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INFLUENCE OF GROWTH REGULATORS AND SUCROSE ON SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM IMMATURE INFLORESCENCE OF *HEVEA BRASILIENSIS*

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A method for somatic embryogenesis and plant regeneration from immature inflorescence of *Hevea brasiliensis* , clone RRII 105 is described. The influence of different growth regulators and sucrose at various stages of this pathway has also been investigated. Among the different growth regulators tested for callus induction, synergistic effect of the two auxins 2,4-D and NAA was observed. Optimum growth regulator combination for callus induction was found to be 2,4-D / NAA / KIN (4.5 /2.7 /2.3 µM). Embryo induction was favoured by both BA and zeatin in the presence of low levels of NAA or 2,4 -D. Significant increase in embryo induction frequency was observed in the presence of GA_3. A higher sucrose level was found to be essential for effective embryo induction as well as maturation. ABA did not show any positive effect on embryo maturation, eventhough lower levels of ABA in the maturation medium enhanced proliferation and further embryogenesis. Plant regeneration frequency was found to be higher on media fortified with BA and GA_3. Lower sucrose level was found to be beneficial for plant regeneration.

Key words : Growth regulators, *Hevea brasiliensis*, Plant regeneration, Somatic embryogenesis, Sucrose.

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INTRODUCTION

Although considerable progress has been made for rubber tree (*Hevea brasiliensis*) improvement during last few decades, the narrow genetic diversity available within the *Hevea* gene pool is a major constraint for conventional breeding. Moreover, long breeding cycle and heterozygous nature of this perennial tree crop make it imperative to seek unconventional methods including gene transfer technology for crop improvement. An efficient plant regeneration system is one of the prerequisites for the feasibility of genetic manipulation studies in *Hevea*. Plant regeneration via somatic embryogenesis has recently become an attractive tool for the production of transgenic plants, because of its single cell origin.

Experiments on somatic embryogenesis of *H. brasiliensis* started as early as the 1970s. Successful plantlet production through somatic embryogenesis was

reported by Wang *et al.* (1980) from anther wall callus. Later Carron *et al.* (1989) reported the formation of somatic embryos from the inner integument of immature fruits of *Hevea*. Since then, several studies have been carried out by different workers on various aspects of somatic embryogenesis of *Hevea* (Asokan *et al.*, 1992 a, b; Etienne *et al.*, 1991, 1993; Montoro *et al.*, 1993). Recently Jayasree *et al.* (1999) described somatic embryogenesis and plant regeneration from immature anther cultures of *H. brasiliensis* (clone RRII 105, the most popular and high yielding Indian clone). Based on experiments involving four different clones, Carron *et al.* (1995) reported that the embryogenic efficiency and performance are highly genotype specific in *Hevea*. According to Montoro *et al.* (1993), the structure of calli and their morphogenetic capacities are not strictly specific to the genotype but rather to a genotype X medium interaction. These studies have revealed the necessity to optimize the culture conditions in order to achieve somatic embryogenesis in each genotype of *Hevea*. Several factors such as the developmental stage of the explant, quantity of growth regulators, basal media components, light intensity etc. appear to play crucial roles in the induction and maintenance of somatic embryogenesis in many plant species including *Hevea*. Sucrose is known to have profound influence on somatic embryogenesis of various species (Ammirato, 1983). The role of auxins and cytokinins during different stages of somatic embryo development has also been reported as critical in many species (Komamine *et al.*, 1992). Immature inflorescence has proved to be a good explant source for successful somatic embryogenesis and plant regeneration in several crops such as sorghum (Boyes and Vasil, 1984), wheat (Ozias-Akins and Vasil, 1982), oil palm (Teixeira *et al.*, 1994) and coconut (Verdeil *et al.*, 1994) because of its juvenile nature. So far, there has been no report of somatic embryogenesis from immature inflorescence of *Hevea*. This paper reports, for the first time, whole plant regeneration via somatic embryogenesis of *H. brasiliensis* by utilizing immature inflorescence as the explant source. Parameters like the nature and concentration of various growth regulators as well as sucrose concentration at different stages of the pathway were optimized.

