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Effect of Hormones on the Organogenesis and the Somatic Embryogenesis of an Endangered Tropical Forest Tree - *Hildegardia populifolia* (Roxb.) Schott. & Endl.

T. Anuradha[1] and T. Pullaiah[1, 2]

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ABSTRACT: Nodal explants from aseptic seedlings of *Hildergardia populifolia* cultured on Murashige and Skoog's (MS) medium supplemented with 2 mgL^{-1} N^6-benzylaminopurine (BAP) generated very few shoots over a period of 30 days. Shoots were rooted *in vitro* on 1/2 strength MS macro salts containing 2 mgL^{-1} naphthalene acetic acid (NAA). A significant achievement in the present investigation was the production of somatic embryos from immature zygotic embryo explants exposed to MS medium supplemented with NAA 3 mgL^{-1} and casein hydrolysate (CH) 1000 mgL^{-1} over a period of 60 days. Subsequent withdrawl of NAA and CH from the medium resulted in the maturation and growth of embryos into plantlets on MS medium. The plantlets were transferred to sand: manure: soil mixture (1:1:1) where they showed no visual abnormalities in morphological characters.

KEY WORDS: ***Hildegardia populifolia*, Endangered tree, Somatic embryogenesis, Nodal regeneration.**

INTRODUCTION

Hildergardia populifolia (Roxb.) Schott & Endl. is a medium sized tree of the family Sterculiaceae confined to a few tropical deciduous forests of Tamil Nadu and Andhra Pradesh in India. The fibre extracted from the bark is used for domestic purposes and the leaf extract is known to have healing properties. As per the information given in the Red Data Book of Indian Plants (Nayar and Sastry, 1990), this narrow endemic species is under great threat due to factors not apparent at present. But it is assumed that anthropogenic interferences, habitat loss and other intrinsic and extrinsic factors might have accounted for their poor regeneration and low seed viability.

In vitro regeneration is an efficient means of *ex situ* conservation of plant diversity (Krogstrup *et al.*, 1992; Fay, 1994) because with this technology many endangered species can be quickly propagated and preserved from a minimum of plant material, and with little impact on wild population. Moreover this technique has the unique advantage of propagating the desired taxon, independent of season, reproductive barriers, germination hurdles and so on. Available literatures (Purohit and Dave, 1996; Pandey, 1998; Sunnichan *et al.*, 1998) reveals that *in vitro* protocol for this threatened taxon has not been elucidated so far and in response to the recommendations made in the Red Data Book of Indian Plants the present task of conserving the taxon has been carried out through two distinct *in vitro* regeneration approaches i.e. caulogenesis and somatic embryogenesis. The present work has great relevance in conservation point of view as it buffers and makes up for the loss.

1. Department of Botany, Sri Krishnadevaraya University, Anantapur 515 003, India.
2. Corresponding author.

MATERIALS AND METHODS

Dry follicles of *Hildergardia populifolia* were procured in the month of February during 1996-99 from Chendrayudikona hills in Anantapur district of Andhra Pradesh. The infertile seeds were discarded by water floatation method and the healthy seeds treated with conc. H_2SO_4 for 30 minutes followed by bruising of the seeds against a wire mesh so as to remove the outer charred hard seed coat. While for somatic embryogenesis, unripe seeds were collected 90, 120, 150 days after flowering. The dry seed as well as unripe seeds were surface sterilised with 0.1% $HgCl_2$ for 5 minutes followed by 3-4 rinses with sterile distilled water. The dry seeds were germinated in 1/2 strength MS medium (Murashige and Skoog, 1962) fortified with 0.5 mgL^{-1} Gibberillic acid (GA_3). The nodal explants from seedlings were cultured on appropriate semi-solid medium with their cut ends inserted in the medium. The immature embryos from the unripe seeds were carefully separated and placed on culture medium. The culture medium in both seedling explant cultures and immature embryo cultures consisted of MS salts supplemented with 4% (W/V) sucrose, a reduced nitrogen source such as Casein Hydrolysate (CH) and glutamine and various auxins (NAA, Indole acetic acid, IAA, Indole butyric acid, IBA, 2,4-dichlorophenoxy acetic acid, 2,4-D) and cytokinins (BAP and Kinetin, KN) at appropriate concentrations, both individually and in combinations. In addition various organic supplements were also provided to see their effect on morphogenesis. Effect of various carbon sources, glucose, sucrose, fructose and cane sugar (3%, W/V) - on embryo induction has been studied. Effect of different concentrations of sucrose (2%, 4%, 6%, 8% and 12%) on embryo induction has also been studied. All plant growth regulators and addenda were added to the medium before autoclaving. The pH of the medium was adjusted to 5.6-5.7, autoclaved at 108 kPa and 121°C for 15 min. A quantity of 20 ml medium was dispensed in rimless culture tubes (150 x 25 mm) closed with aluminium foils. Cultures were incubated under a 16-h photoperiod with a light intensity of 2000 lux at 25° ± 2° C. A minimum of 20 culture tubes were raised for each combination and all experiments were repeated thrice. The nature and percentage of response were recorded at an interval of 5 weeks. Subculturing was periodically carried out at 3-week interval. The regenerated shoots were isolated and subjected to rooting experiments. Shoots with an average length of 2 cm and above were excised from the explant and placed in half strength B_5 medium variously amended with IAA, IBA and NAA (0.1, 1, 2 mgL^{-1}) both individually and in combinations supplemented with 2-isopentyl adenine (2-ip).

