In Vitro Cell.Dev.Biol.—Plant (2012) 48:539–545
DOI 10.1007/s11627-012-9462-z

PLANT TISSUE CULTURE

Plant regeneration through somatic embryogenesis from immature and mature zygotic embryos of *Musa acuminata* ssp. *burmannica*

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Received: 14 September 2011 / Accepted: 13 August 2012 / Published online: 13 September 2012 / Editor: J. Forster

Abstract A simple and efficient protocol has been developed for *in vitro* regeneration of *M. acuminata* ssp. *burmannica* (AA) plants. Somatic embryos were produced when immature and mature zygotic embryo explants were cultured on Murashige and Skoog medium supplemented with plant growth regulators 2,4-dichlorophenoxyacetic acid; (2,4-D), picloram or benzyl adenine and indole acetic acid. In general, immature embryos responded better than mature embryos. Callus proliferation was highest in medium supplemented with 2,4-D (4.5 μM). Subsequent transfer of callus to fresh medium produced rapidly proliferating embryogenic calli. Embryogenic calli were maintained in complete darkness for 15 d followed by cycles of 8 h dark and 16 h light, under white fluorescent lamps with a light intensity of 3,000 lm/m^2 and at temperature of 28±2°C. Regeneration of embryogenic calli into plantlets was higher for immature embryos (76.6%) than for mature embryos (50.6%). This plant regeneration protocol using mature or immature zygotic embryos, *via* somatic embryogenesis, has significant potential to improve germination efficiencies of hybrid progenies used in conventional breeding strategies. Furthermore, tests on seed storage showed that seed viability rapidly decline after harvesting and was negligible after 9 mo of storage. This indicates using freshly harvested seeds as explant material is necessary for maximizing the tissue culture response.

Keywords Banana and plantains · Seed storage · *Musa acuminata* ssp. *burmannica* · Plant regeneration · Somatic embryogenesis · Zygotic embryo

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Introduction

Banana and plantains (*Musa* spp.) form a major staple food for millions of people living in the tropics and subtropics. The two wild progenitors of edible bananas *viz*., *Musa acuminata* and *Musa balbisiana* produce seeds freely while most of the edible clones are seedless, with a few notable exceptions such as 'Pisang Awak' sub-group (ABB; Simmonds 1966). Owing to the parthenocarpic and sterile nature of edible bananas, strategies aimed at banana improvement are extremely complex and time consuming. Therefore, to complement conventional breeding programs new strategies need to be developed utilizing tissue culture and genetic engineering technologies. Limited and variable seed germination exhibited by *Musa* spp. (Simmonds 1952 and 1959) may be due to seed mortality caused by lack of endosperm. Embryo culture prior to embryo abortion has the potential to rescue many useful crosses. Therefore, *in vitro* embryo culture represents a convenient tool for improving recovery of hybrid germplasm in a short time (Cox *et al.* 1960).

Banana seeds are orthodox in nature and different types of dormancy have been reported (reviewed by Fortescue and Turner 2011). Although banana seeds germinate immediately after extraction, they exhibit secondary dormancy upon storage; this can be overcome by seed treatments using various plant growth regulators (PGRs). Improvement of commercial banana varieties through conventional breeding, despite sterility, polyploidy, and its parthenocarpic nature has become possible through embryo culture and embryo rescue (Uma *et al.* 2009). These techniques have proven advantageous in rescuing many hybrid progenies in similarly recalcitrant crops. Botti and Vasil (1983), for example, developed embryogenic cell suspensions from excised zygotic embryos from pearl millet. Escalant and Teisson (1988) generated somatic embryos from the seeds of banana fruit by culturing them on basal medium supplemented with

PGRs. Further research by Escalant and Teisson (1989) found that calli from immature zygotic embryos and somatic embryos were suitable for development of embryogenic cell suspension cultures. Cronauer-Mitra and Krikorian (1988) also reported regeneration of plants *via* somatic embryogenesis in the seeded diploid banana *Musa ornata* Robx. Although plants have been regenerated successfully from zygotic embryos in banana, there remain some inherent problems with induction of somatic embryogenesis from zygotic embryos before it can become a routine procedure. Understanding the effect of physiological maturity on germination and regeneration of somatic embryos has led to the development of an improved protocol in *M. acuminata* ssp. *burmannica*.

