Plant Cell Biotechnology and Molecular Biology 6(1&2): 61-64

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IN VITRO GERMINATION OF *HEVEA* SOMATIC EMBRYOS : EFFECT OF CYTOKININS

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ABSTRACT

A successful procedure for somatic embryo germination and plant regeneration from immature anther derived callus of RRII 105, a high yielding clone of *Hevea brasiliensis*, has been described. Of the four cytokinins (BA, ZEA, KIN, TDZ) tested, TDZ at 0.25 mg/l concentration was found to be superior for embryo germination as well as plant regeneration response followed by BA and ZEA. On medium supplemented with KIN, maximum response occurred with 2.0 mg/l however, the response was found to be low compared to BA and ZEA. Initial growth of plants in terms of height, shoot length and number of leaves was found to be higher for TDZ than plants derived with BA and ZEA.

Keywords: Immatue anther, *Hevea brasiliensis,* Plant regeneration, Somatic embryogenesis

Abbreviations : TDZ - Thidiazuron; BA - 6-Benzylaminopurine; ZEA – Zeatin; KIN- Kinetin; 2,4-D - 2,4-Dichlorophenoxyacetic acid; NAA - α-Naphthaleneacetic acid; MS - Murashige and Skoog's medium.

INTRODUCTION

Rubber tree (*Hevea brasiliensis)*, a member of Euphorbiaceae family, is originated in Brazil and is now widely grown in tropical regions of Asia. The efficiency of conventional breeding techniques for the incorporation of specific traits in this crop becomes rather slow and quite difficult, as in many other tree crops. Genetic engineering could be an alternative, however, requires an efficient *in vitro* plant regeneration system. In *Hevea,* where the other *in vitro* techniques remains difficult, somatic embryogenesis may offer new possibilities for crop improvement via genetic transformation.

Somatic embryogenesis in *Hevea* has been successfully reported from different explants (Wang et al., 1980; Carron and Enjalric, 1985; Kumari Jayasree et al., 1999; Sushamakumari et al., 2000). Although substantial progress has been achieved in the recent past (Thulaseedharan et al., 2000), the embryo germination and subsequent conversion into viable plantlets still remains an enigma for most of the researchers worldwide. This low germination and further regeneration is one of the major constraint limiting the application of this system in clonal propagation (Cailloux et al., 1996; Linossier et al., 1997) and in genetic transformation studies. Our efforts are thus being concentrated in overcoming this limitation by the manipulation of culture media, particularly, with cytokinins which are identified as an important media component during germination in many plant species like rose wood (Muralidhar Rao, 1996), *Acacia catechu* (Rout et al., 1995), Swietentia *macrophylla* King (Maruyama and Ishii, 1999). In the present study, we reported the results of the detailed investigation on the response of four cytokinins, namely BA, ZEA, KIN and TDZ on germination and plant regeneration from *Hevea* somatic embryos. Comparative response of TDZ with BA and ZEA derived plants in terms of morphological characters is also discussed.

MATERIALS AND METHODS

After sterilization with 0.5% sodium hypochlorite, immature anthers (before microsporogenesis), were

Corresponding Editor: Dr. P. B. Kavi Kishor

dissected out under microscope and transferred to liquid callus induction medium for 10 days and then placed on solid callus induction medium containing 2.0 mg/l 2,4-D and 0.5 mg/l KIN (Kumari Jayasree et al., 2001). Induced calli were subcultured onto embryo induction medium containing the growth regulators - NAA (0.2 mg/l), KIN (0.7 mg/l) and 7% sucrose for 5 weeks. The medium was enriched with glutamine (200 mg/l), casein hydrolysate (400 mg/l) (Kumari Jayasree et al., 2001) and GA_3 (2.0 mg/l) (Kumari Jayasree and Thulaseedharan, 2001). Differentiated embryos were kept on the same medium for another three weeks. Cotyledonary stage embryos were then separated and used for the study.

In order to study the effect of different cytokinins on germination, four cytokinins in the following concentrations were tested - BA (0, 0.5, 1.0, 1.5, 2.0 mg/l), KIN (0, 0.5, 1.0, 1.5, 2.0 mg/l), ZEA (0, 0.5, 1.0, 1.5, 2.0 mg/l) and TDZ (0, 0.25, 0.5, 0.75, 1.0 2.0 mg/l). Mature embryos were placed on germination medium consisted of 2.0 mg/l GA_3 (Kumari Jayasree and Thulaseedharan, 2001) coupled with the above combinations. The pH of all media was adjusted to 5.6 before autoclaving at 121^0 for 15 min. Cultures were maintained at 25° C under16h photoperiod (40 $\mu E\ m^{-2}\ s^{-1}$). Rooted plantlets from different treatments were removed from the culture tubes, washed off the medium in running tap water and transplanted to small polybags filled with sand and soil (1:1v/v) and later to large polybags (35 × 65 cm) for hardening. The acclimatized plantlets were maintained in shadehouse.