RESULTS AND DISCUSSION

Nodes and shoot apex were the only explants amenable to shoot regeneration treatments. However, the number of shoots induced from both cotyledonary node and nodal explants was very low (Table 1). Among the two cytokinins employed, a somewhat better response was elicited by BAP (2 mgL^{-1}). KN, on the otherhand was less effective than BAP in inducing shoots. There was no improvement in shoot regeneraton efficiency by the combined presence of these two adopted cytokinins. A similar observation was made by Sunnichan *et al.* (1998) in *Sterculia urens*. Other hormone permutations i.e. BAP in combination with either NAA, IAA or 2,4-D induced a single shoot and promoted shoot elongation to a limited extent. The average length of shoots induced from cotyledonary node was more or less 1.5 cm; whereas that attained by cultured shoot apex was ca. 3 cm, while that from node was ca. 2 cm long. All the individual shootlets were excised and placed in fresh BAP (0.1 mgL^{-1}) medium for elongation. During this process, occasionally, a new shoot made its appearance from the node.

Table 1. Effect of BAP in combination with auxins (NAA, IAA and 2,4-D) on adventitious shoot regeneration from shoot-tip (St), nodal (N) and mesocotyl (MC) explants of *Hildedegardia populifolia*.

BAP (mg L^{-1})	Auxins (mg L^{-1})	NAA						IAA						2,4-D					
		1			2			1			2			1			2		
		St	N	MC	St	N	MC	St	N	MC	St	N	MC	St	N	MC	St	N	MC
0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	0	0	1±0
	0.50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	0	0	1±0
0.1	0.01	0	75	85	0	0.70± 0.01	1.12± 0.08	0	0	0	0	0	1.11± 0.08	0	65	75	0	1±0	1±0
	0.50	0	75	85	0	0.70± 0.10	1.07± 0.90	0	70	75	0	1.00± 0.00	1.11± 0.08	0	65	75	0	1±0	1±0
2	0.01	0	80	80	0	1.06± 0.06	1.07± 0.09	0	75	60	0	1.10 ± 0.02	1.11± 0.08	0	75	70	0	1±0	1±0
	0.50	0	80	80	0	1.06± 0.06	1.07± 0.09	0	70	65	0	1.06 ± 0.06	1,11± 0.08	0	50	70	0	1±0	1±0
5	0.01	0	70	65	0	1.00± 0.00	1.00± 0.00	0	65	65	0	1.00 ± 0.00	1.07± 0.07	0	70	65	0	1±0	1±0
	0.50	0	60	65	0	1.00± 0.00	1.00± 0.00	0	70	65	0	1.00 ± 0.00	1.00± 0.00	0	70	65	0	1±0	1±0

1: Percentage of adventitious shoot regeneration.
2: No. of adventitious shoots per explant.
(Values represent mean ± S.E.)