Materials and Methods

Plant material. Seeds of *Musa acuminata* ssp. *burmannica*, an indigenous (AA) wild donor parent with profuse seed set, were used as a model banana species. Plants were maintained in the field gene bank of National Research Centre for Banana (NRCB), Tiruchirappalli, India, were seeds were harvested and used as explant material. Newly opened flowers were manually self-pollinated using pollen from same clump and the bunch was covered to avoid cross pollination. Fruits were harvested at 78 and 110 d-after-flowering corresponding to 70% and 100% maturity, respectively (Fig. 1), determined previously (Uma *et al.* 2011). The seeds were carefully separated from the pulp by continuous washing in tap water. Harvested seeds were subjected to viability and germination tests at 60-d intervals for a period of 9 mo. Varying concentrations of gibberellic acid (0.5, 1.4, 2.0, or 2.8 μM) were also employed to assess the promotional effects of exogenous gibberellic acid on germination.

Embryo excision and culture initiation. Embryo excision and culture initiation were conducted according to Uma *et al.* (2010). Embryos were extracted from seeds using a dissecting microscope under aseptic conditions. A longitudinal incision was used to open the seeds; embryos were excised carefully and cultured in test tubes (25 mm×150 mm), with one embryo per tube that contained 10–15 ml media.

Culture media and conditions. The culture medium consisted of Murashige and Skoog (1962) (MS) salts and Morel vitamins (Morel 1950) supplemented with varying concentrations of PGRs. Media composition and culture conditions are shown in Table 1. The pH of the media was adjusted to 5.8 with NaOH or HCl prior to autoclaving for 20 min at 120°C. Cultures were initially maintained in complete darkness for 15 d followed by cycles of 8 h dark and 16 h light, under white fluorescent lamps with a light intensity of 3,000 lm/m^2, and at temperature of 28±2°C.