MATERIALS AND METHODS

Plant material and culture media

Immature inflorescences (Fig. 1a) were collected from mature trees of *Hevea brasiliensis* (clone RRII 105). The explants were washed in running tap water for 10 minutes and then surface sterilized with 0.1% (w/v) mercuric chloride solution containing two drops of Tween 20 for three minutes followed by several rinses in sterile distilled water. The basal medium used throughout the experiment was M S (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968) and 5% coconut water. Activated charcoal (0.2%) was included in all media except in callus induction medium.

Effect of growth regulators

Callus induction

Callus inducing ability of five different auxins namely 2,4 - dichlorophenoxyacetic acid (2,4 - D), 1 - Naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA), Indole-3 - acetic acid (IAA) and 4 - amino-3,5,6 - trichloropicolinic acid (picloram) were evaluated using various concentra-

tions ranging from 1.0 - 20.0 µM . Among the five auxins used, 2,4-D and NAA in the range 2.5 - 9.0 µM and 1.0 - 5.0 µM respectively were selected for further optimization individually as well as in combination with N-6-benzyl adenine (BA) or kinetin (KIN) (2.3 and 4.6µM). Four explants (2-3 mm in length) were placed into individual culture tubes and subculturing was done at 3 week intervals. Percentage of callus induction was evaluated in all the experiments.

Embryo induction

Various combinations of the different growth regulators namely, 2,4-D, NAA, BA, KIN, zeatin (ZEA) and giberellic acid (GA_3) at concentrations ranging from 1.0 - 10.0 µM were tested in order to optimize the most suitable combination for effective embryo induction. Approximately 100 mg of friable callus was transferred to individual tubes and the cultures were subcultured once in a month. Percentage of embryo induction was assessed after 3 months in culture.

Embryo maturation

Embryo clusters from the embryo induction medium were transferred to the maturation medium containing different concentrations of abscisic acid (ABA) (0.1-10.0 µM). Observations were recorded after one month of culture.

Embryo germination and plant regeneration

Mature embryos were cultured on media supplemented with various concentrations of BA, KIN and GA_3 (0.5 - 25.0 µM) or without any growth hormones for embryo germination and plantlet development. Subculturing was done at 3 week intervals.

Effect of sucrose

To study the effect of sucrose concentrations on somatic embryogenesis, 58 - 350 mM sucrose were incorporated into the media with the optimized growth regulator combinations for various stages.

In all the experiments twenty replicates were prepared for each combination and each experiment was repeated thrice. The pH of the medium was adjusted to 5.6, solidified with 0.2% phytagel and autoclaved at 121°C for 15 min. The growth regulators namely, KIN, GA_3, ZEA and IAA were filter sterilized and added to the medium after autoclaving. Cultures were grown in 2.5 x 15 cm glass culture tubes with 20 ml nutrient medium per tube and incubated at 25°C in a growth room either in continuous dark for callus and embryo induction or under cool, white fluorescent light (40 µE/m^2/s) under a 16 h photoperiod for embryo maturation and plant regeneration. Regenerated plants were transferred to 175 ml glass jars containing 60 ml of the same medium for further growth and development. For hardening, well developed plantlets were transferred to small polybags containing soil mix and maintained in the green house for about 1 to 2 months for hardening following which they were transplanted to large polybags, kept under shade and later established in the field. The data were analysed statistically using completely randomized design with two factors.

RESULTS AND DISCUSSION

Growth regulators

Callus induction

The effect of various auxins on callus induction is illustrated in Table 1.

Table 1. **Effect of different auxins on callus induction from immature inflorescence of *H. brasiliensis***

Auxin (μM)	Picloram	2,4-D	IBA	NAA	IAA
0.0*	—	—	—	—	—
1.0	—	—	—	—	—
2.0	—	+	—	+	—
4.0	—	++	+	+	—
5.0	—	++	+	+	+
10.0	—	+++	+	++	+
15.0	—	+++	++	++	+
20.0	—	++++	++	++	+

Callus rating was done as
— = No callus induction
\+ = 0-20% callus induction
++ = 20-40% " "
+++ = 40-60% " "
++++ = 60-80% " "
* Twenty replicates per treatment and each treatment was repeated thrice.

