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ORIGINAL PAPER

Embryo rescue and plant regeneration in banana (*Musa* spp.)

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Abstract An efficient regeneration protocol for zygotic embryos at varying maturity stages was developed for wild banana (Pisang Jajee (AA)). Embryo ontogeny was studied to determine the best maturity stage for embryo rescue, suitable media and culture conditions (light and dark) for germination and regeneration. The conversion of endosperm from transparent fluid into a semi-solid state was followed by visible embryo development, which commenced only after 70% embryo maturity. Zygotic embryos of Pisang Jajee at different maturity levels were excised and cultured on medium fortified with different concentrations of 6-benzyl adenine (BA) and indole acetic acid (IAA). Zygotic embryos produced callus or plantlets 25 days after initiation. The frequency of callus induction was greater in immature embryos irrespective of the media composition and decreased with increasing maturity. Fully matured embryos regenerated directly into plantlets without producing callus. Immature embryos required medium supplemented with plant growth regulators (PGRs) for successful regeneration. Although the culture conditions had no influence, dark conditions favoured callus induction and plant regeneration.

Keywords Banana · Embryo maturity · In vitro culture · Zygotic embryo · Callus · Regeneration

Abbreviations

BA	6-benzyl adenine
IAA	Indole acetic acid
PGRs	Plant growth regulators

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Introduction

Bananas and plantains are giant monocotyledonous perennial herbs, belonging to the order Zingiberales (Scitamineae) and family *Musaceae*. Banana is one of the few fruit crops in which all of the cultivated varieties are human selections. Its recalcitrance to improvement through breeding is mainly due to parthenocarpy and male and/or female sterility among the most cultivated varieties. Diverse genomic constitutions like AA, AB, AAB, ABB etc. with varied ploidy levels (2×, 3× and 4×) have aggravated the problem of improvement through classical breeding. These incompatibility problems have manifested into improper seed formation, poor embryo development, endosperm malformation etc. The success of a breeding programme depends on compatibility among the parents and the production of a good number of fertile seeds. More often, the banana seeds, although complete in terms of embryo, endosperm and seed coat, fail to germinate due to various reasons (Chin 1996). Under such circumstances, embryo culture becomes vital. Failure of embryo germination is attributed to the activation of growth inhibitors at the advanced maturity stage of seed development, leading to seed coat dormancy (Le Page-Degivry and Garello 1973; Bewley and Black 1982; Fenner 1985). Therefore, it becomes essential to rescue the embryos before maturity in order to salvage the maximum number of hybrid progenies in a breeding programme.

Occasionally, some banana seeds readily germinate after harvest and show no sign of seed dormancy, while others become dormant after drying (Chin 1996). At least 3–6 weeks is required for the initiation of seed germination in soil and germination occurs either in a flush or intermittently over a 3–15-week period. The germination percentage differs between harvest lots (Simmonds 1952, 1959;

Stover 1959), which depend on fruit maturity at the time of harvest, post-harvest, physiological age of the seed and storage conditions (Simmonds 1952, 1959). Seed germination is also hindered by physical barriers such as the impermeability of seed coat, along with chemical barriers like growth inhibitor-induced dormancy.

These barriers may break down over a period of time or by soaking the seeds in water, acid scarification or the manual excision of embryos and their culture in an appropriate medium. These pre-treatments enhanced the germination rate in *Musa* hybrid seeds, but the germination percentage was very low (4–10%). Studying the banana seed morphology (structure) will help to understand the complexity in seed germination and facilitates the creation of a better physical environment for enhanced germination (Chin 1996).

The success of embryo culture and regeneration depends on the normal seed development. The germination of excised embryos is mainly influenced by two factors, viz. the maturity of the embryo at excision and culture medium (Johri and Rao 1984). Knowledge on the seed anatomy, its various structures and their function in embryo development has an important role in developing improved embryo culture strategies through manipulations in growth conditions and culture media. Very little information is available about the factors affecting the seed germination in the genus *Musa*, except that germination is extremely variable and relatively difficult to obtain under artificial conditions. Hence, this trial was carried out to study the various factors influencing the success of embryo rescue in wild banana, Pisang Jajee (AA).