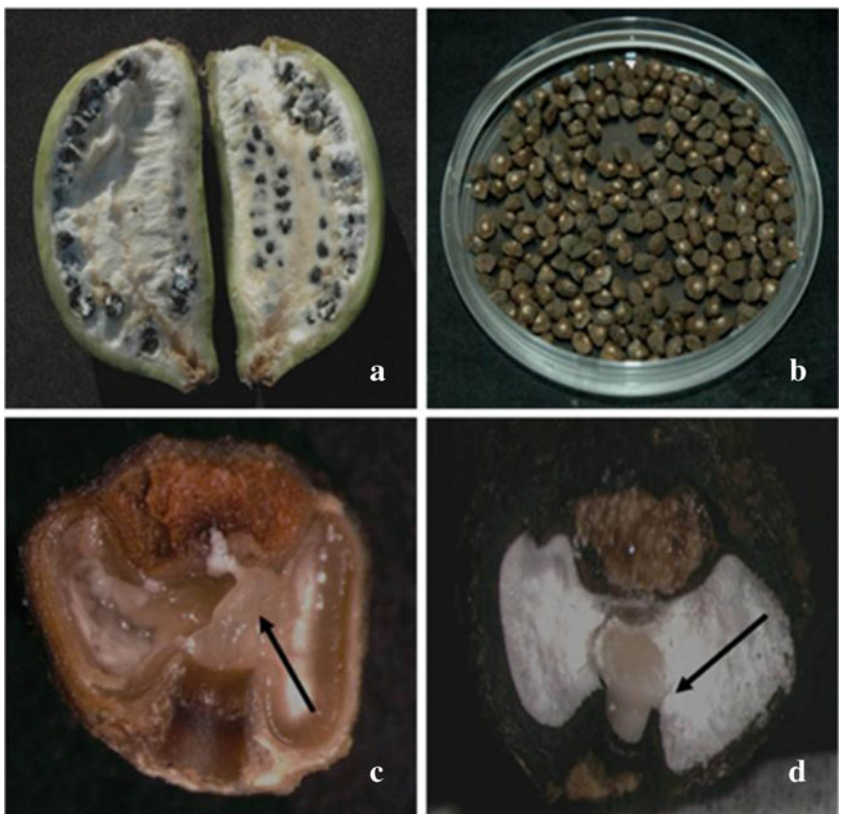

Figure 1. Explant material. (*a*) Dissected fruit, (*b*) Mature seeds of *M. acuminata* ssp. *burmannica*, (*c*) Immature embryo with watery endosperm (initial sign of embryo formation), (*d*) Mature seed and fully formed embryo with rich endosperm.

Table 1. Media composition used for *in vitro* culture of banana embryos

Component	Culture medium							
	M1	M2	M3	M4	M5	M6	M7	M8
BA (μM)	–	2.2	–	–	–	–	–	–
IAA (μM)	–	5.8	–	–	–	–	–	–
2,4-D (μM)	–	–	1.1	2.2	4.5	–	–	–
Picloram (μM)	–	–	–	–	–	0.8	2.07	4.1

Components of basal medium: MS, Morel vitamins, sucrose (30 g/l), pH 5.8, phytagel (2 g/l)

BA benzyl adenine, *2,4-D* 2,4-dichlorophenoxyacetic acid, *IAA* indole acetic acid.

Establishment of callus and plant regeneration. Calli were initiated and subcultured every 3 wk on the same medium (M1–M9; Table 1), using Petri dishes (60 mm diameter) to facilitate rapid proliferation. After three subsequent subcultures, somatic embryos were initiated from embryogenic calli which later developed into shoots. The proportion of callus was calculated as the number of embryos producing calli over the total number of embryos initiated, expressed as a percentage. The level of embryogenic callus formed was calculated as the number of embryos producing embryogenic calli over the total number of embryos producing calli, expressed as a percentage. The somatic embryo germination rates were calculated as the number of petri dishes with germinating somatic embryos over the total number of petri dishes with embryogenic calli, expressed as a percentage. Shoots obtained were rooted in MS medium supplemented with 9.8 μM IBA, 5.3 μM NAA, and 0.1% (*w*/*v*) activated charcoal. Thereafter, plants with well-developed shoots and roots were transferred to black polyethylene bags (150 gauge) containing sand, red soil, and farm yard manure in 2:1:1 ratio and maintained under 75% shade net with 60–70% relative humidity for hardening.

Seed storage and germination. After an initial viability test using tetrazolium chloride, seeds were stored in clear polyethylene bags (250 gauge) at room temperature for a period of 9 mo. Embryos extracted from stored seeds were subjected to viability tests at 60-d time intervals. From the composite seed samples, ten seeds were randomly selected to estimate viability status at bi-monthly intervals. The seeds were soaked in distilled water for 24 h before staining for complete hydration. Later, seeds were bisected longitudinally to expose the embryo and were stained with 1% (*w*/*v*) solution of triphenyl tetrazolium chloride (TCC). The seeds were placed in a Petri dish (60 mm diameter) containing Whatman No.1 filter paper soaked with 1% TTC. Petri dishes were covered with aluminum foil and incubated at 35±1°C in the dark for 36 h.

Table 2. Effect of media composition on plant regeneration through somatic embryogenesis in immature zygotic embryos of *M. acuminata* ssp. *burmannica*

Medium	Number of embryos survived	Callus induction %	Embryogenic calli %	Somatic embryo germination (%)
M1	31	35.6±0.6a	27.1±0.6a	21.5±0.7a
M2	32	74.9±0.7d	69.7±0.5e	54.7±0.5d
M3	32	71.4±0.6c	63.2±0.8d	62.6±0.6e
M4	34	85.9±0.7e	79.7±0.5g	76.6±0.6h
M5	31	93.4±0.4f	54.3±1.2c	49.5±0.8c
M6	35	57.1±0.5b	55.6±0.6c	47.4±0.7b
M7	36	75.1±0.6d	77.6±0.8f	70.5±0.9f
M8	32	84.0±0.4e	48.4±0.6b	46.5±0.9b

