SOMATIC EMBRYO GERMINATION IN *HEVEA BRASILIENSIS*: EFFECT OF EMBRYO DESICCATION, PHYTOHORMONES AND PHLOROGLUCINOL

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Somatic embryo induction was achieved earlier with a high frequency (>70%) from leaf explants of glass house grown, bud grafted plants of *Hevea brasiliensis* (clone RRII 105). Experiments were carried out to improve quality of germinating embryos, enhance rooting and improve embryo-plant conversion. White, opaque embryos at the cotyledonary stage were cultured for two weeks in maturation medium after which they were given different desiccation treatments. Three desiccation treatments such as slow and fast desiccation in empty petri dishes and water stress by PEG (10, 15 and 20 g L^{-1}) were provided. Embryos after desiccation were cultured in maturation medium where the IAA/GA_3 ratio was optimized. The effect of phloroglucinol (0-200 mg L^{-1}), in the germination medium containing the optimized level of phytohormones was also studied. Among the desiccation treatments tried, slow desiccation of embryos in sealed Petri dishes for 3 days, after an initial two week culture in maturation medium, improved embryo quality and rate of germination. It was observed that optimizing the ratio of IAA/GA_3 in presence of ABA (0.1 mg L^{-1}) in the germination medium, favoured embryo germination after the desiccation treatment. Phloroglucinol (100 mg L^{-1}) enhanced rooting by lateral root induction in 40 per cent of germinating somatic embryos. Germination response was evaluated in terms of both root-shoot apex induction and conversion to plantlets. The effect of embryo desiccation and phloroglucinol in presence of phytohormones, on somatic embryo germination and embryo-plant conversion in *Hevea* is discussed.

Keywords: Embryo desiccation, Germination, Phloroglucinol, Phytohormones

INTRODUCTION

Hevea brasiliensis belonging to the Euphorbiaceae family is the major commercially cultivated species as a source of natural rubber. Commercial propagation in this cross pollinated tree is by bud grafting, since the seedlings are highly heterozygous. Systems for plant regeneration through somatic embryogenesis from inner integument, immature anthers and immature inflorescence have been reported earlier in *H. brasiliensis* (Carron and Enjalric, 1982; Jayasree *et al.*, 1999; Sushamakumari *et al.*, 2000). Etienne *et al.* (1993) has reported that, relatively poor germination of somatic

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embryos and their low rates of development into functional plants are limiting steps for the widespread use of somatic embryogenesis in *Hevea* improvement programmes. A protocol for somatic embryogenesis and plant regeneration was also developed from leaf explants of glass house grown bud grafted plants (Kala *et al.*, 2007). Abnormalities were observed during germination of leaf derived embryos which led to reduced plant regeneration. The germination rate of mature somatic embryos of several conifer species is improved by slow drying under high relative humidity before the transfer to germination medium (Malabadi and van Staden, 2005). Phloroglucinol (PG) (1,3,5-trihydroxybenzene), which is a degradation product of phloridzin, has growth-promoting properties. When added to rooting media together with auxin, phloroglucinol further stimulates rooting, because PG and its homologues act as auxin synergists particularly indole-3-acetic acid (IAA). In most of the studies, PG was added as an additive to a defined medium, usually in addition to phytohormones. Phloroglucinol has been used in *in vitro* propagation studies and approximately 60 per cent of the studies resulted in improved rooting and about 30 per cent in enhanced shoot induction (Teixeira da Silva *et al.*, 2013). Somatic embryo germination is also found to be influenced by gibberellin/auxin ratio in the germination medium. In *Camellia japonica* this was achieved when isolated somatic embryos were cultured in medium with GA_3 at 5.0 mg L^{-1} and IAA at 1.0 mg L^{-1} (Vieitez *et al.*, 1991).

The aim of this study was to improve the germination and plant recovery of *Hevea* somatic embryos induced in embryogenic lines derived from leaf explants. Experiments were done to evaluate the effect of partial desiccation and water stress to enhance plant recovery from somatic embryos previously cultured in maturation medium. The effect of IAA and GA_3 on termination of desiceated embroyes and effect of phloroglucinol in the presence of IAA on enhancing rooting and lateral root induction was also investigated.