RESULTS AND DISCUSSION

Immature anthers, precultured for 10 days in liquid medium, induced pale yellow callus after 25 days of culture on solid medium. Upon transferring the calli onto embryo induction medium, the emergence of embryoids was seen followed by the maturation of cotyledonary stage embryos (Fig. A). By transferring normal mature embryos into plant regeneration medium, irrespective of the type and concentration of cytokinins used, the germination (Fig B) started within 7-10 days of culture followed by primary leaf emergence (Fig. C). After 10 days of culture, embryo germination frequency was significantly higher (70%) for medium containing 0.5mg/l BA. However with 1.0 mg/l, although the germination was decreased, the full plant development was slightly increased. When BA was increased from 1.5-2.0 mg /l, the full plant recovery was decreased (Table 1). By replacing BA with ZEA, maximum embryo germination occurred on 2.0 mg (Table 1). On the contrary, lower concentrations of ZEA was required for somatic embryo germination in *S. macrophylla* King (Maruyama and Ishii, 1999). The present work revealed though BA and ZEA showed more or less similar response on germination, the requirement was different. Overall, BA induced higher germination than ZEA although the difference was very marginal. Response of KIN in terms of germination was less compared to BA and ZEA and the concentration required for maximum response was also higher (2.0 mg/l). Medium supplemented with 0.5mg/l KIN, percentage of germinated embryos without shoot (only root) was very higher. However, even after 50 days of maintenance on the same medium, shoot apex was never emerged on these shootless embryos (Fig. D). However, in *Acasia catechu*, the development of plantlets via somatic embryogenesis was achieved from immature cotyledon derived callus when medium supplemented with 13.9 um KIN along with 2.7 um NAA (Rout et al., 1995). By the addition of TDZ, maximum germination was occurred when medium was supplemented with 0.25 mg/l (Table 2). When the concentration was slightly raised to 0.5 and 0.75 mg/l, the embryo germination was reduced. All concentrations above this level have a retarding effect suggesting that TDZ was required only in lower concentrations. This is attributed to the hypothesis that TDZ is active at lower concentrations than the aminopurine cytokinins (Mok et al., 1987). Plantlets (Fig. E) produced by TDZ showed a maximum height of 11-12 cm and were vigorous in growth compared to BA and ZEA derived plants (Table 3) suggesting that TDZ might have exhibited a dual role as both auxin and cytokinin like activity. After hardening, the hardened plants (Fig. F) were transplanted into big polybags (Fig. G). The acclimatized plantlets were phenotypically normal in appearance.

During *in vitro* culture, many factors were found to affect somatic embryo germination. Our results revealed that in all treatments tested, regardless of the cytokinin type and concentration, some embryos remained nongerminated and some induced only root but no shoot. This may probably due to the most sensitivity of shoot apical meristem to the cultural conditions than root pole during somatic embryo germination. Also in the present study, all germinated embryos were not converted into fully developed plantlets. Since germination and full plant recovery was defined as two different stages during morphogenesis (Stuart and Strickland, 1984), the reason for our results reflected the fact that although germination occurred, sometimes the germinated embryos did not have a viable apical meristem or well developed epicotyl formation or cotyledonary