Picloram, at all concentrations tested, was totally ineffective in callus initiation, but explant swelling was observed in its presence. Soft and translucent callus was produced on medium containing IBA or IAA, whereas, hard callus was developed in the presence of NAA (10 - 20 μM). However, they were found to be non-embryogenic. Although compact callus was induced in lower concentrations of NAA (2.0 - 5.0 μM), the proliferation rate was low. On the other hand, 2,4-D at 5.0 to 10.0 μM produced highly proliferating, friable callus. Callus induced at higher concentrations of 2,4-D (above 15.0 μM) was hard and nonembryogenic. A factorial experiment with 2,4-D and NAA at concentrations 2.3 to 9.0 μM and 1.0 to 5.0 μM respectively was tried in the presence of 2.3 and 4.6 μM of BA or KIN. A synergistic effect of these two auxins was observed, the maximum being at 4.5 to 7.0 μM 2,4-D and 2.7 μM NAA in the presence of 2.3 μM KIN (Table 2). The other level of KIN and both

Table 2. **Combined effect of 2,4-D and NAA along with 2.3 μM KIN on callus initiation**

		A				
	NAA (μ M)	2,4-D (μM)				
		2.3	4.5	7.0	9.0	Average
	0	8.33	11.66	13.33	18.33#	12.91*
	1.0	15.00	33.33	40.00	38.33	31.66
B	2.7	25.00	56.66	55.00	48.33	46.24
	4.3	16.66	46.66	41.66	40.00	36.24
	5.0	15.00	31.66	33.33	30.00	27.49
Average		15.99	35.99	36.66	34.99	

A - **, B - **, AB - ** ** indicates significance for P≤ 0.01 CD (A) = 2.44 CD (B) = 2.72 CD (AB) = 5.45
- Percentage of compact, nodular callus induced after 8 weeks in culture.
* Values are average of twenty replicates and each experiment was repeated thrice.

Table 3. **Effect of various cytokinins and GA_3 on embryo induction in combination with 1.0 µM NAA**

Cytokinin (µM)	GA_3 (µM)				
	0	1.45	2.9	5.8	Average
N-6-benzyl adenine					
1.1	1.66	13.33	20.00	8.33#	10.83*
2.2	5.00	25.00	21.66	8.33	14.99
4.4	6.66	30.00	31.66	13.33	20.41
8.8	3.33	16.66	15.00	6.66	10.41
Average	4.16	21.24	22.08	9.16	
	A - **; B - **; AB-NS; CD (A) =3.61; CD (B)=3.61				
Zeatin					
1.2	8.33	16.66	11.66	11.66	12.07
2.3	6.66	26.66	30.00	13.33	19.16
4.6	11.66	33.33	28.33	11.66	21.24
9.2	10.00	16.66	15.00	8.33	12.49
Average	9.16	23.33	21.24	11.24	
	A - **; B - **; AB - **; CD (A) = 3.09; CD (B) = 3.09; CD(AB) = 6.18				
Kinetin					
1.2	3.33	5.00	8.33	6.66	5.83
2.3	5.00	5.00	10.00	6.66	6.66
4.6	6.66	11.66	11.66	5.00	8.74
9.2	5.00	3.33	10.00	5.00	5.83
Average	4.99	6.24	8.74	7.0	
	A - NS; B - NS; AB - NS				

- Percentage of embryo induction; * Values are average of twenty replications and each experiment was repeated thrice. ** indicates significance for $P \leq 0.01$

the levels of BA did not show any such effect. A combination of 2,4-D/NAA/KIN (4.5/2.7/2.3 µM) was found to favour induction of creamy white, compact, nodular calli with embryogenic potential. About 50% of the explants responded positively and profuse calli were formed within 2 to 3 months of culture.

Embryo induction

Somatic embryo induction started with the appearance of yellow, friable, embryogenic callus (Fig. 1b) which later turned into clusters of globular pro-embryos (Fig. 1c). Evaluation of different experiments at this stage was done by determining the percentage of cultures showing embryogenesis which was calculated as the number of tubes giving rise to embryogenic calli as well as pro-embryo clusters in relation to the total number of tubes cultured. Table 3 shows the effect of various growth regulators on embryo induction. Addition of GA_3 (1.45 - 2.9 µM) strongly stimulated the embryogenesis process. Percentage of cultures showing embryos in the presence of KIN was significantly lower compared to the other two cytokinins. Response of cultures on media fortified with BA or ZEA was more or less similar. Statistical analysis showed that the interaction between ZEA and GA_3 on embryo induction was significant whereas the combination of BA and GA_3 was not statistically significant. ower levels of NAA or 2,4-D (1.0 µM) was found to be essential for embryo induction (Data not shown).