Shoots with an average length of 2 cm and above having two nodes were placed in 1/2 strength MS basal medium supplemented with NAA (2 mgL^{-1}) and 2-ip (0.5 mgL^{-1}) for rooting. However only 16% of the shoots could be rooted. Treatment with all other root inducing hormones i.e. IAA, IBA, failed to stimulate rooting at any concentration. Full strength MS medium supplemented with same combination of NAA and 2-ip, indicated previously, induced callus at the cut end of the shoots. The promotive effect on rooting of shoots by low salt and reduced sugar concentration has been reported by Purohit and Dave (1996) and Sunnichan *et al.* (1998) in *Sterculia urens*. The rooted shoots were transferred to soil. Of 35 plantlets transferred, 32 survived with subsequent establishment.

Immature embryos 2, 5 and 10 mm in length excised from seeds collected 60, 120, 180 days after flowering were cultured on MS, B_5 (Gamborg *et al.,* 1968) and Woody Plants Medium (Lloyd and McCown, 1981). None of these media in the absence of growth regulators could induce callus from the embryos. However, with the incorporation of growth regulators, callus induction was noticed (Fig. 1A). Initially, callus formation was confined to the radical end of the embryo but subsequently it over grew the white embryo. In most of the treatments, three callus morpho types were obtained.

When incubated on agar solidified MS medium supplemented with 3 mgL^{-1} NAA and 1000 mgL^{-1} CH, amidst brown cell aggregates, light green globular shining masses which later developed into somatic embryos were visible after about 8 weeks in culture. Based on various concentrations (1 - 10 mgL^{-1}) of NAA tested, 3-5 mgL^{-1} were found to be optimal for embryo induction (Table 2). Outside this range the cultures showed a tendency to produce non- embryogenic callus. Other auxins were not amenable for embryo induction (Table 3). Similarly of all the CH levels, 1000 mgL^{-1} was potentially inductive (Table 4). At lower concentrations of CH, less than 100 mgL^{-1}, all cultures altogether lost the tendency to form embroygenic callus itself. Among the various carbon sources, sucrose at 4% concentration was found to be better suited for embryo induction (Figs. 2B, C).

Among the auxins tested at 3 mgL^{-1} concentration, IAA gave the lowest and slowest callus production (Fig. 1C) (Table 3). IAA, induced compact nodular callus, as contrary to IBA which was also noticed in the studies on *Sterculia urens* (Sunnichan *et al.*, 1998) and *S. alata* (Pandey, 1998). In comparison with IAA, the callus resulting from IBA was very watery, sponge-like and unorganized. On the other hand with 2,4-D the callus was more compact than that with NAA, but growth was not as fast. As per the two cytokinins (BAP and KN) examined, both individually and in combination with IAA, the effect was not as distinct as the individual auxins, although, when used in combination with NAA, the calluses was rather friable. Here, as comparison the degree of friability KN was lesser than that with BAP and the embryos showed elongation at the radical end (Fig. 1B).

The results signify the embryogenic role of NAA and CH in *Hildegardia populifolia*. Rangaswamy (1986) reported that reduced form of nitrogen was absolutely essential, especially at the inductive phase of somatic embryogenesis. Reduced nitrogen in general and CH in particular allows vigorous organ development over a broader range of growth regulators (Murashige and Skoog, 1962). Wen and Kinsella (1991) reported a high incidence of somatic embryogenesis in *Theobroma cacao* on MS medium supplemented with NAA and CH. Gluatmine too, had the effect on embryogenesis, but to a lesser extent. However, the reports of Pandey (1998) emphasized the potentially embryogenic role of gluatmine in *Sterculia alata*. Other reduced nitrogen sources such as Yeast extract (YE), Malt extract (ME), Coconut milk (CM), could not be substituted for either CH or gluatmine (Fig. 2D). They were just able to improve quantitatively the formation of non-organogenic callus.