Values are means±SE of at least five experiments. Within a *column*, values with different *letters* are significantly different ($P \leq 0.05$). Media are listed in Table 1

To study the efficacy of gibberellic acid in enhancing germination, the seeds stored at various intervals *viz* 1, 3, 5, 7, or 9 mo were transferred to 150 ml conical flasks containing water in which gibberellic acid is incorporated in various concentrations (0.5, 1.4, 2.0, or 2.8 μM). Flasks were agitated at 80 rpm in a rotary shaker for 24 h. Seeds were surface sterilized and cut open to remove the embryos. The embryos were cultured on MS basal medium supplemented with 45 g/l of sucrose and solidified with 2 g/l phytagel and percent embryo germination was recorded.

Experimental design and data analysis. The experiment was designed using a completely randomized design with five replications per treatment. Data were analyzed by

Table 3. Effect of media composition on plant regeneration through somatic embryogenesis in mature zygotic embryos of *M. acuminata* ssp. *burmannica*

Medium	Number of embryos survived	Callus induction %	Embryogenic calli %	Somatic embryogermination (%)
M1	34	14.5±0.6a	5.8±0.6a	2.7±0.4a
M2	34	52.9±0.5d	51.5±0.8f	50.6±0.6g
M3	36	38.8±0.5c	34.5±0.7d	39.4±0.5e
M4	35	37.1±0.6c	33.6±0.9d	31.4±0.8d
M5	33	27.1±0.5b	19.5±0.5b	23.5±0.7c
M6	33	54.4±0.5e	47.4±0.9e	48.4±0.9f
M7	33	42.2±0.6d	30.4±0.8c	26.6±0.7c
M8	32	35.6±0.8c	22.5±0.6b	14.6±0.5b

Values are means±SE of at least five experiments. Within a *column*, values with different *letters* are significantly different ($P \leq 0.05$). Media are listed in Table 1

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

Results and Discussion

In monocots, the selection of appropriate explant material is considered more important than the plant genotype (Krishnaraj and Vasil 1995). During seed development, cells differentiate early and rapidly which is accompanied by a loss of mitotic and morphogenetic ability. In addition, levels of endogenous hormones which differ among organs, tissues, and cells, may also influence regeneration responses (Brown and Thorpe 1995; Krishnaraj and Vasil 1995). According to Johri and Rao (1984), the regeneration of excised banana embryos is influenced primarily by two factors: embryo maturity at excision and the *in vitro* culture medium.

In the present investigation, morphological changes, callus induction, cell proliferation, and the regeneration capacity of both mature and immature zygotic embryos of *M.*

