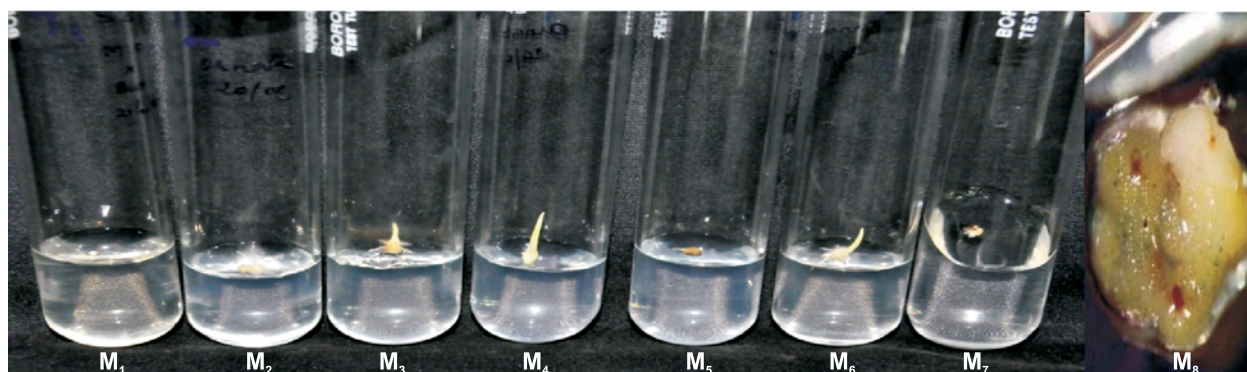
Fig. 3. *In vitro* response of embryos from seeds of 80% maturity in various media combinations

augmented in the initiation medium. Auxin is known to induce callus formation under *in vitro* conditions. White, friable callus was induced in the presence of IAA at different concentrations (Rambabu *et al.,* 2006). In 100% mature embryos, M_4 exhibited good plantlet production followed by M_6 and M_3, indicating that cytokinin in the media has influenced the plantlet production.

Maturity status of embryos play a key role in direct and indirect regeneration (Uma *et al.,* 2012). After 70% maturity, the fluid endosperm gets converted slowly into semi-solid state which helps in development of embryos in banana (Uma *et al.*, 2011). Even though the germination process was stimulated but the germinated embryos failed to regenerate into complete plantlets. According to Sandra (2005), immature embryos are heterotrophic in nature (provide the essential substance in the medium for embryo growth). But mature embryos are autotrophic in nature (embryo has ability to synthesise the essential substances for their growth). Our results corroborate this as the results are based on the maturity status of embryos at 60, 80 and 100%. It is suggested that 100% mature embryos alone were regenerated into complete plantlets, while 60 and 80% embryos developed only into callus. Generally, a fully matured embryo is considered as miniature plant which can develop into a normal plant without requirement

of any plant growth regulator. Basal MS medium is sufficient for 100% mature embryo germination and regeneration process (Uma *et al.*, 2011). But in our present experiment, 100% mature embryos have not fully regenerated into plantlets (Table 5). This could be attributed to species specific response of embryos for regeneration into plantlets. But our results corroborated with the results of Mathias and Simpson (1986), who reported the effect of genotype was much stronger than the presence of complex organic additives in the medium.

Carbon source plays a vital role in the embryo culture and embryo rescue. Embryos of 60% maturity failed to germinate and regenerate in different media. Embryos of 80% maturity showed highest per cent of germination and regeneration through only callus formation. But embryos of 100% maturity showed moderate germination and regenerated both by callus formation and plantlet development. This is supported by Raghavan (2003) who reported the requirement of a high osmotic concentration in the medium for successful growth of mature embryos, as they generally failed to grow in a mineral salt medium unless supplied with a concentration of sucrose far above that required as a carbon energy source. High and low osmotic concentrations in the medium prevented or encouraged the precocious germination and supports normal embryonic development, respectively.