MATERIALS AND METHODS

Somatic embryo induction, maturation and germination

High frequency somatic embryo induction was obtained from leaf explants in medium earlier standardized (Kala *et al.*, 2007). White, opaque, cotyledonary somatic embryos were separated from the embryogenic callus and cultured in standardized maturation medium. After 3 weeks of culture in maturation medium, embryos with well developed cotyledons were transferred to light, for root shoot apex induction. Germinated embryos were transferred to MS (Murashige and Skoog, 1962) medium containing myoinositol (100 mg L^{-1}), organic supplements and sucrose (30 g L^{-1}) for plant regeneration. Phytohormones used were BA (1.0 mg L^{-1}) and GA_3 (0.5 mg L^{-1}) along with IBA (0.3 mg L^{-1}) and the medium was solidified with 0.35 per cent phytagel.

Effect of desiccation on somatic embryo germination

After fifteen days culture in maturation medium, the embryos were given different desiccation treatments. For slow desiccation, the well developed, white, opaque cotyledonary embryos were placed in sterile empty Petri dishes sealed with parafilm and dark incubated for different time periods (1 to 5 days). For fast desiccation, the embryos were placed in open Petri dishes

and kept in laminar flow hood for different time intervals (1-5 hrs). Embryo desiccation was also provided with PEG at three different concentrations (10, 15 and 20 g L^{-1}) in the maturation medium. After the desiccation treatments, the embryos were again cultured in maturation medium along with control and the effect on germination was studied. The germination ability of the embryos under the experimental conditions was determined using three replicates, with 10 somatic embryos per replicate and experiment was repeated thrice.

Optimization of IAA/GA_3 ratio

Single cotyledonary embryos after desiccation were transferred to embryo germination medium and incubated under dark conditions. Germination of embryos was carried out in Woody Plant medium (Lloyd and McCown, 1962) containing MS minor salts, 36.7 mg L^{-1} NaFeEDTA, 100 mg L^{-1} myoinositol, B_5 vitamins (Gamborg, 1968), amino acids such as glutamic acid (150 mg L^{-1}), arginine (40 mg L^{-1}) and glycine (2.0 mg L^{-1}), organic supplements such as CW (5%), malt extract (100 mg L^{-1}) and casein hydrolysate (300 mg L^{-1}), sucrose (60 g L^{-1}) and phytohormones BA (0.5 mg L^{-1}) and IBA (0.2 mg L^{-1}) (Kala *et al.*, 2007). Since there are several reports that lateral root induction and germination of embryos can be influenced by the ratio of IAA/GA_3, optimization of their concentration in the germination medium was attempted. A two way factorial experiment was carried out with different concentration of IAA (0-1.0 mg L^{-1}) and GA_3 (0-2.0 mg L^{-1}) in the germination medium to identify the optimal IAA/GA_3 ratio enhancing embryo germination. The phytohormones were added by filter sterilization to the autoclaved medium and poured into sterile Petri plates inside the laminar flow hood.

Effect of phloroglucinol on root enhancement and improving germination

After identifying the suitable desiccation method and phytohormone combination favouring embryo-plant conversion, the desiccated embryos were cultured for germination. Phloroglucinol was supplemented in the germination medium containing the optimized concentration of phytohormones IAA and GA_3. The effect of addition of different concentrations of phloroglucinol (0-200 mg L^{-1}) in the germination medium was also studied. Phloroglucinol was added directly to the germination medium and pH was adjusted to 5.7 before autoclaving at 121 °C for 15 minutes at 15 lb pressure.

RESULTS AND DISCUSSION

Maturation and germination of somatic embryos

Sub-culture of embryogenic callus to fresh medium at 40 days interval helped in callus proliferation and subsequent embryo induction. From the initial translucent pro-embryos at the globular stage, the embryos gradually became white, opaque and cotyledonary in the same medium. Between 30-45 days, globular and heart shaped embryos became clearly visible. Embryos cultured individually and maintained in the dark, matured with apex induction. The embryos changed colour gradually becoming pink and then green with the root-shoot induction in the earlier standardised medium. Most of the mature embryos (70%) were of normal morphology with both root and shoot apex while 30 per cent showed abnormalities with deformed, single or fused cotyledons having either shoot/root apices or without them (Fig. 2A). Mature normal embryos germinated without any pre-treatment. Problems encountered