Materials and methods

Explant source

Pisang Jajee, a seeded wild banana, was evaluated for its biometric traits at the National Research Center for Banana (NRCB), Trichy, India, for the last 3 years and is being used in the breeding programme. In the present investigation, morphological changes, callus formation and regeneration capacity of the zygotic embryos at various maturity stages were studied after selfing. The days taken for bunch maturity were calculated from the day of pollination to full maturity. On an average, it takes 110 days to complete fruit maturity. Based on this, days for 70, 80, 90, 95 and 100% maturity of the embryos were calculated. The newly opened flowers were pollinated using the pollen from a different plant of the same species and the bunch was covered to avoid cross-pollination. Self-pollinated hybrid seeds thus obtained were used for the study. The fruits were collected at 77, 88, 99, 105 and 110 days after pollination, corresponding to 70, 80, 90, 95 and 100% maturity.

Seed extraction

Seeds were separated from the pulp by continuous washing in tap water. Washed seeds were transferred to a beaker containing water for 15 min. Only sunken seeds were used, since most of the floating seeds are devoid of either endosperm and/or embryo.

Preliminary seed treatments

Seed disinfection was performed under sterile conditions in a laminar hood. Seeds were treated with 5% sodium hypochlorite for 15 min, followed by 0.1% mercuric chloride for 15 min. Before and after each treatment, the seeds were rinsed with sterile distilled water 2–3 times. Finally, the seeds were transferred to a sterile petri plate and used for embryo isolation.

Extraction and initiation of embryos

Embryos were extracted using a stereoscopic microscope in a chamber under laminar flow. A longitudinal fissure was made in each seed and the whitish, mushroom-shaped embryo was removed. The excised embryos consisted of a haustorium and a meristematic stalk (Fig. 1f) and were cultured in medium consisting of Murashige and Skoog salts (1962) and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Different concentrations of 6-benzyl adenine (BA) and indole acetic acid (IAA) were used to study the effect on embryo germination. BA concentrations were 2.2 and 4.4 μM. A combination of BA and IAA was also tried (BA 4.4 μM and IAA 2.8 μM).

Callus induction and plantlet regeneration

The embryos after initiation were observed for callus formation. The calli were sub-cultured at regular intervals (15 days) in fresh media and shifted to a 16/8-h (light/dark) photoperiod after 1 month. The frequency of callusing and regeneration of plants were recorded after 1 month and analysed.

Culture conditions

Each treatment contained 100 embryos, one per culture tube. A set of 50 embryos from each treatment were maintained in a 16/8-h (light/dark) photoperiod under white fluorescent lamps with a light intensity of 3,000 lux and another set of 50 embryos were incubated under