MA-1 (M_8) medium is a callus induction medium used in Musa species which contain various auxins. During developmental stages, the cells are immature in nature and rapid cell division occurs inside. Polar auxin transport was involved to modulate the cells and localize region of the globular embryo where cotyledons

Table 4. Effect of media on various maturity levels of zygotic embryo germination

Medium	60%	80%	100%
M1	25.03±0.63[b]	83.06±0.32[b]	63.10±0.51[c]
M2	42.4±0.45[a]	83.08±0.62[b]	45.00±0.48[d]
M3	25.01±0.62[b]	83.08±0.50[b]	45.05±0.52[d]
M4	0	50.23±0.56[d]	72.04±0.61[b]
M5	25.06±0.53[b]	66.16±0.58[c]	45.09±0.45[d]
M6	12.13±0.50[c]	99.97±0.52[a]	50.00±0.53[c]
M7	0	83.04±0.62[b]	44.05±0.94[d]
M8	25.01±0.46[b]	66.13±0.38[c]	90.17±0.49[a]

Values are means ± SE of five replications. Means in the same columns followed by different letters are significantly different (*P*≤0.05) using the Student's t-test.

Table 5. Regeneration of plantlets from 100% mature embryos

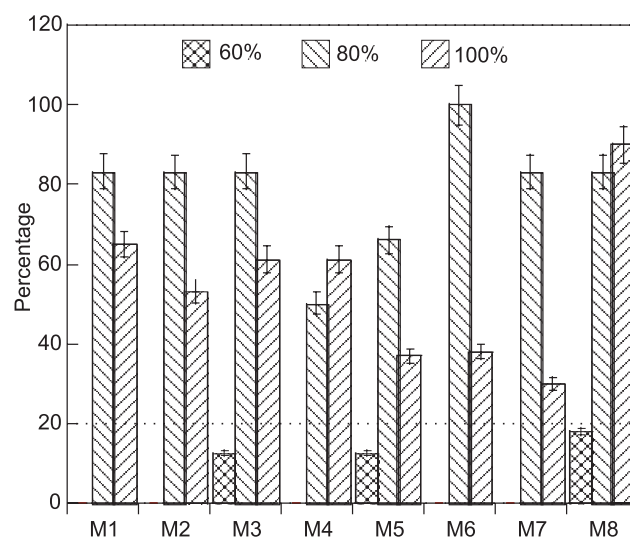| Treatments	Plantlets (%)	Callus (%)
M1	0.0[d]	65±0.31[b]
M2	7±0.70[c]	44±0.70[c]
M3	15±0.70[b]	44±0.70[c]
M4	46±1.04[a]	15±0.54[f]
M5	7±0.70[c]	30±0.31[d]
M6	15±0.70[b]	23±0.54[e]
M7	0.0[d]	30±0.31[d]
M8	0.0[d]	90±0.83[a]

Fig. 4. Regeneration response of zygotic embryos

arise (Raghavan 2003). This statement supports the present result as 60 and 80% mature embryos showed good quality friable callus compared to callus from 100% mature embryos. Only indirect regeneration through callus was obtained in this treatment. Similar results are also reported earlier by Purnhauser *et al.* (1987). The auxins which are essential for callus induction are reported to play a negative role in plant regeneration and are generally reduced or excluded from culture media used for shoot regeneration.

BAP and kinetin were used in this experiment at two different concentrations (0.1 mg/L, 0.5 mg/L respectively). At 60% maturity, embryos responded only for lower BAP concentrations *i.e.*, 0.1 mg/L (M_3 and M_5). High amount of cytokinins might have suppressed the germination process. These results are in accordance with Sandra (2005) who also reported that low level of cytokinin stimulated growth of inter specific hybrid embryos of *Trifolium*. Embryos of 80% maturity responded positively for all four concentrations of cytokinin. More than 50% of initiated embryos responded to cytokinin supplemented medium. 100% mature embryo responded well in cytokinin containing medium and regenerated into complete plantlets. This result was similar to results of Komalavalli and Rao (2000), who reported that shoot sprouting in *Gymnema* was initiated in all concentrations of BAP and Kinetin (alone and in combinations).

From the present study, it was observed that in seeds of *M. ornata,* there was a decrease in endosperm component in the seed during its maturity from 80 to 100 per cent. This could have contributed for the decrease of per cent sunken seeds in fully matured fruits and hence for the decrease in germination. Generally endosperm gets dehydrated at maturity level in orthodox seeds. Similar observation was noticed in *M. ornata* seeds also. At 80% maturity, embryos are expected to have attained physiological maturity leading to good germination and regeneration. Therefore, embryos can be rescued at 80% maturity for better regeneration of plantlets. Embryo rescue of these seeds can be done successfully on most of the media compositions and M_6 (kinetin 0.5%) gave the best regeneration. In general, medium rich in auxins led to callus formation at all maturity levels.

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