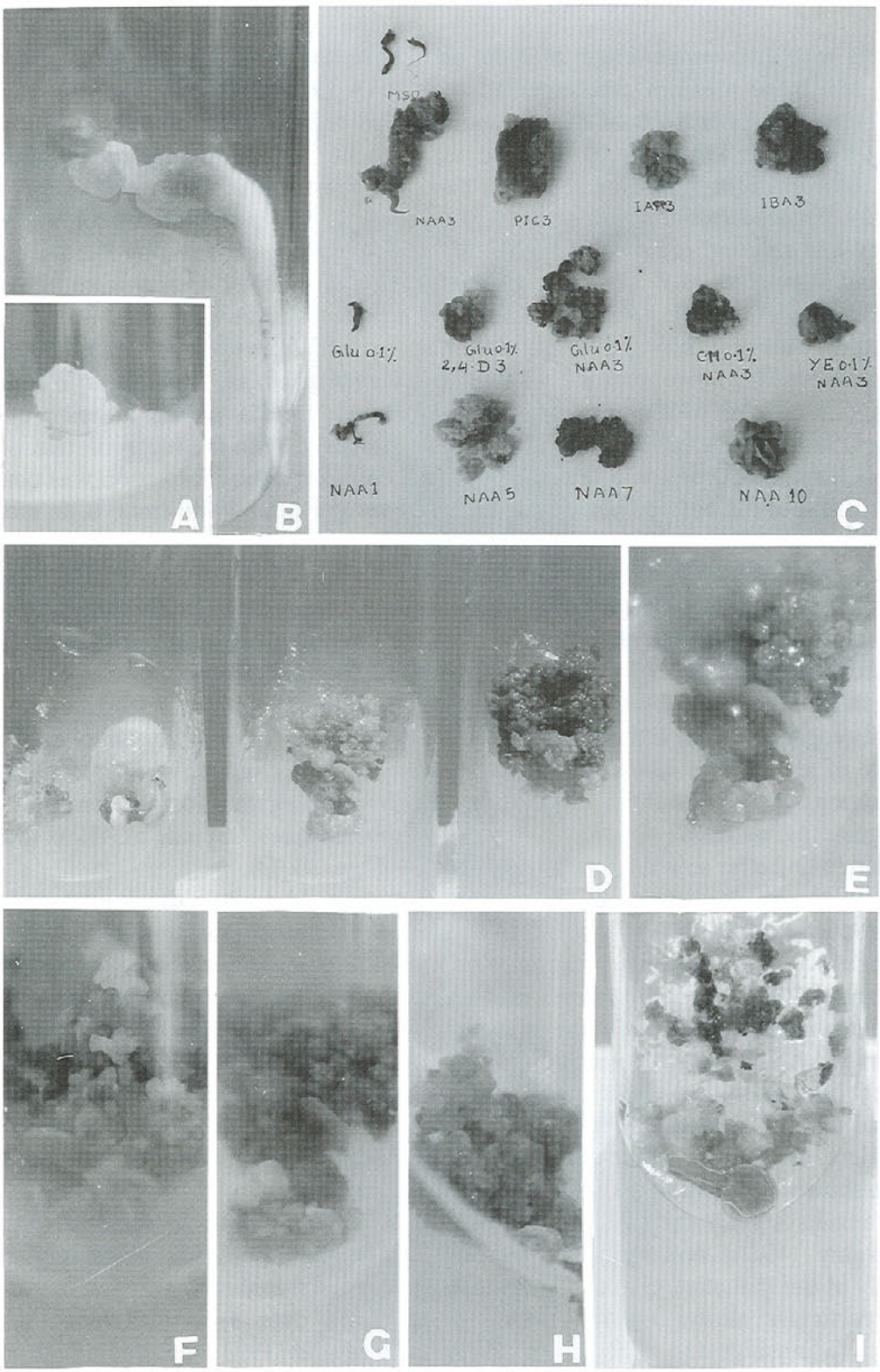

Fig. 1. Immature embryo culture and somatic embryogenesis in *Hildegardia populifolia*. A. Immature embryo in BAP (3 mgL^{-1}) + CH (0.1%) supplemented medium. B. Immature embryo in NAA (3 mgL^{-1}) + KN (3 mgL^{-1}). C. Response of immature embryos to different concentrations and categories of auxins and organic supplements. D and E. Developmental stages of embryogenic callus. F. Heart shaped somatic embryos. G. Recurrent embryogenesis from somatic embryo in induction medium. H and I. Somatic embryos showing asynchronous growth.

Table 2. Interaction of stage/length of explants with NAA concentrations on frequency of somatic embryogenesis from immature zygotic embryos of *Hildegardia populifolia.*

Conc. of NAA in mgL^{-1} + CH (0.1%)	% of response		
	Heart-shaped embryo (< 5 mm)	Early cotyledonary stage (5-7 mm)	Cotyledonary stage (> 7 mm)
1	0	0	0
2	5	5	0
3	10	30	5
4	10	25	0
5	15	25	0

CH: Casein Hydrolysate.