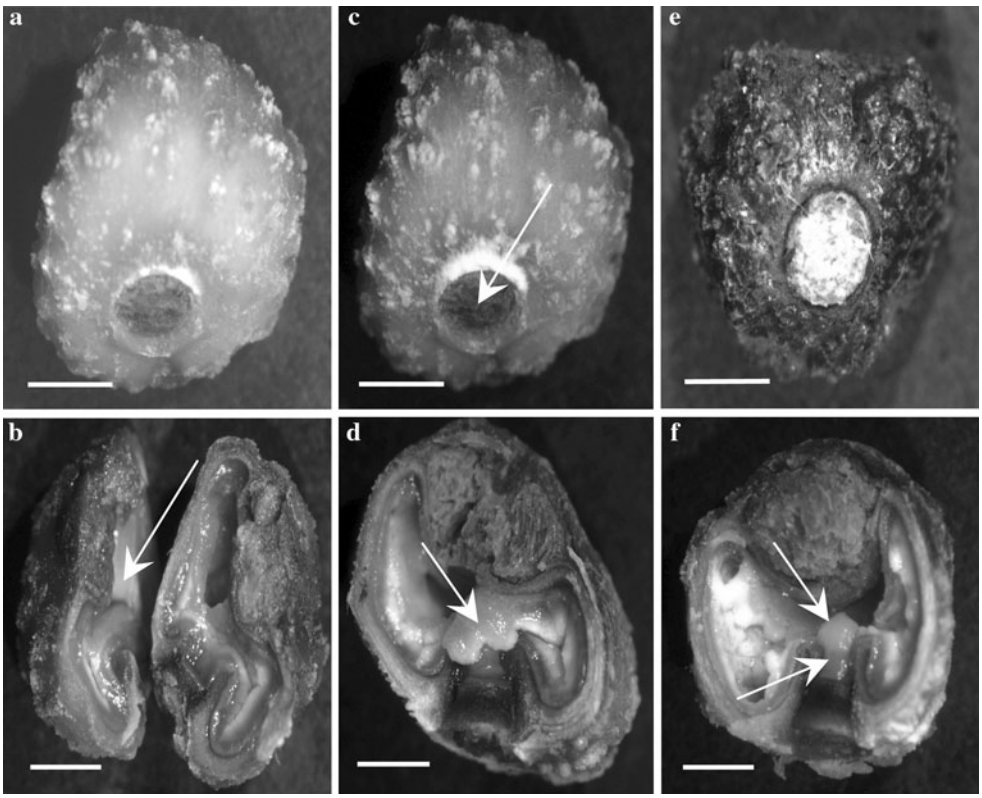

Fig. 1a–f Stages of seed and embryo development. **a**, **c** and **e** 60, 70 and 90% mature seeds showing micropyle. **b** Lacking embryo and endosperm at 60% maturity. **d** 70% mature seed with watery endosperm covering partially formed embryo. **f** 90% mature seed with haustorium and meristematic stalk. The *bar* represents 5 mm

complete darkness. All cultures were maintained at 28 ± 2°C. The experiments were repeated thrice and analysed statistically by Student's *t*-test.

Results and discussion

Embryo developmental studies

The age of the embryo is a crucial factor in embryo rescue studies. A preliminary study on seed and embryo development indicated that, until 70% maturity, no embryo formation was recorded and the endosperm was more or less a transparent fluid. After 70% maturity only, it was observed that the fluid endosperm has partially converted into a semi-solid state and early indications of embryo formation were noticed. This coincided with the thickening of the outer and inner integuments.

Embryos at various stages of development were initiated on MS medium fortified with different plant growth regulators (PGRs). The results indicated that the effect of embryo maturity on callus induction was statistically significant ($P = 0.023$). The frequency of callus induction was high in immature embryos (70%) irrespective of the media composition and it decreased with embryo maturity. Similar results of callusing from immature banana zygotic embryos were reported by Escalant and Teisson (1989). All mature embryos failed to induce callus but regenerated directly into plantlets irrespective of the media composition. Raghavan and Srivastava (1982) reported that low concentrations of auxins favoured normal growth, whereas higher concentrations either proved inhibitory or favoured unorganised callus growth from cultured embryos. But the results of this study emphasised that the maturity state of explants plays a key role in direct or indirect regeneration.

Response of embryo maturity status on plant regeneration

The embryo maturity statuses on direct regeneration into plantlets were found to be highly significant. Plant regeneration exhibited a gradual increase up to 90% embryo maturity, beyond which there was a steep increase. Media composition has no significant influence on the percentage of plantlet regeneration among various stages of embryo maturity. The maximum plantlet regeneration (30.21%) was observed in the medium with BA and IAA followed by basal Murashige and Skoog (MS) with no PGRs. A similar

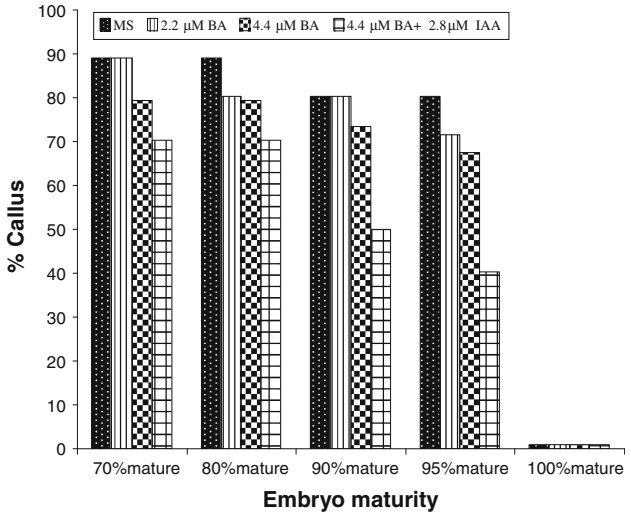

Fig. 2 Effect of maturity status and medium composition on callus induction in zygotic embryos of Pisang Jajee

result of IAA augmentation for better regeneration of banana hybrid embryos has been reported by Bakry (2008). Chi (2000) has found that media composition greatly influenced the embryo regeneration in combination with embryo maturity status. The younger the embryo, the more complex the media is required for successful regeneration. Generally, in embryo culture, embryos with more than 90% maturity, basal MS with no PGR had been successfully used in many crop species (Sharma et al. 1996). Our observations in the present study also suggested that 100% mature embryos induced direct organogenesis along with complete plant regeneration.

In embryos with less than 90% maturity, it was observed that growth regulators did not significantly influence the plant regeneration. This is also supported by the findings of de Oliveira e Silva et al. (1999) in selected hybrid embryos. The interaction effect between embryo maturity and media composition was also statistically non-significant ($P = 0.121$) (Fig. 2).

