Effect of cytokinins on *in vitro* germination of *Hevea* somatic embryos

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Abstract:

In Hevea brasiliensis somatic embryogenesis may offer new possibilities for crop improvement programs via. genetic transformation when the other in vitro techniques remain difficult. In the recent past, although substantial progress has been made in Hevea somatic embryogenesis, the embryo germination and subsequent development into viable plantlets still remain an enigma for most of the researchers worldwide. A detailed investigation on the response of various cytokinins on germination was carried out using benzyl adenine (BA), zeatin (ZEA), kinetin (KIN) and thidiazuron (TDZ). Irrespective of the type and concentration of cytokinins used, germination started within 7-10 days of transfer to plant regeneration medium. TDZ proved to be superior to BA and ZEA while KIN showed the least response. 70% germination and 83% plant development occurred with BA while ZEA resulted in 67% germination and 80% plant recovery. Maximum germination and plant development (80% and 82% respectively) was obtained when the medium was supplemented with TDZ. A low germination rate of 53% was obtained with KIN. The initial growth of plantlets in terms of height, shoot length and number of leaves was higher for plantlets produced with TDZ when compared to BA and ZEA derived plants. In the present paper an efficient method for achieving a high frequency germination and plant recovery from somatic embryos have been described for the clone RRII 105 using immature anthers as explants.

Introduction

Genetic improvement of Hevea brasiliensis, like many other woody crops, by traditional breeding methods is a slow and difficult process. The major need for a successful plant genetic transformation for crop improvement programs is the availability of an efficient plant regeneration protocol through somatic embryogenesis. Due to the high proliferation potential and low risk of chimeric plant development, somatic embryogenesis is a desirable component of plant genetic transformation and further regeneration protocols in plants (Ammirato 1989). In Hevea, considerable work has been made worldwide in the last decade on somatic embryogenesis and plant development. However, the response in terms of plantlet regeneration is poor due to low germination rate of somatic embryos limiting the application of this system in crop improvement. In the present study, to enable the efficient recovery of plantlets, a detailed study on germination and subsequent plant development was carried out with four cytokinins - benzyl adenine (BA), zeatin (ZEA), kinetin (KIN) and thidiazuron (TDZ).

Material and methods

Young inflorescences were collected from *Hevea* plants (Clone RRII 105). After surface sterilization in 0.5% sodium hypochlorite solution for 5 min and extensive washing in sterile distilled water, immature anthers before microsporogenesis were dissected out, transferred to liquid callus induction medium and maintained for 10 days followed by culturing on solid callus induction medium containing 2.0 mg/l 2,4-D, 0.5 mg/l KIN and 5% v/w sucrose (Kumari Jayasree et al. 1999). Induced

calli were then subcultured to embryo induction medium containing NAA 0.2 mg/l and KIN 0.7 mg/l and 7% sucrose for 5 weeks. Medium was also enriched with 150 mg/l glutamine and 400 mg/l casein hydrolysate (Kumari Jayasree et al 2001) and 2.0 mg/l GA_3 (Kumari Jayasree and Thulaseedharan 2001). Embryos developed were kept on the same medium for maturation and cotyledondary stage embryos were cultured for germination on medium containing 2.0 mg/l GA3 and various cytokinins in the following concentrations BA (1, 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). Well developed plantlets were transplanted to small polybags filled with sand and soil and kept for hardening in greenhouse. Cultures were maintained at 25° C under darkness upto embryo initiation phase and a 16 hour photoperiod (40 $\mu E\ m^{-2}\ s^{-1}$) in the germination phase. All experiments were repeated three times with 10 replications for each treatment. The rate of germination was recorded after 10 days and the percentage of germination and full plant recovery was evaluated after 60 days.

Results and discussion

Immature anther derived callus on transferring to embryo induction medium initially changed to brown and subsequently friable embryogenic callus was produced.

Development of globular embryos followed by heart and torpedo to cotyledonary stage was obtained after 5 weeks of culture and the cotyledonary stage embryos had reached maturation after 3 weeks maintenance on the same medium. After 60 days of culture, embryo germination frequency was significantly higher (70%) for medium containing 0.5mg BA and 80% germinated embryos developed into full plantlets. However, by increasing the concentration from 1.5 - 2.0 mg/l, the full plant recovery was decreased to 66 – 56 %. Similarly BAP (0.5-1.0 mg/l) requirement were reported for germination and conversion of plantlets in rose wood (Muraleedhar Rao 1996), bipolar differentiation of somatic embryos in coconut palm (Verdeil et al. 1994), germination of secondary embryos in Cassava (Sofiari et al. 1997). In previous other studies on *Hevea*, BA alone enhanced germination of somatic embryos derived from integumental tissues (Veisseire et al. 1994). By replacing BA with ZEA, maximum embryo germination occurred with 1.5 and 2.0 mg. On the contrary, Maruyama and Ishii (1999) reported a favorable effect of ZEA on somatic embryo germination in *Switiana macrophylla* (big–leaf mahogany) where lower concentrations were required. In the current study, BA and ZEA responded more or less equally but the requirement was different. In the case of KIN, maximum response was obtained only at higher concentrations (2.0mg/l) in contrast in *Acasia catechu*, plantlet regeneration was achieved by supplementing with 13.9 µm KIN and 2.7µm NAA (Rout et al. 1995). By the addition of TDZ, embryo germination started after 7 days of culture and maximum germination was occurred when the medium supplemented with 0.25 mg. When the concentration was raised to 0.5 and 0.75 mg, the embryo germination was reduced and beyond this level showed a decreased effect. Similar observations were reported in white ash (Bates et al. 1992), in eastern black walnut (Neumann 1992), in pigeonpea (Sreenivasu 1998) and in *Vitis vinifera* (Matsuta and Hirabayashi 1989). Plantlets produced by TDZ showed a maximum height of 11-12 cm and were vigorous in growth when compared to BA and ZEA derived plants. The initial growth of plant in terms of height, shoot length, number of leaves and survival rate after hardening was higher for plants produced with TDZ when compared to BA and ZEA treatment.