Table 3. Effect of different auxins and cytokinins on non-embryogenic callus formation from immature zygotic embryos of *Hildegardia populifolia.*

MS + CH 10 (0.1%) + Auxin at 3 mgL^{-1} conc.	Callus morphology	Callus scoring
IBA	Friable, watery, soft, creamish white callus	+
2,4-D	Less friable or compact pale brown	+++
IAA	Compact, shining surface pale yellow	+
ABA	Compact, hard or dry pale brown	++
KN	Compact shiny surface creamish	+
BA	Soft, watery or wet white-creamy callus	+

Callus induction frequency: + 1-20%; ++ 21 - 50%; +++ 51 - 80%.
Data scored after 2 months of culture.

Table 4. Effect of different concentrations of casein hydrolysate (CH) in combination with NAA (3 mgL^{-1}) on induction of somatic embryogenesis in *Hildegardia populifolia.*

Conc. of CH (mgL^{-1})	Callus browning	Embryo formation
0	Less	Not seen
250	Moderate	Unfrequent formation
500	Moderate	Sporadic formation
750	Abundant	Sporadic formation
1000	Abundant	Frequent development

In the inductive medium consisting of NAA (3 mgL^{-1}) and CH (1 gL^{-1}) the explant transformed to brown, rather heterogeneous callus within 3 weeks of incubation. Generally, two types of calluses were formed at the radical end of the responsive explant in induction medium. These were pale yellow, soft callus that differentiated light green embryos over a 60-day period amidst brown nodular callus that perpetually remained non-embryogenic. Occasionally, a third type of callus, white and translucent also appeared.

Usually, the different forms of callus formed at the radical end of the explant were thoroughly intermixed with each other. As a rule, every bit of nodular/friable callus was removed at the time of transfer to multiplication medium. Irrespective of the concentration of NAA, the 20 - 30% cultures showed only friable callus. Such cultures were discarded in the initial stage itself. This was necessary not only because friable callus never differentiated into embryos, but also because it multipled faster than embryogenic callus severely impairing the growth of the latter (Saxena and Dhawan, 1999).

Subcultures were performed at an interval of 4 weeks. Globular masses made their appearance by the end of 1st subculture itself. During successive subcultures, several organized greenish globular structures emerged (Figs. 1D-G). After about 8 weeks of incubation, some cell aggregates started to turn brown and slightly greenish globular structures began to emerge amidst the brown cell aggregates. Some of the globular structures developed a rudimentary root when these differentiated structures were subcultured onto a fresh basal medium. If, incubated on a fresh induction medium, a second generation of somatic embryos ensues (Figs. 1H, I). Occurrence of secondary somatic embryogenesis was also reported in onion (Eady *et al.*, 1998) but here a weekly subculture enhanced the frequency of embryo production.

Nearly 25-30% of the cultures formed embryogenic callus. Although callus became visible within 10-12 days of cultures, a substantial quantity of callus / embryos were apparent only in 8-week cultures. The number of embryos formed varied between 15-25. Initially embryos were globular in shape. They were pale or off-white in the early stages of development but gradually turned into green heart-shaped structures as they attained maturity. However, the growth of the embryos was clearly asynchronous, with embryos of apparently different developmental stages i.e. from globular - heart shape (Fig. 2A). Such an asynchrony in the growth of somatic embryos was also observed in grapevine (Jayasankar *et al.*, 1999) and several other perennial species (Litz and Gray, 1992).

A number of key parameters were identified during the course of this investigation. First is the culture duration in induction medium. If the cell aggregates were transferred into hormone free medium within 8 weeks i.e. before the globular structures were formed, embryoid development was poor. In addition, embryos did not germinate in the germination medium, unless they had a distinct embryo like appearance. On the otherhand, if the globular structures were left in the induction medium too long without subculturing, cell death occurred. The globular structure with a rudimentary root appeared to be the correct stage for transferring into the hormone free medium for further embryo development. Formation of embryo clusters typically occurred in about 25 to 30% of the cultures.