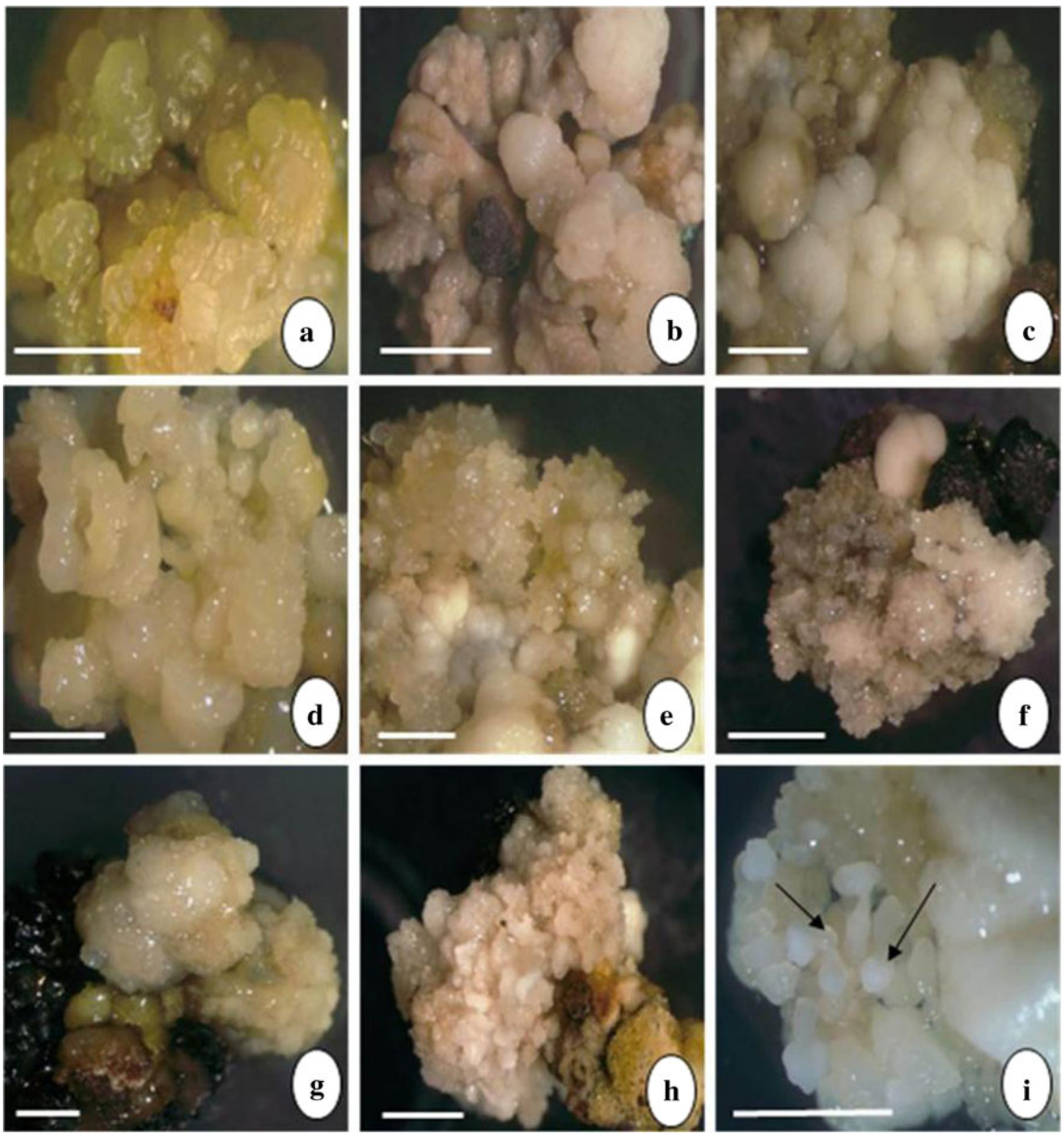

Figure 2. *In vitro* culture of *M. acuminata* ssp. *burmannica.* (*a–c*) Compact homogenous non-embryogenic callus, (*d*) yellow watery callus, (*e–g*) heterogenous friable callus, (*h*) friable embryogenic callus, (*i*) embryogenic callus with translucent proembryos (*arrows*). *Bars* 1 mm.

acuminata ssp. *burmannica* were studied. Developing banana seeds at 70% maturity contained some endosperm and embryos with an undefined structure. Embryos excised from seeds before 70% maturity, on the other hand, had poorly developed embryos with a reduced level of watery endosperm (Uma *et al.* 2011). de Oliveira e Silva *et al.* (1999) also reported that embryos produced from seeds with no endosperm failed to germinate, whereas it was possible to obtain plants from seeds with some endosperm. On the other hand, immature zygotic embryos produced more somatic embryos compared to mature zygotic embryos when cultured *in vitro* (Table 3), similar to results obtained by Walker *et al.* (1978) and Walker and Sato (1981). This is consistent with the observation that less differentiated and younger tissues are more amenable to *in vitro* tissue culture (Uma *et al.* 2010).