Table 1. Effect of cytokinins on germination and full plant development (Data were recorded from 60 days of culture; based on 30 replications from three experiments).

Cytokinin	**Concentration- mg/l**	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
BA	Germination(%)	40	70	60	60	47
	Plant recovery(%)	50	80	83	67	57
	Concentration(mg/l)	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
ZEA	Germination(%)	37	40	53	60	67
	Plant recovery(%)	45	50	56	72	80
	Concentration –mg	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
KIN	Germination(%)	40	40	47	50	53
	Plant recovery(%)	42	42	50	47	56
	Concentration – mg	**0.0**	**0.25**	**0.5**	**0.75**	**1.0**	**2.0**
TDZ	Germination(%)	43	80	60	60	50	30
	Plant recovery(%)	38	83	67	67	53	40

Table 2. Evaluation of morphological characters of plantlets produced with TDZ, BA and ZEA (Observations were made on 60 days of culture (survival rate after 4 month of acclimatization).

Cytokinin (mg/l)	Plant height (cm)	Shoot height (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

Conclusion

The present paper reports a successful protocol for obtaining high germination rate and plant regeneration (80-83%) from somatic embryos by using the cytokinin TDZ which was found superior to BA and ZEA. Irrespective of cytokinin type and concentration, some embryos remained nongerminated or produced only root. Although it was unclear further studies will be required at molecular level.

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References

AMMIRATO PV (1989). Recent progress in somatic embryogenesis. Intl. Association of Plant Tissue Culture News1. 57, 2-16.

BATESS S, PREECE JE, NAVARRETTE NE, VAN SAMBEEK JW, GAFFNEY GR (1992). Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white *ash (Fraxinus americana* L.). Plant Cell Tissue Organ Cult 31: 21-30.

KUMARI JAYASREE P, ASOKAN MP, SOBHA S, SANKARI AMMAL L, REKHA K, KALA RG, JAYASREE R, THULASEEDHARAN A (1999). Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell.) Arg. Current Sci 76: 1242-1245.

KUMARI JAYASREE P, VINOTH THOMAS, C.K.SARASWATHYAMMA, THULASEEDHARAN A (2001). Optimization of parameters affecting somatic embryogenesis in *Hevea brasiliensis* (in press) Indian journal of Natural Rubber Research.

KUMARI JAYASREE P, THULASEEDHARAN A (2001). Gibberellic acid regulated embryo induction and germination in *Hevea brasiliensis* (Muell). Arg. (Communicated) Indian journal of Natural Rubber Research.

MARUYAMA E, ISHII K (1999). Somatic embryogenesis in Big-leaf Mahogany In: Somatic embryogenesis in woody plants (eds Jain SM, Gupta PK, Newton RJ) Kluwer academic Publishers, The Netherlands, pp 5: 45-62.

MURALEEDHAR RAO M, LAKSHIMI SITA G (1996). Direct somatic embryogenesis from immature embryos of rose wood (*Dalbergia latifolia Roxb.*). Plant Cell Rep 15: 355-359.

MURASHIGE T, SKOOG F (1962) A revised medium for rapid growth and bioassays with tabacco tissue culture. Physiol Plant 15: 473-497.

NEUMAN MC, PREECE JE, VAN SAMBEEK JW, GAFFNEY GR (1992). Somatic embryogeesis and callus production from cotyledon explants of eastern black walnut (*Juglans nigra* L.). Plant Cell Tiss. Org. Cult. 32: 9-18.

ROUT GR, SAMANTARAY S, DAS P (1995). Somatic embryogenesis and plant regeneration from callus cultures of *Acasia catechu*, a multi purpose leguminous tree. Plant Cell Tissue Organ Cult 42: 283-285.

SOFIARI E, RAEMAKERS CJJM, KANJU E, DANSO K, VAN LAMMEREN AM (1997). Comparision of NAA and 2,4-D induced somatic embryogenesis in cassava. Plant Cell Tissue Organ Cult.50: 45-56.

VEISSEIRE P, LINOSSIER L, COUDRET A (1994). Effect of absciscic acid and cytokinins on the development of somatic embryos in *Hevea brasiliensis*. Plant Cell Tissue Organ Cult 39(3): 219-223.

VERDEIL JL, HUET C, GROS DEMANGE F, BUFFARD- MOREL J (1994). Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.) evidence for somatic embryogenesis. Plant Cell Rep 13: 218-221.