After the completion of each multiplication cycle, embryos at different stages of development were selected and processed according to their state of maturity. While immature embryos were transferred to a fresh multiplication medium, mature embryos were placed in germination medium consisting of half MS with GA_3 (0.5 mgL^{-1}) and gluatmine (250 mgL^{-1}). Any deviation in the selection process of embryos resulted in significant variation in the multiplication and germination rates.

Sometimes, a few embryos, less than 3% showed precocious germination on the multiplication medium itself and formed roots. Generally, here shoots were not well developed. At the end of the multiplication cycle, incipient embryo-derived plantlets of less than 1 cm were transferred to germination medium for further development. Similarly somatic embryos in grapevine germinated precociously and had to be transferred to germination medium for further development (Jayasankar *et al.*, 1999).

A number of studies have shown that abscissic acid (ABA) could induce normal embryo development (Wen and Kinsella, 1991; Pence, 1992; Bespalhok and Hattori, 1998). By contrast, in the present study the incorporation of ABA in the medium did not assist embryo development. Another important factor in embryo development was sucrose concentration. Some authors speculate that a higher osmotic concentration stimulates the endogenous ABA production (Kong and Yeung, 1995). However, in the present investigation higher levels of sucrose (Figs. 2E-G), which stimulate ABA production, proved inhibitory to the

