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GIBBERELLIC ACID-REGULATED EMBRYO INDUCTION AND GERMINATION IN *HEVEA BRASILIENSIS* (MUELL. ARG.)

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Effect of gibberellic acid (GA_3) on embryo induction and germination during somatic embryogenesis of *Hevea brasiliensis* with respect to clone RRII 105 was studied. Immature anthers were inoculated on callus induction medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) and the induced calli were then transferred to the embryo induction medium. Incorporation of GA_3 up to 2.0 mg/l increased the embryo induction. Germination percentage was significantly enhanced by higher concentrations, however, further plant development was affected by increasing GA_3 levels. A reduction in response to both embryo induction and germination was observed by co-autoclaving of GA_3.

Key words : Anther culture, Gibberellic acid, *Hevea brasiliensis*, Somatic embryogenesis.

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INTRODUCTION

During *in vitro* culture, the cells possess or acquire competence for morphogenesis and undergo a permissive pattern of development. Although the success of such pattern of development is affected by various factors, the effect of constituents of medium is very crucial for production of embryo from somatic cells (Ammirato, 1986). In *Hevea*, somatic embryogenesis using different explants has been reported from China, Malaysia, France and India (Wang *et al.*, 1980; Wan *et al.*, 1982; Carron and Enjalric, 1985; Asokan *et al.*, 1992; Jayasree *et al.*, 1999). Somatic embryogenesis remains problematic due to low germination and plant conversion rate although enough attention has been focussed on its induction phase (Linossier *et al.*, 1997).

Gibberellins (GA) are known to regulate many aspects of growth and development of plants (Hooley, 1994). Gibberellic acid (GA_3) is a potent growth regulator influencing embryo induction and germination. Although conflicting reports are existing for the influence of GA_3 on embryo induction and germination in many crops, a comprehensive study in *Hevea* is lacking. Therefore, it was of interest to investigate the influence of GA_3 on somatic embryogenesis in *Hevea*.

MATERIALS AND METHODS

Floral buds were collected from *Hevea brasiliensis*, clone RRII 105. After ascertaining the developmental stage, buds at the diploid stage were selected and surface-sterilized with 0.5 per cent hypochlorite solution for 5 min and then washed with sterile distilled water (Jayasree *et al.*, 1999). Immature anthers were dissected out and cultured on modified callus induction medium (Murashige and Skoogs, 1962) containing 2,4-D (2.0 mg/l), KIN (0.5 mg/l) and sucrose (3%). Cultures were incubated under darkness at 25 ± 2°C. Calli induced were then subcultured on embryo induction medium supplemented with glutamine (200 mg/l), casein hydrolysate (400 mg/l), α-napthaleneacetic acid (NAA) (0.2 mg/l), KIN (0.7 mg/l) and sucrose (7%).

Effect of GA_3 on embryo induction

Filter sterilized stock solutions (100 mg/l) of GA_3 at final concentrations of 0.0 – 10 mg/l with an increment of 1.0 mg/l were added to the above mentioned basal medium after autoclaving. Calli were subcultured on the GA_3 supplemented media and maintained in darkness at 25 ± 2°C. Efficiency of embryogenesis was calculated on the basis of embryos produced after seven weeks of subculture.

Influence of GA_3 on embryo germination

Mature embryos which originated from medium containing 2.0 mg/l GA_3 were transferred to plant regeneration medium containing 3 per cent sucrose and 0.2 per cent activated charcoal (Jayasree *et al.*, 1999). The effect of exogeneous GA_3 on germination was tested by adding filter-sterilized GA_3 at 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l to the autoclaved medium. Cultures were incubated at 25 ± 2°C under 16 h photoperiod (40 $mE/m^2/s$). Efficiency of germination was calculated by scoring the greening of cotyledon, formation of the primary root and shoot followed by the emergence of a leaf. In order to study the effect of GA_3 on co-autoclaving with the medium, in separate experiments GA_3 (1.0, 2.0 and 3.0 mg/l) was added to the medium before autoclaving.

All media were adjusted to pH 5.6, gelled with 0.25 per cent gelrite and autoclaved for 10 min at 120°C. The experiments were repeated thrice with 3 replications for each treatment and data were subjected to analysis of variance. Regenerated plants were transplanted to small polybags initially and then to larger polybags (35 x 65 cm). Hardened plants were maintained under shade till field-planting.

RESULTS AND DISCUSSION

Effect of GA_3 on embryo induction

Calli were induced on callus induction medium containing 2,4-D and KIN after 35 days of culture. Upon subculturing these calli to embryo induction medium, discolouration of callus was noticed initially followed by production of friable embryogenic calli and emergence of embryoids (Fig. 1).