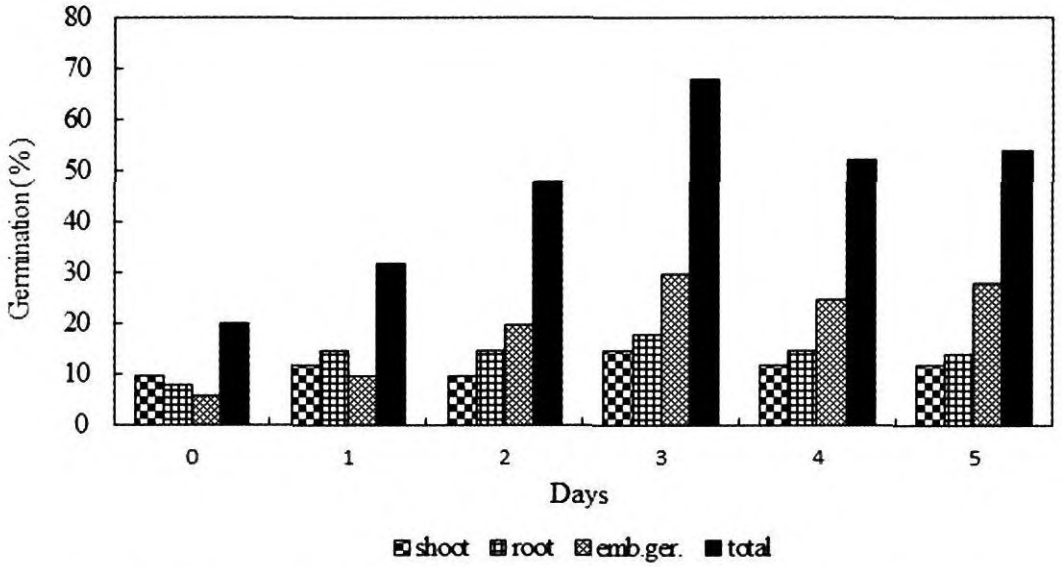

Fig. 1. Germination response of embryos after slow desiccation for different time intervals

during embryo-plant conversion *in vitro* were impaired leaf development and scarce lateral root induction in the developing embryos (<10%), though most of them had healthy and long tap root.

Effect of embryo desiccation

The desiccated embryos showed marked changes in rate of germination as well as quality of embryos. The embryos that were cultured without desiccation germinated at a slow pace compared to the desiccated ones (Fig. 2B). The desiccated embryos germinated with induction of shoot and root apex within ten days of culture and were healthier with larger size compared to the ones without desiccation (Fig. 2C). Among the three methods of desiccation tried, slow desiccation in sealed Petri plates was found more suitable. The embryos that underwent slow desiccation were found to be healthier and germinated faster than the ones that underwent fast desiccation. In addition, the desiccation time also had an impact on the germination and conversion of somatic embryos into plantlets. The response of desiccation on further germination of embryos in terms of shoot and root induction and total growth is represented in Figure 1. The germination response of embryos after both fast and slow desiccation is shown in Figure 2. (C&D). Similar results were reported by Etienne *et al.* (1993a) that rapid desiccation of immature somatic embryos of clone PR 107 derived from inner integument tissue of *Hevea* improved their germination capacity, but their continued development into plantlets was low. In contrast, slow desiccation led to enhancement of germination and was more effective in stimulating conversion into plantlets. Slow desiccation resulted in substantial accumulation of starch and protein reserves required for continued development of