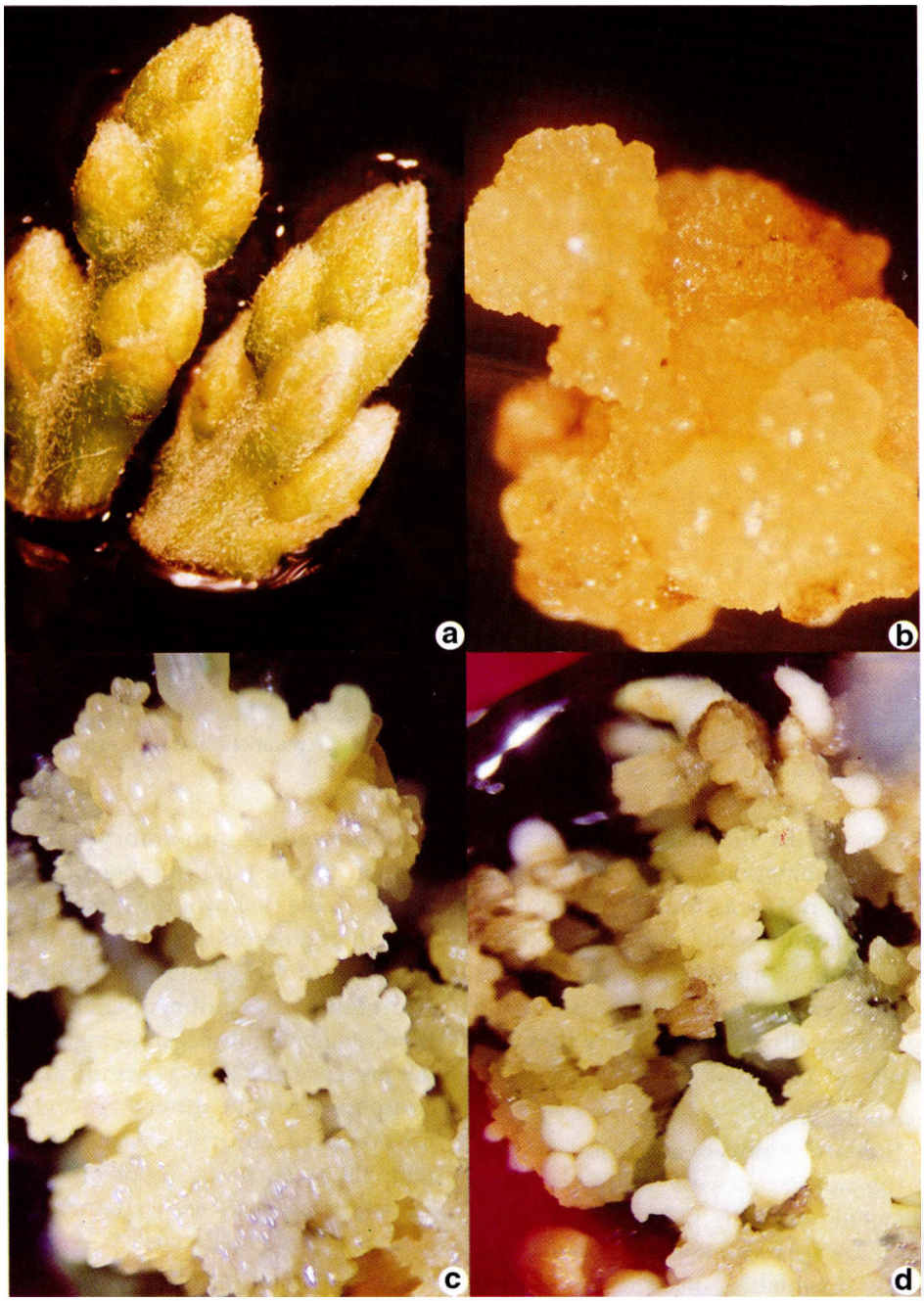

Fig. 1. Somatic embryogenesis from immature inflorescence of *Hevea brasiliensis* : a. Immature inflorescence used as initial explant; b.Embryogenic callus formation; c. Clusters of globular embryos emerged from the embryogenic callus; d. Mature embryos (white) on the maturation medium.