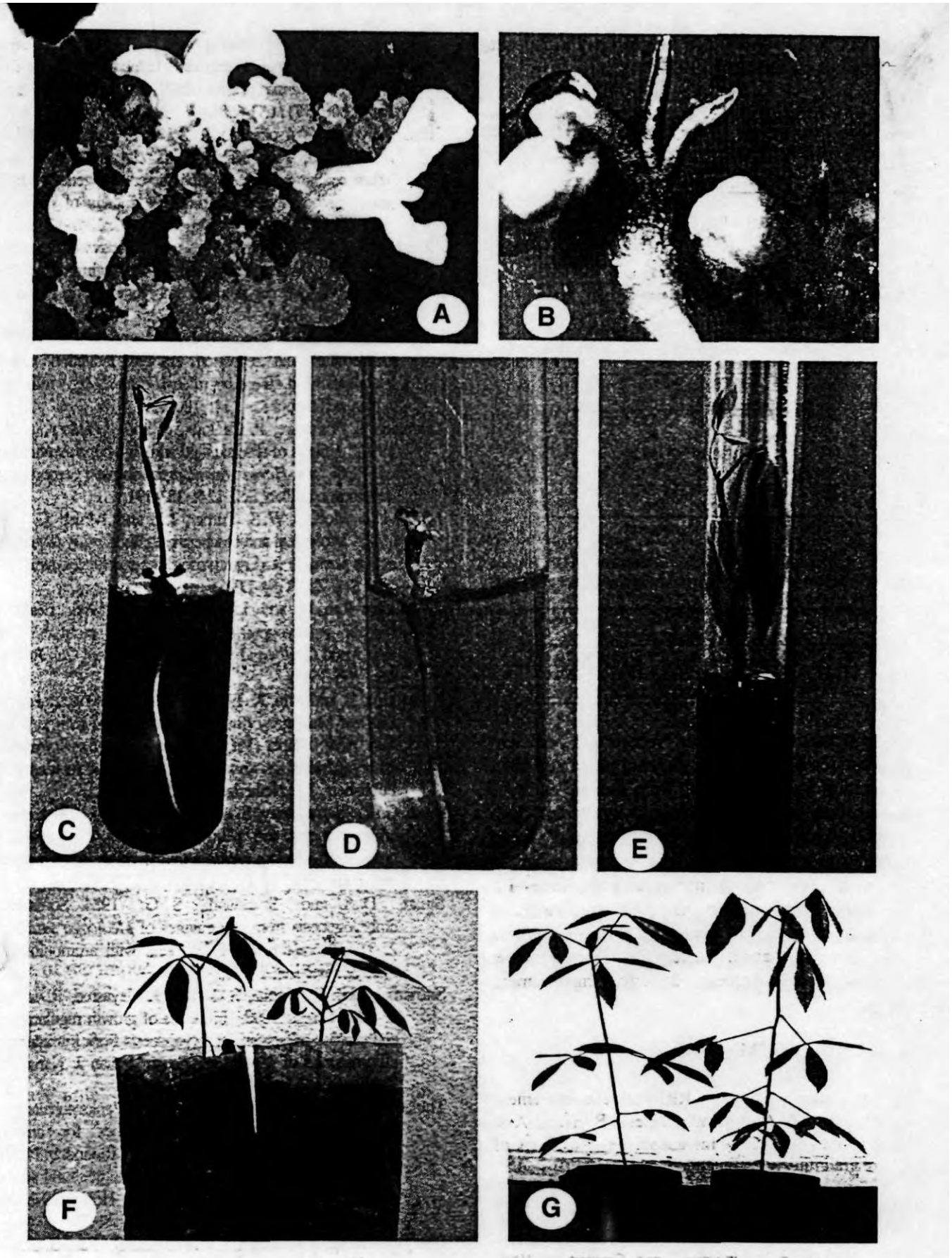

Fig. A-G *In vitro* germination of *Hevea* somatic embryos. A. mature cotyledonary stage embryos; B. root and shoot pole differentiated embryo; C. germinated embryo; D. embryo having only root; E. fully developed plantlet; F-G. hardened plants growing in small and big polybags.

structures with or without a radicle on the opposite end (Maruyama and Ishii, 1999).

Table 1. Effect of BA and ZEA on germination and full plant development. Data were recor ded from 60 days of culture based on 30 replications from three experiments

BA (mg/l)	GE (%)	P (%)	ZEA (mg/l)	GE (%)	PD (%)
0.0	40	50	0.0	37	45
0.5	70	80	0.5	40	50
1.0	60	83	1.0	53	56
1.5	60	67	1.5	60	72
2.0	47	57	2.0	67	80

GE-Germinated embryos; PD-Plant development

Table 2. Effect of KIN and TDZ on germination and full plantdevelopment. Data were recorded from 60 days of culture based on 30 replications from three experiments

KIN (mg/l)	GE (%)	PD (%)	TDZ (mg/l)	GE (%)	PD (%)
0.0	40	42	0.00	43	38
0.5	40	42	0.25	80	83
1.0	47	50	0.50	60	67
1.5	50	47	0.75	60	67
2.0	53	56	1.00	50	53
			2.00	30	40

GE-Germinated embryos; PD-Plant development

Table 3. Evaluation of morphological characters on TDZ, BA and ZEA derived plants (Observations were made on 60 days of culture) (Values are mean ±SD)

Cytokinin (mg/l)	Plant height (cm)	Shoot length (cm)	Leaves (nos)
TDZ - 0.25	11.45 ± 0.30	6.29 ± 0.19	3.21± 0.17
BA - 0.5	10.38 ± 0.32	5.56 ± 0.21	2.81± 0.18
ZEA - 2.0	8. 47 ± 0.32	4.91 ± 0. 21	2.56± 0.18

In conclusion, this paper reports an efficient procedure for achieving a high germination and plant regeneration frequency (80%) from somatic embryos and involves the use of a single cytokinin TDZ. However, more attention is to be required during hardening process and the work is now in progress. The system may provide numerous possibilities for the better understanding of the molecular and physiological control of somatic embryogenesis and plant development and ultimately for the effective use of somatic embryogenesis in crop improvement programs.

ACKNOWLEDGEMENTS

Dr. N.M. Mathew, Director, RRII, his encouragement to carry out this study and Ramesh B. Nair, Asst Director, Statistics, RRII, his assistance in analysis of data are gratefully acknowledged.

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