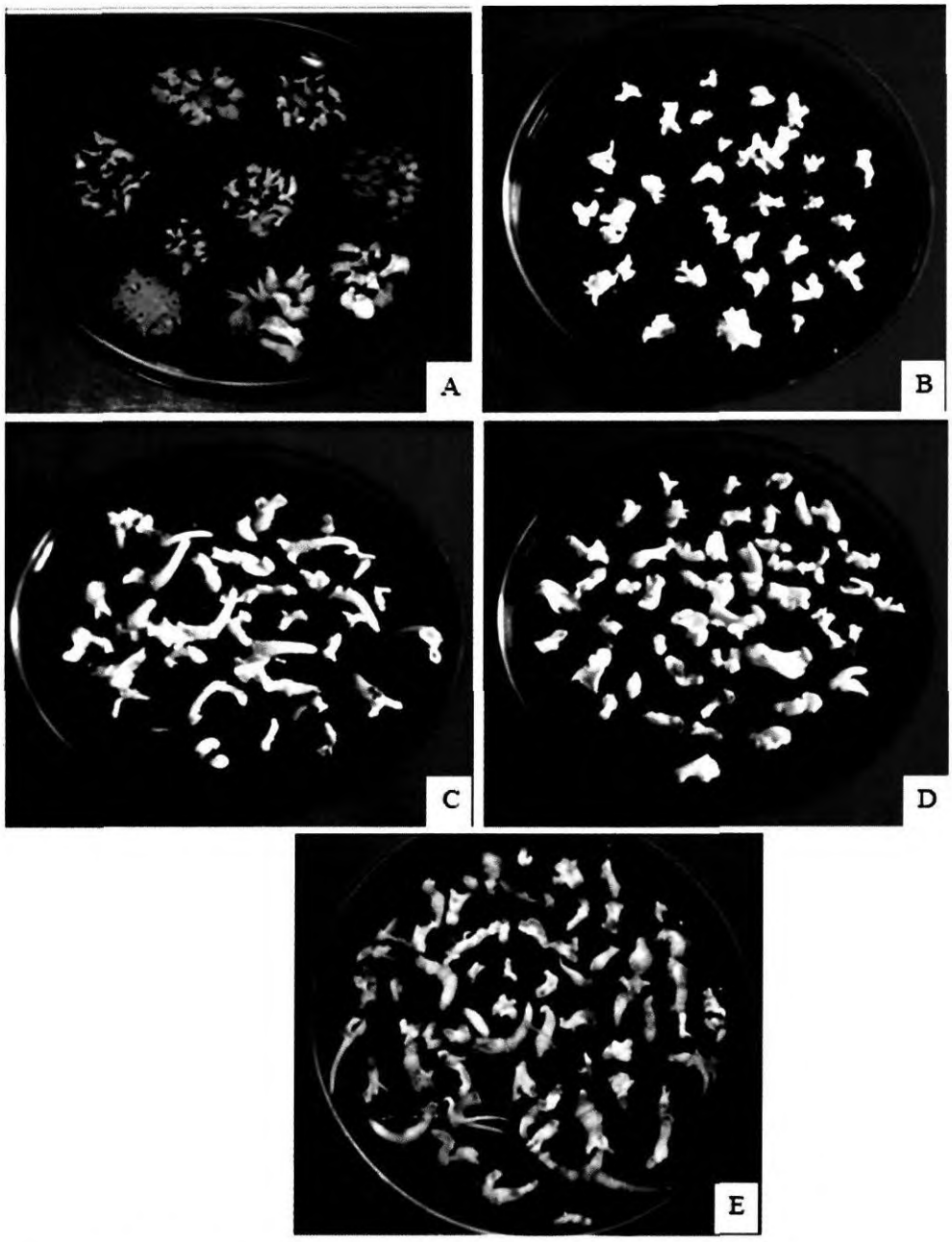

Fig. 2. Embryos cultured in germination medium after desiccation
A- Different types and growth stages of somatic embryos, B- Without desiccation, C- After slow desiccation D- After fast desiccation, E- Embryos with lateral root induction in medium containing PG