Embryo maturation

The effect of various levels of ABA on embryo maturation is depicted in Table 4. Hormone-free medium was used as the control. Percentage of maturation was calculated as the number of embryos belonging to the cotyledonary or late torpedo stage (Fig. 1d) against the total number of embryos present in the medium. ABA was observed to have no significant effect on the maturation of the embryos. The number of mature embryos recovered from control cultures as well as from cultures supplemented with various levels of ABA was almost similar. However, in contrast to the hormone-free control medium, on media fortified with lower levels of ABA (0.01- 0.1 µM) proliferation of the cultured mass yielding more embryogenic callus and embryo clusters was observed. Higher ABA levels (above 0.3 µM) adversely affected the development of the embryogenic clusters by inhibiting their further growth and finally leading to senescence.

Table 4. **Effect of ABA on maturation of somatic embryos**

ABA(µM)	Maturation (%)
0.00	10.63*
0.01	10.04
0.03	10.22
0.05	12.09
0.1	7.24
0.3	4.55
0.5	0.00
1.0	0.00

CD = 6.04 ; VR = 5.73**

* Values are average of twenty replicates and each experiment was repeated thrice.

** indicates significance for P ≤ 0.01

Embryo germination and plant regeneration

The mature somatic embryos belonging to the cotyledonary/late torpedo stage germinated on the regeneration medium, showing normal development with bipolar organization (Fig. 2a) eventually resulting in normal plantlets (Fig. 2b). Conversion frequency of somatic embryos into plants was calculated based on the total number of well developed plants obtained in each treatment in relation to the total number of mature embryos cultured. Frequency of plant regeneration was low on hormone free medium whereas the highest frequency (around 36%) was achieved with the combination of 8.8 µM BA and 1.45 µM GA_3 (Table 5). Rest of the embryos exhibited

Table 5. **Effect of various levels of GA_3 and BA on germination of mature embryos**

GA_3(µM)	BA (µM)						
	0	0.88	2.2	4.4	8.8	17.6	Average
0	3.33	6.66	3.33	10.00	6.66	3.33 #	5.55
0.29	3.33	10.00	13.33	13.33	16.66	6.66	10.55
0.58	6.66	13.33	16.66	26.66	30.00	13.33	17.77
1.45	6.66	13.33	23.33	33.33	36.66	16.66	21.66
2.9	3.33	6.66	10.00	30.00	26.66	20.00	16.10
5.8	0	3.33	13.33	16.66	23.33	13.33	11.66
Average	3.88	8.88	13.33	21.66	23.33	12.21	