Effect of culture conditions on callus induction

The effect of culture conditions (light and dark) on the frequency of callus induction was studied in embryos at various maturity stages and it was found to be maturity-dependant (Table 1). The culture conditions have no influence on the callus induction and was statistically non-significant. However, more calli was observed under dark than in light. This is supported by the finding that, in banana somatic embryogenesis, complete darkness is maintained for several months for callusing (Strosse et al. 2006) but initial dark conditions favoured callusing in cotton (Mehetre and Aher 2004) and also in other crops (Diro and Van Staden 2003). The interaction effect between maturity stages and culture conditions was also statistically not significant.

Effect of culture conditions on plant regeneration

The effect of culture conditions on plantlet regeneration was studied among various stages of embryo maturity. The effect of embryo maturity on the percentage of plant regeneration was highly significant irrespective of the culture conditions and it increased with embryo maturity.

However, under dark conditions, regenerated plants were more vigorous than those regenerated in light. Embryos grown in the dark produced longer shoots (3.9 cm) and roots (5.0 cm) than light-grown embryos. A higher percentage of embryo germination in dark was also reported by earlier researchers in hybrid banana embryos (de Oliveira e Silva et al. 1999) and in *M. velutina* (Pancholi et al. 1995).

Effects of plant growth regulators

The use of PGRs exhibited differential response in terms of plantlet production among various maturity stages and light and dark conditions. Irrespective of the culture conditions, the combination of BA (4.4 µM) and IAA (2.8 µM) gave the highest regeneration of plantlets (Figs. 3 and 4). Our results are also supported by Bakry (2008), who reported that BA alone and in combination with IAA influenced the germination of hybrid embryos derived from various crosses. Basal MS without PGRs was found efficient for direct regeneration of plantlets (Fig. 5). Though media without PGR produced higher regeneration frequency, the response was noticed only with 95 and 100% mature embryos. This might be due to the adequate/sufficient nutrient reserves in the fully matured embryos. In embryos with 70–95% maturity, plantlets could be regenerated through callus, but at a very low frequency. The regeneration response of embryos among different maturity status did not differ within the BA treatments (2.2 and 4.4 µM).

Irrespective of the culture conditions and PGRs tested, the extent of plant regeneration depends on the maturity of excised embryos. Although the culture conditions did not have a significant influence, dark conditions induced better callus and plant regeneration. Rijven (1952) reported that light during the first few days of culture may be avoided, as it induced precocious germination, while high light intensity may suppress it. Our findings have also shown that maintaining the embryos under complete dark conditions gave comparatively better results than in light and dark cycles. de Oliveira e Silva et al. (1999) also reported high embryo germination when banana embryos were maintained in darkness for 2 weeks before the plantlets were taken into the light. The difference was more prominent when a combination of growth regulators was used (9.2%).