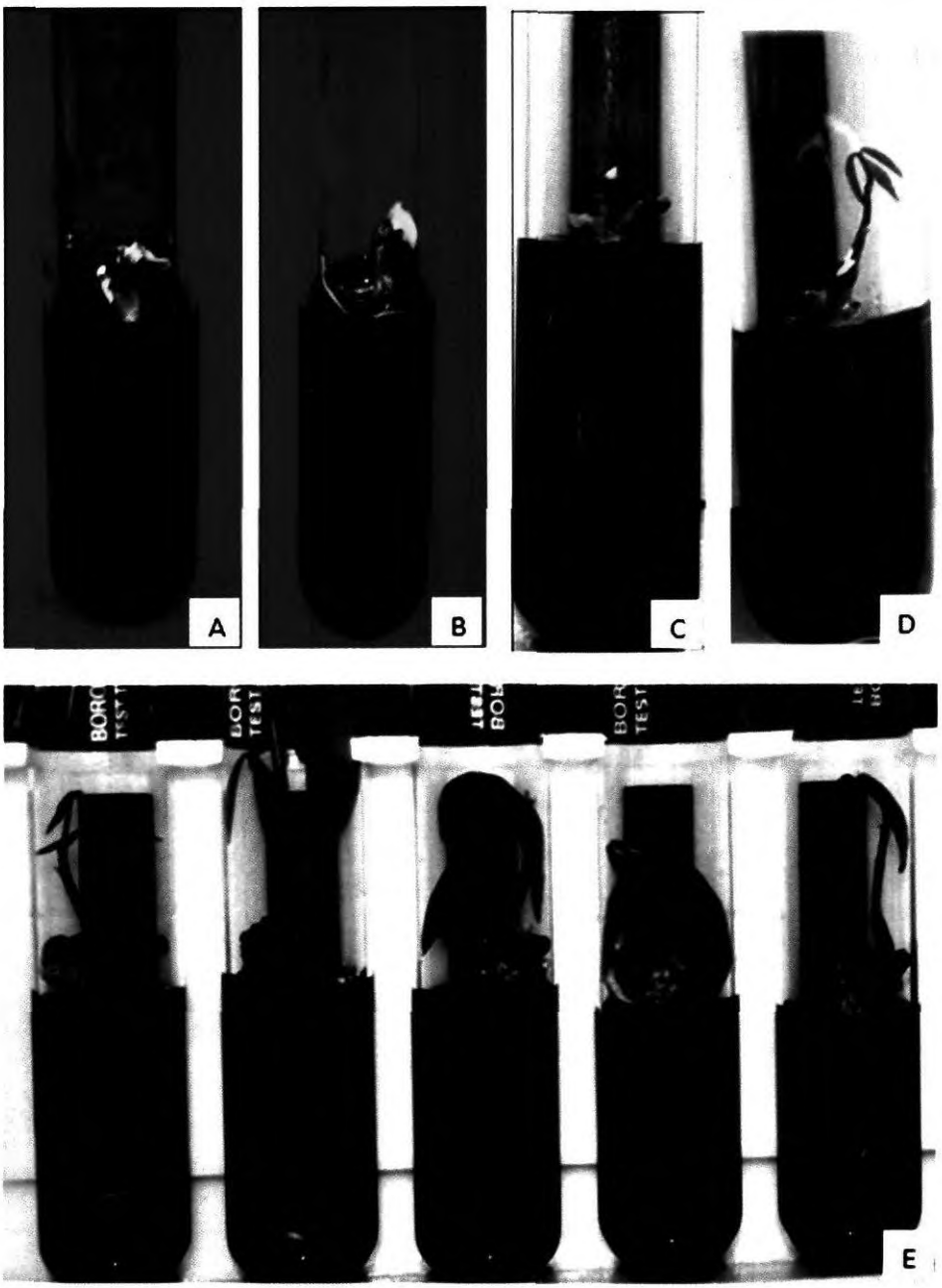

Fig. 3. Embryo germination and plant regeneration
A- Embryo in germination medium with lateral root induction, B&C- Germinating embryos, D - Regenerating plantlet with lateral root, E - Plant regeneration

Table1. **Effect of IAA and GA_3 on embryo germination**

GA_3(mg L^{-1})	IAA (mg L^{-1})					
	0	0.2	0.4	0.6	0.8	1.0
0	24.47(29.58)	33.33(35.19)	33.33 (35.19)	31.11(33.80)	34.44(35.90)	37.78(37.92)
0.25	31.11(33.87)	42.22(40.51)	50.00(45.00)	44.44(41.77)	57.78(49.53)	53.33(47.07)
0.5	51.11(45.65)	60.00(50.92)	87.77(69.84)	81.11(64.25)	76.66(61.21)	60.00(63.64)
1.0	70.00(56.81)	71.11(57.70)	81.11(64.37)	79.99(64.28)	73.33(59.02)	77.77(61.98)
1.5	75.55(60.59)	71.11(57.55)	72.22(58.23)	71.11(57.52)	77.78(62.25)	73.33(59.02)
2.0	70.00(56.81)	77.77(62.13)	81.11(64.75)	78.89(62.66)	76.66(61.15)	74.44(59.76)
CD (P≤0.05)	7.85					

Values in parenthesis are arcsine transformed values.

immature embryos in comparison with rapid dehydration. Therefore, desiccation could be used to enhance germination when the embryo approaches physiological maturity (Etienne *et al.*, 1993a). For many species, a salient feature of the later stages of the development of zygotic embryos is desiccation, which plays a role in the transition between embryo maturation and germination (Kermode, 1990). Water stress provided by PEG was also found to promote embryo germination. Accordingly, water stress provided by 10 g L^{-1} PEG gave maximum response in terms of germination but the embryo-plantlet conversion was found to be reduced. It has been reported that, water stress caused by both PEG and high concentrations of ABA is essential for somatic embryo development and inhibition of precocious germination (Stasolla and Yeung, 2003). PEG enhanced somatic embryo maturation in several species, including *P. glauca* (Attree *et al.*, 1995) and *Abies* hybrids (Salaj *et al.*, 2004).