Table 1. **Influence of gibberellic acid on embryo induction**

GA_3(mg/l)	Mean number of embryos		
	Normal	Abnormal	Total
0	24	0	24
1	31	0	31
2	46	2	48
3	27	4	31
4	18	5	23
5	11	5	16
6	5	9	14
7	3	6	9
8	1	4	5
9	0	2	2
10	0	0	0
VR	82.74**	10.90**	76.17**
CD (P=0.05)	4.95	2.55	4.92

The present study revealed the stimulatory effect of GA_3 at lower levels (Table 1). Though embryo induction occurred even in the absence of GA_3, it was higher at 1.0 mg/l and maximum at 2.0 mg/l (Fig. 2). A progressive reduction was noticed

Table 2. **Effect of gibberellic acid on germination of embryo**

GA_3 (mg/l)	Cotyledon greening (%)	Root formation (%)	Embryo germination (%)
0	100	100	29.22
1	100	100	39.56
2	100	100	41.78
3	100	100	46.56
4	100	100	50.22
5	100	100	52.33
VR			77.16**
CD (P=0.05)			2.66

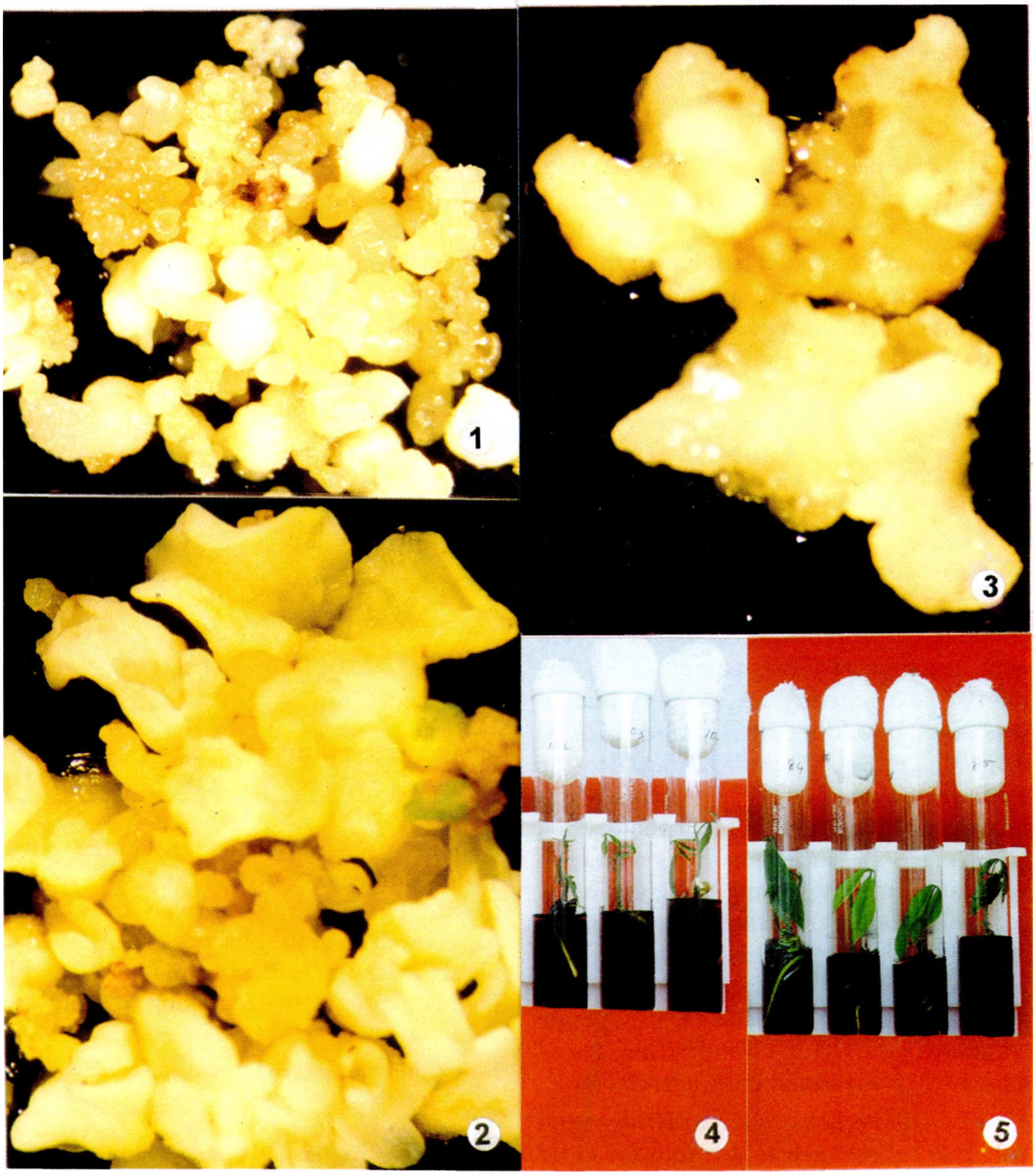

Fig.1-5. Embryogenesis and plant conversion in *Hevea* : 1. Emerging of embryoids from friable callus; 2. Differentiation of various stages of embryos; 3. Embryos with morphological abnormality; 4. Germinated somatic embryos; 5. Fully developed plants.