A - **, B - **, AB - * ; CD (A) = 3.84 CD (B) = 3.84; CD (AB) = 9.39

* indicates significance for P ≤ 0.05 ; ** indicates significance for P ≤ 0.01

\# - Percentage of plant regeneration

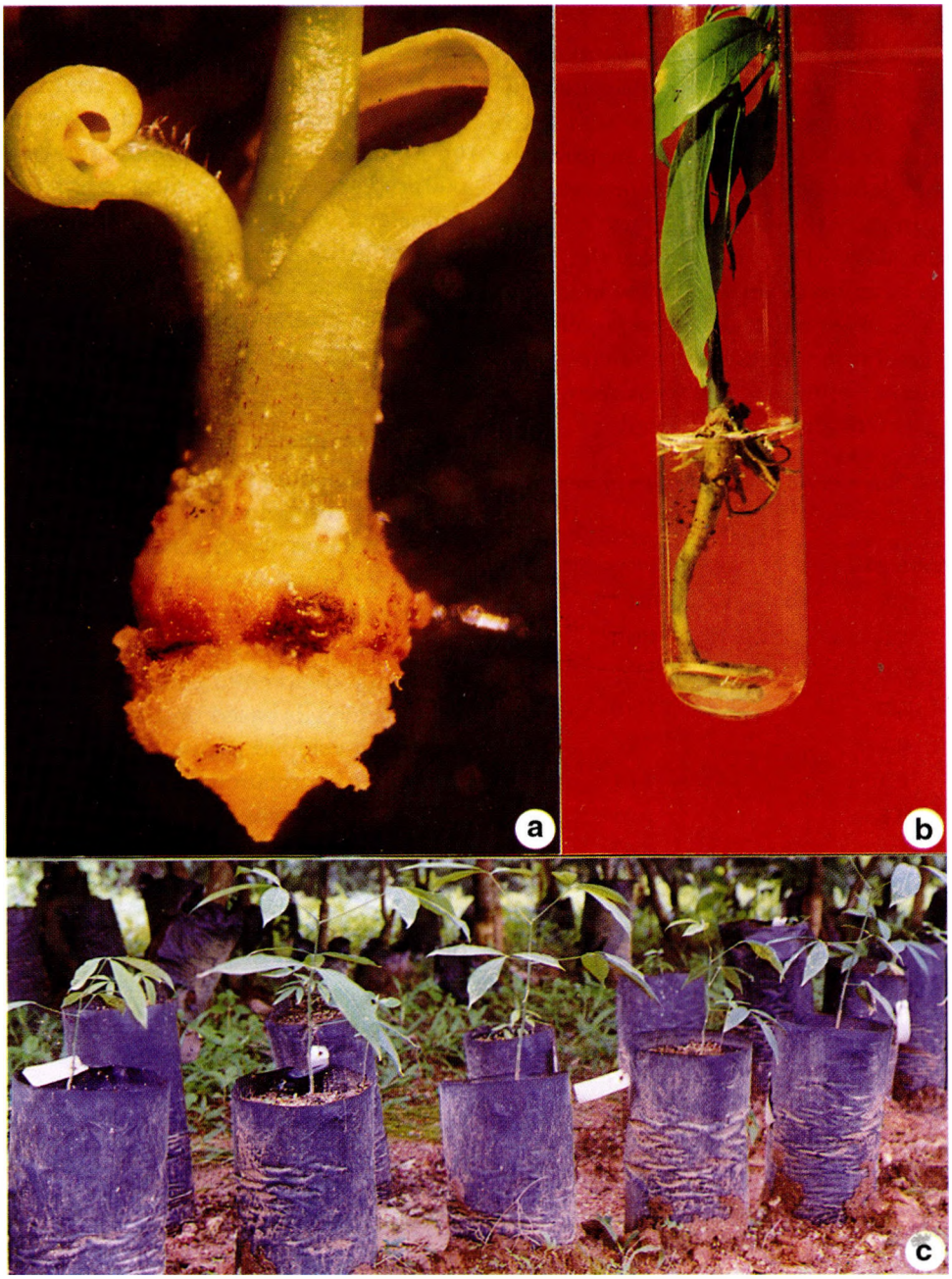

Fig. 2. Germination and plant regeneration from somatic embryo: a. Germinating cotyledonary embryo showing bipolar differentiation; b. Full plantlet regenerated from somatic embryo; c. Acclimatised somatic embryo derived plants in polybags maintained in the shade house.

growth with distorted, fused or multiple cotyledons and non-extended hypocotyls or remained dormant and shootless with only roots. Regenerated plants got acclimatized and established well in polybags (Fig. 2c) within two to three months.

Sucrose

Figure 3 shows the influence of sucrose concentration at various stages of somatic embryogenesis pathway. Sucrose level had a significant effect on the induction of callus as well as embryos and also on embryo maturation and plant regeneration. A lower level of sucrose (87 mM) enhanced callus initiation whereas at higher levels the frequency declined. Embryo induction was increased with increase in sucrose concentration, the optimum being 234 mM. When the sucrose concentration was increased beyond 234 mM, the embryo induction frequency decreased. Maturation of embryos was considerably enhanced at higher sucrose concentrations (234-292 mM). The highest frequency of embryo germination was observed at 87 mM sucrose concentration, whereas it was decreased at higher levels of sucrose.