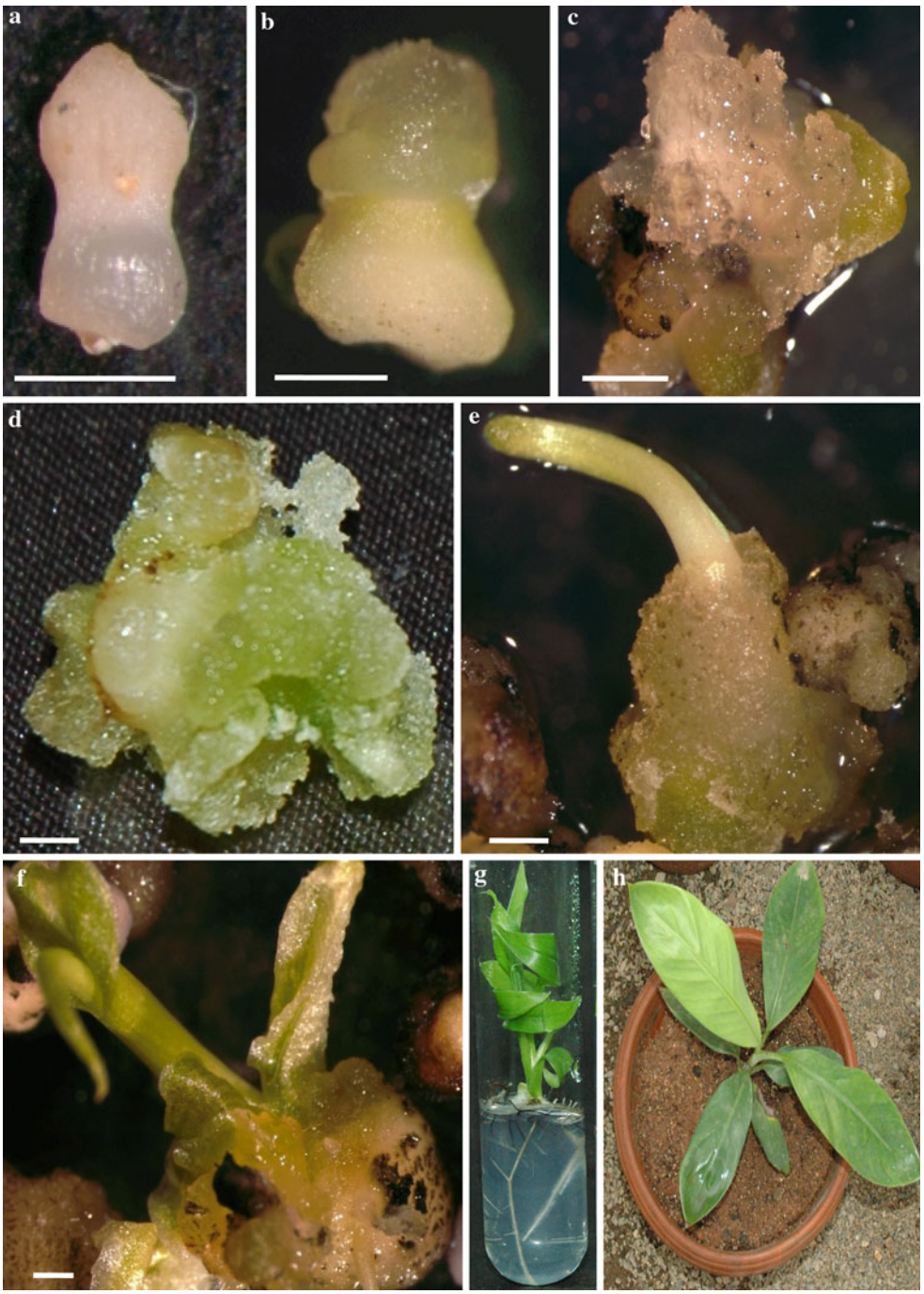

Fig. 3a–f In vitro culture and plant regeneration via indirect organogenesis from Pisang Jajee. **a** 70% mature zygotic embryo before culture. **b** Primary response of embryo. **c** Induction of callus. **d** Callus proliferation. **e** Monopolar regeneration. **f** First leaf emergence. **g** Mature plant ready for rooting. **h** Pisang Jajee plant regenerated in vitro growing in soil. The *bar* represents 1 mm

This finding is similar to earlier reports that embryo germination in darkness was higher than cultures under different photoperiodic regimes. In general, dark conditions were superior over light/dark conditions for the growth rate and shoot length of germinated plantlets (Ahmed et al. 2006). Irrespective of the mode of plant regeneration (direct or indirect), the response of embryos for plant regeneration was more evident across all embryo maturity stages, along with BA and IAA supplements. Diro and Van Staden (2003) also observed improved germination of *Ensete ventricosum* embryos with the use of PGRs, viz. BA (2.2 μM) and IAA (1.1 μM). Even 70% matured embryos recorded 10% plantlet recovery under dark conditions and root was first formed in embryos maintained in the dark.

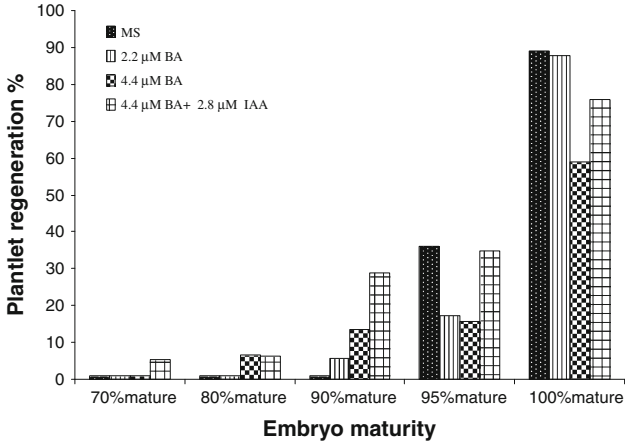

Fig. 4 Effect of embryo maturity and media composition on plantlet regeneration from callus tissue derived from zygotic embryos

A report by Asif et al. (2001) also suggested that dark conditions delayed shoot emergence (8.9 days) as compared to light, whereas roots appeared earlier in the dark as compared to the light (7.7 days).