above 2.0 mg/l with cessation of embryogenesis at 10.0 mg/l. Morphological abnormality (Fig. 3) was frequently noticed in all treatments above 1.0 mg/l. When the level exceeded 5.0 mg/l, the number of abnormal embryos was higher than normal. For somatic embryogenesis, GA_3 at low levels (0.5 to 2.0mg/l) for sandal wood (Sita *et al.*, 1979; Sita, 1986) and 1.0 mg/l for fennel petioles and *Eryngium foetidum* (Hunault and Maatar, 1995; Ignacimuthu *et al.*, 1999) has been found to be optimum. However, enhancement in somatic embryogenesis at 34.6 mg/l for spinach (Xiao and Branchard, 1993) and at 10 mg/l for *Rumwex acetosell* (Culafic *et al.*, 1987) has also been reported. In contrast, GA_3 appears to suppress embryogenesis in carrot (Tisserat and Murashige, 1977a & b), soybean (Phillips and Collins, 1981), citrus (Kochba *et al.*, 1978) and spinach (Zdravkovic and Neskovic, 1999).

Effect of GA_3 on germination and plant regeneration

Upon transferring of embryos to hormone-free medium, 29 per cent embryo germination occurred (Jayasree *et al.*, 1999). Germination frequency increased with increasing GA_3 concentrations (Table 2), however, low level (2.0 – 3.0 mg/l) was found as optimum for both germination and subsequent full plant recovery (Figs. 4 & 5). The embryo germination was more than 50 per cent at 5.0 mg/l which was statistically on par with that at 4.0 mg/l. But full plant development was affected at these concentrations. Though cotyledon greening and root formation was independent of GA_3 levels, shoot development and leaf appearance were dependent. Retardation of growth at higher concentration of GA_3 was accompanied by the arresting of shoot elongation and appearance of leaf senescence. In most cases, new leaf development from these plants was delayed or inhibited eventually resulting in the death of the plant. If higher concentrations of GA_3 was supplied, transfer of germinated embryo to hormone-free medium was found as very essential for further development of plants (data not shown). This suggests that once shoot apex is differentiated, GA_3 is no longer necessary for further growth. It has been reported that in *Eschscholzia californi*, addition of GA_3 (3.0 mg/l) though led to an increase in conversion frequency, resulted in moderate to severe hyperhydricity (Park and Facchini, 1999). The growth suppression of germinated embryos of *Hevea* may also be due to the hyperhydricity. Embryos showing morphological abnormality were also able to germinate in GA_3 containing medium. GA_3 has been reported to enhance germination of somatic embryos in *Runnex acetosella* (Culafic *et al.*, 1987), in grapevine (Mullins and Srinivasan, 1976; Rajasekaran and Mullins, 1979) and in *Panax ginseng* (Chang and Hussing, 1980; Choi *et al.*, 1999). Although the exact mechanism is not fully clear, the ultrastructural studies carried out by Choi *et al.* (1999), in ginseng, showed that somatic embryos developed *in vitro* may be dormant after maturing and thus required a dormancy-breaking treatment.

GA_3 is a heat sensitive component that can be partly destroyed during autoclaving (Henderson, 1960). In the present study, no significant difference was noticed between addition of filter-sterilized GA_3 to the autoclaved medium and co-autoclaving of GA_3 for the induction of embryogenesis. Although reduction in the embryo induction was observed at lower concentrations, a slight enhancement was noticed with autoclaved GA_3 at 3.0 mg/l (Table 3). However, germination was lower with autoclaved GA_3 at all concentrations. The marginal reduction in both embryo induction and germination in autoclaved medium may be due to the partial degradation of GA_3 during autoclaving. Hunault and Maatar (1995) also observed that autoclaved GA_3 was

Table 3. **Effect of methods of sterilization of GA_3**

GA_3 (mg/l)	Embryo induction (Mean)		Embryo germination (%)	
	Filter-sterilized	Autoclaved	Filter-sterilized	Autoclaved
1.0	31.89	30.89	40.11	35.56
2.0	48.22	45.00	42.56	40.33
3.0	32.22	36.44	47.22	44.56
CD (P=0.05)	1.85	NS	1.96	1.60
CD (interaction)	2.61	NS		

as effective as filter-sterilized, for somatic embryogenesis of fennel.

In conclusion, lower levels of GA_3 was observed to foster induction and germination of somatic embryos and subsequent development of full plants. Slight reduction in response was observed for both induction and germination of embryos by autoclaving GA_3.

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