Optimization of IAA/GA_3

Single cotyledonary embryos when transferred to embryo maturation medium and incubated under dark condition initiated germination after two weeks. The embryos cultured in germination medium containing IAA and GA_3 after desiccation treatment, showed more vigour. From Table 1, it can be inferred that maturation medium containing IAA (0.4 mg L^{-1}) and GA_3 (0.5 mg L^{-1}) gave 87 per cent embryo germination. It could also be seen that higher concentration of GA_3 was essential for improving germination, though IAA at elevated levels did not show much influence. The embryos were healthy and started germination within two weeks with some of the germinating embryos showing lateral root induction also. Hence, this was identified as the optimum

Table 2. **Effect of phloroglucinol on lateral root induction**

Phloroglucinol (mg L^{-1})	Root induction (%)
0	9.75 (11.08)
50	33.75 (35.48)
100	43.75 (41.40)
150	43.75 (41.40)
200	41.25 (39.96)
CD (P≤0.05)	7.03

Values in parenthesis are arc sine transformed values.

concentration for germination. In grape wines, the conversion rate was higher when somatic embryos were germinated in culture medium containing IAA and GA_3 (Lopez *et al.*, 2006). The role of IAA in the release from dormancy and plant regeneration has been discussed by Faure *et al.* (1998). Improvement in embryo germination by the addition of IAA to the embryo germination medium suggest that IAA plays a much stronger role than PG in root induction, although PG serves to enhance the effect of IAA (Dobránszki and Teixeira da Silva, 2010).

Effect of phloroglucinol

The embryos cultured in medium supplemented with phloroglucinol started germination with induction of lateral root primordium (Fig.1E). It was observed that addition of phloroglucinol (100 mg L^{-1}) to the medium enhanced shoot growth and also induced lateral roots in 40 per cent of germinating embryos (Fig. 3. A-D). At higher concentrations of phloroglucinol, increase in rate of lateral root induction were not observed (Table 2). Complete plant regeneration was obtained after one month (Fig. 3E). De Klerk *et al.* (2011) has evaluated the effect of phenolic compounds on root formation from apple stem slices. When IAA was used, most phenolics increased rooting, and a 5-fold increase in the maximum number of roots with 3 µM IAA and 1 mM PG was obtained. Indeed, PG is essential for enhancing root initiation and subsequent development in the presence of an auxin, confirming the notion that PG is an auxin promoter, although its effectiveness is strongly dependent on genotype (Zimmerman, 1984; Magyar- Tábori *et al.*, 2010). PG was a critical ingredient for the rooting of *Asparagus racemosus*, a medicinal plant of high value. The inclusion of PG enhanced the rooting frequency from 41-85 per cent (Bopana and Saxena, 2008). PG also enhanced the effects of IBA in the rooting medium and induced shoot growth (James, 1979; James and Thurbon, 1981).

CONCLUSION

This paper has focused on the effect of physical factors such as embryo desiccation and water stress by PEG on germinating embryos. The influence of the chemical environment by optimization of IAA/GA_3 and addition of phloroglucinol in the maturation medium on embryo-plant conversion in *Hevea* was also evaluated. Slow desiccation of embryos for three days in sealed petri plates enhanced embryo vigour and germination. Desiccated embryos when transferred to germination medium containing optimized IAA/GA_3 ratio and supplemented with phloroglucinol (100 mg L^{-1}) germinated within two weeks with 40 per cent lateral root induction. Plants could be regenerated *in vitro* successfully after one month. The results show that the combination of desiccation treatments along with optimized IAA/GA_3 increased the conversion rate of embryos to healthy plantlets in *Hevea*. Enhancement of rooting by increase in root number and lateral root induction may also result in continued growth of plantlets by enhanced uptake of nutrients which would further improve plant survival during hardening.

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