Table 1 Effect of culture conditions on callus induction and plant regeneration on *Musa* spp. of embryos at different maturity stages

Embryo maturity (%)	Callus induction (%)		Plant regeneration	
	Light	Dark	Light	Dark
70	81.4[a]	84.6[a]	0.9[c]	2.5[c]
80	75.8[a]	80.2[a]	0.9[c]	13.3[c]
90	60.8[b]	75.8[a]	8.4[c]	19.4[c]
95	62.6[b]	62.6[b]	26.1[b]	33.8[b]
100	0.9[c]	0.9[c]	88.5[a]	88.8[a]

Means within the same column followed by different letters are significantly different ($P \leq 0.05$) using Student's *t*-test

In summary, for matured embryos, basal MS medium was sufficient for successful and direct regeneration, whereas immature embryos could be rescued and regenerated indirectly through callus in MS medium supplemented with PGRs. In general, the regeneration capacity depends on the maturity of the explant, while culture conditions have no significant influence.

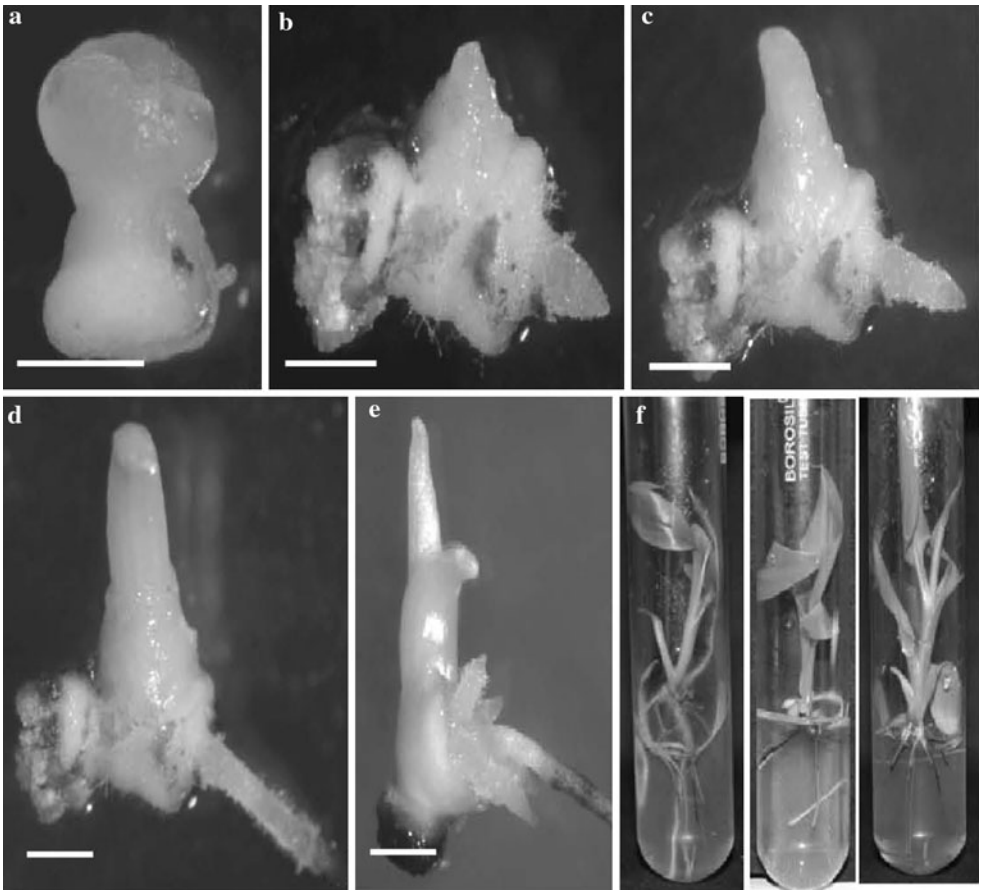

Fig. 5a–e Germination of zygotic embryos and establishment of plantlets. **a** Response of 100% mature zygotic embryo 3 days after initiation. **b** Development of shoot and root primordia after 5 days of culture. **c**, **d** Elongation of shoot and root. **e** First leaf emergence after 16 days of initiation. **f** Regenerated plantlets from zygotic embryo. The *bar* represents 1 mm

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