Effect of media on germination of somatic embryos. The presence of an established haustorium and stalk is related to seed maturity, and becomes visible in zygotic banana embryos at 78 d-after-flowering when embryos are 70% mature (Uma *et al.* 2011). Results obtained from immature and mature zygotic embryos inoculated on different media are provided in Tables 2 and 3 respectively. Change in the color of embryos from white to yellowish 10–15 d after inoculation in the culture media was considered as the initial growth response. Callus induction was highest in M5 (93.4%),

Figure 3. Somatic embryogenesis and plant regeneration from zygotic embryos of *M. acuminata* ssp. *burmannica.* (*a*) Emergence of chlorophyllous plumule (*arrow*), (*b*) monopolar origin of shoot tip (*arrow*), (*c*) germinating embryo with base covered with somatic embryos, (*d*) plantlet with well-developed root and shoot system, (*e*) complete plantlet, (*f*) plantlet in acclimatization phase in growth chamber, (*g*) plantlet in secondary hardening stage. *Bars* 1 mm.

followed by M4 (85.9%), and M8 (84%) media, whereas the lowest percentage was observed on M1 (35.6%) medium. Initiated embryos produced heterogenous calli 20–30 d after initiation (Fig. 2), which could be maintained by regular transfer to fresh medium at 2-wk intervals.

The morphology of the calli varied among treatments, but it was uniform within a single treatment. Vigorously proliferating milky white callus was observed when the medium was supplemented with 2.2 μM BA or 5.8 μM IAA (M2), whereas yellow friable callus was obtained in media supplemented with 2,4-D at a range of concentrations (Fig. 2). The comparative effect of picloram *versus* 2,4-D showed that frequency of callus induction was less in media supplemented with picloram (72%, average for M6, M7, and M8 media) than 2,4-D (83.5%, average for M3, M4, and M5 media). Moreover, heterogenous calli was more often observed in medium supplemented with picloram (M6–M8).

After three subcultures, explants produced embryogenic calli which later formed whitish translucent embryos, as also reported by Côte *et al.* (1996). Typically, 3–5 somatic embryos were formed, while a maximum of 15 could be obtained from a single immature zygotic embryo. Further subculturing of embryogenic calli in the same media subsequently led to shoot formation (Fig. 3). The highest germination percentage (76.6%) was recorded from the explants cultured on medium supplemented with picloram, whereas the lowest percentage (21.5%) was recorded on MS basal medium (M1) with no supplementation (Table 3).

Mature embryos. Mature seeds had embryos with a well-developed stalk and haustorium surrounded by a rich endosperm. The effect of various media on regeneration from mature embryos showed that medium supplemented with PGRs initially exhibited only calli formation. Calli morphologies were similar to immature embryos while germination percentages differed. As shown in Table 3, callus induction was highest in embryos cultured on M6 (54.4%) followed by M2 (52.9%) medium, whereas the lowest percentage was observed in M1 (14.5%) media. It was also observed that subculture periods of more than 2 wk resulted in accumulation of phenolic compounds in the medium leading to necrosis. Hence, the calli were subcultured on same media every 21 d for four cycles. It was observed that a minimum of three subcultures was required to obtain an embryogenic response from calli cultured on any of the media tested.

At higher concentrations of 2,4-D or picloram, the explants formed highly proliferating callus, but the germination capacity was reduced compared to immature embryos (Tables 2 and 3). Highest germination percentage for mature embryos (50.6%) was recorded for M2 medium (BA 2.2 μM and IAA 5.8 μM), as reported by Bakry and Horry (1992) and Pillay *et al.* (2011) for wild tetraploid species and East African highland bananas, respectively.

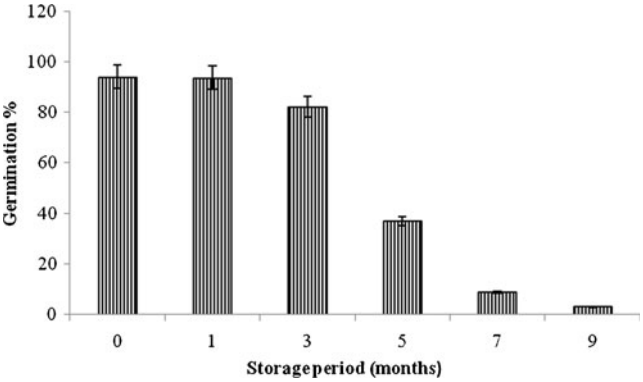

Figure 4. Effect of seed storage on germination capacity of mature embryos.