The present experiments have demonstrated that callus initiation from immature inflorescence of *H. brasiliensis* is strongly dependent on the nature and concentration of growth regulators. 2,4-D has been reported to be essential for callus formation from other explants of *H. brasiliensis*. The present study also confirms this observation. Moreover, it has been observed that the combination of 2,4-D and NAA induced nodular and

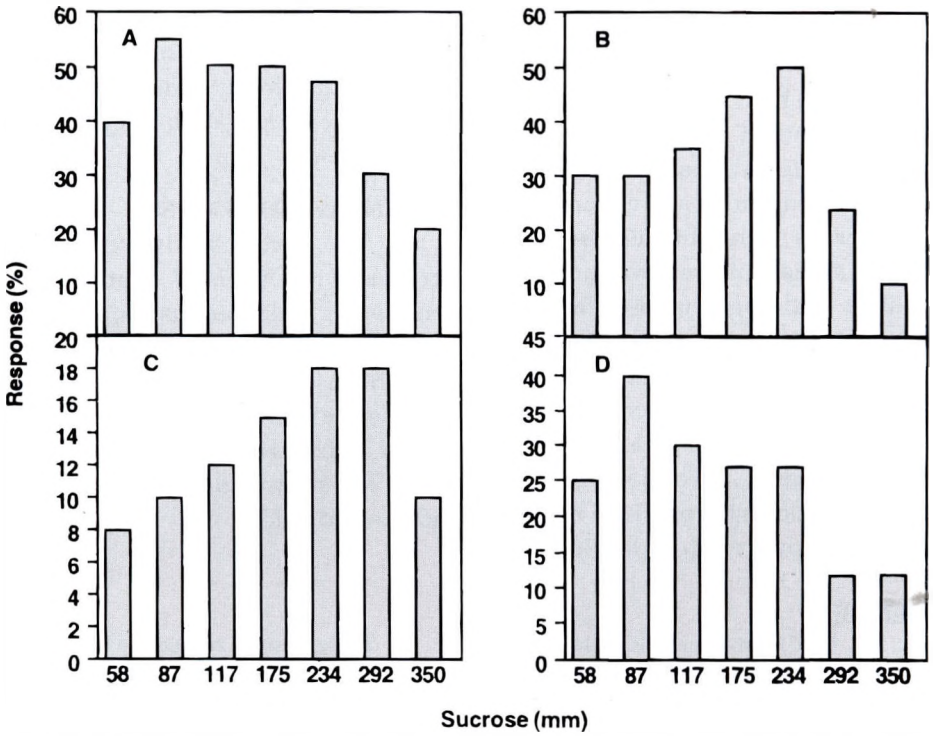

Fig. 3. Influence of sucrose on embryogenesis and plant regeneration : A. Callus induction; B. Embryo induction; C. Embryo maturation; D. Plant regeneration

compact embryogenic callus. Eventhough callus initiation is possible in the presence of the two auxins alone, supplementation of kinetin in the callus induction phase is absolutely essential for embryogenesis in the subsequent stages. Dedifferentiation medium described by Chen (1984) for anther culture also contained this growth regulator combination but at different concentrations. Stimulation of embryo induction by the addition of GA_3 in the embryo induction medium has been reported earlier (Chen *et al.*, 1984). In the present study, GA_3 (1.45 or 2.9 µM) not only enhanced the embryo induction frequency but also improved the normal growth of these embryos. Requirement of high sucrose levels for bringing about effective embryo induction and subsequent maturation has been established in this study. Linossier *et al.* (1997) observed enhancement of somatic embryo development in the presence of ABA and high concentration of PEG in *Hevea*. In contrast, in the present study, ABA was found to have no significant effect on embryo maturation. More number of mature embryos were obtained from embryogenic material cultured over hormone-free medium with high sucrose concentration (234-292 mM). The probable reason may be an osmotic or water stress imparted to the embryos at higher sucrose concentration. At lower sucrose levels in the maturation medium, precocious germination of the somatic embryos which retarded its further growth into plantlets was observed. Similar results were obtained in several other woody plants. A low concentration of sucrose in the maturation medium reduced somatic embryo development in *Picea abies* (Jain *et al.*, 1988). Elevated sucrose increased the rate of somatic embryo development of *Theobroma cacao* (Pence *et al.*, 1981) and *Pinus strobus* (Finer *et al.*, 1989).

CONCLUSION

Somatic embryo induction and further plant regeneration from immature inflorescence of *H. brasiliensis*, clone RRII 105, has been achieved. Various stages of this pathway are strongly dependent on the type and concentration of growth regulators as well as the concentration of sucrose. This method could be exploited for genetic transformation techniques aimed at crop improvement and can be extended to other genotypes also. After appropriate refinements, this system can be utilized for mass scale propagation of elite cultivars of *H. brasiliensis*.

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