The next best treatment was M6 (4.1 μM picloram) with 48.4% somatic embryo germination, which was also reported to have a positive response on regeneration rates (Escalant and Teisson 1989). The lowest germination rate (2.7%) was observed using basal medium without supplementation (M1).

Effect of seed storage on germination. Freshly harvested mature seeds without any pre-treatment showed a decline in germination with storage. The highest germination percentage (91.1%) was recorded from freshly harvested seeds whereas it was negligible from seeds stored for 9 mo (Fig. 4).

Seed viability was also determined at 0, 3, 5 7 and 9 mo using a TCC test. Viable embryos stain bright red while embryos colored grayish-red to purple indicated reduced viability (Aslam *et al.* 2010). Freshly harvested embryos stained bright red in color and the intensity gradually reduced with increase time in storage. Embryos from seeds stored for 9 mo remained unstained. The present result showed that prolonged storage of seeds significantly

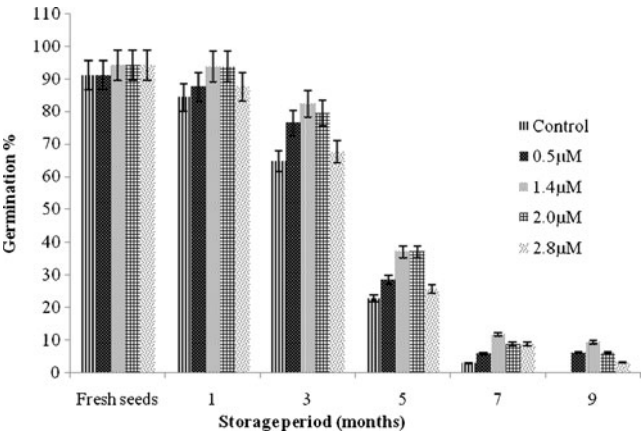

Figure 5. Effect of soaking *M. acuminata* ssp. *burmannica* seeds in GA_3 solution prior to germination of zygotic embryos excised from stored seeds.

reduced the viability and germination percentage, which is typical for orthodox seeds (Fig. 4). The results also imply that *in vitro* culture of embryos should be performed using freshly harvested material.

Effect of gibberellic acid on seed germination. In order to determine whether seed germination frequencies could be increased, seeds stored for various lengths of time were pre-soaked in solutions containing GA_3 (0, 0.5, 1.4, 2.0, or 2.8 μM) prior to germination on MS media. Results indicated that all concentrations of GA_3 were effective in enhancing *in vitro* germination efficiency of zygotic embryos compared to control after storage for 3 mo or more (Fig. 5). Among the various concentrations tested, 1.4 μM GA_3 was the most effective and plantlets were successfully developed from the seeds stored as long as 9 mo. Germination rates improved with increasing GA_3 concentrations from 0.5 to 1.4 μM, beyond which it failed to show significant effect on plantlet recovery (Fig. 5). Similar results of GA_3-enhanced germination has been reported in *Musa velutina* (Pancholi *et al.* 1995), *M. balbisiana* (Afele and de Langhe 1991), and in hybrid banana seeds (NRCB 2011).

Conclusions

Embryogenic calli was obtained by culturing immature and mature zygotic embryos, and successful regeneration of plantlets through somatic embryogenesis has been reported in diploid *M. acuminata* spp. *burmannica*. The success of somatic embryogenesis using immature zygotic embryos can be exploited in the regeneration of hybrid progenies where mature seeds/embryos exhibit poor germination. The embryogenic calli obtained can be used as a base material for the establishment of embryogenic cell lines and production of multiple hybrid plantlets. Complementing embryo rescue and somatic embryogenesis with conventional breeding programs is expected to improve hybrid regeneration efficiencies in otherwise sterile crop like banana.

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