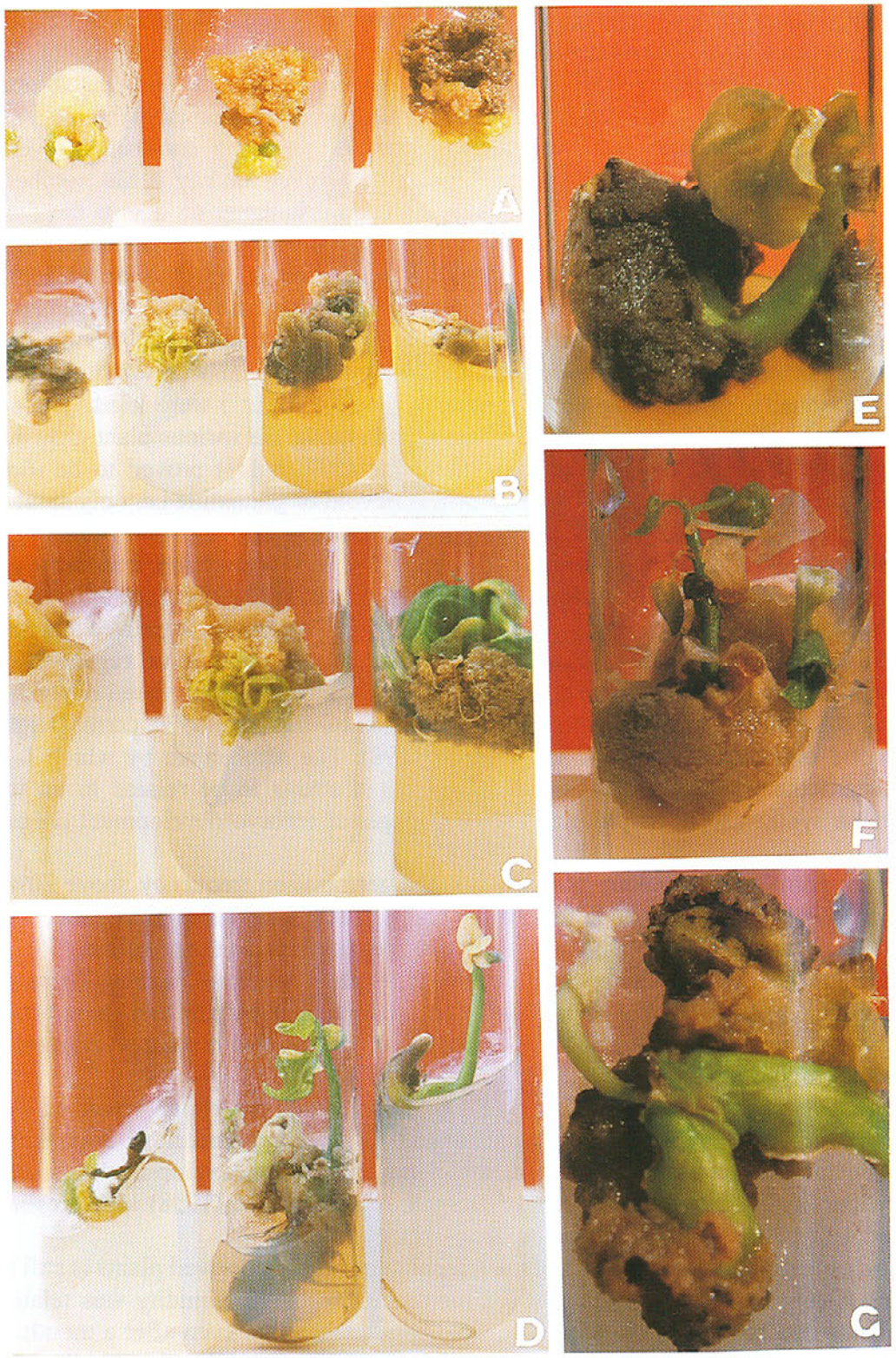

Fig. 2. Response of immature zygotic embryos of *Hildegardia populifolia* to different treatments and germination of somatic embryos. A. Developmental stages of embryogenic callus in NAA + CH supplemented MS medium. B. Effect of glucose, sucrose, cane sugar and fructose (3% conc.) on embryo induction. C. Sucrose concentration 2%, 4% and 6% on embryo induction. D. Effect of CM, CH and YE (0.1% conc.) on embryo germination. E-G. Abnormalities in somatic embryo conversion at 4%, 8% and 12% sucrose concentration.

developmental process of embryos which may presumably be due to the passive role of ABA in the development of somatic embryos. This is contradictory to the reports of *Theobroma cacao* (Wen and Kinsella, 1991; Pence, 1992) and *Tagetes erecta* (Bespalhok and Hattori, 1998), where ABA plays an active role in the maturation of somatic embryos.

An attempt has been made to produce artificial seeds by encapsulating embryos at cotyledonary state in sodium alginate (Fig. 3A). However, no/negligible number of these seeds were converted to plantlets (Fig. 3B). It may presumably be due to the fact that the embryos encapsulated might not have reached physiological maturation stage. Two 'bottlenecks' that have limited the use of encapsulated somatic embryos as artificial seeds are difficulty in concentrating and purifying embryos at a stage suitable for encapsulation; low conversion rates when encapsulated somatic embryos are planted in soil (Herman, 1995).

For the germination of embryos, the hormones under each class i.e. auxins, cytokinins and GA_3 individually, in the same concentration range (0.5 mgL^{-1}) were tried. The conversion frequency of somatic embryos was rather poor and among the various plant growth regulator therapy, half MS + GA_3 (0.5 mgL^{-1}) + glutamine (500 mgL^{-1}) proved to be ideal for the germination of embryos. When the medium was devoid of glutamine i.e. only stout shoot and root developed (Fig. 3C). Lateral roots did not develop on primary root. Similarly shoot did not grow beyond first pair of leaves (Fig. 3C). The low conversion frequency of somatic embryos has also been confirmed from the studies of Pandey (1998) in *Sterculia alata.*

The addition of *L*-glutamine at concentrations of 500 mg/L improved regeneration of somatic embryos (Fig. 3D). However, a decline in the number of regenerated somatic embryos was observed when the concentration of glutamine was increased beyond 500 mg/L (Fig. 3E). Similar results were obtained in *Geranium* (Gill *et al.,* 1993), carrot (Higashi *et al.*, 1996) and marigold (Bespalhok and Hattori, 1998). The mechanism by which *L*-glutamine stimulates embryo development is unknown, but it seems to be related to an increase in glutamine synthetase activity during the later stages of embryo development (Higashi *et al.*, 1996).

In the germination medium not only was the germination frequency above 20%, but also nearly 50% of these developed into healthy plants. After 4 weeks of incubation, the plantlets grew to a length of 5-7 cm with a well developed root system. In the remaining 50% either the conversion frequencies were low or the regenerated plants were not healthy. There were some which rooted vigorously, but their shoots were rudimentary and did not grow beyond 3 cm. These somaclonal variables might be attributed to the genetic mutation/variation along the course of embryo development.

Perhaps due to physiological immaturity, a few embryos either remained dormant or produced only roots, with rudimentary shoot on the germination medium. Such embryos with roots when transferred to fresh germination medium, failed to develop further, instead turned pale within a few days. The studies of Bespalhok and Hattori (1998) also proved the poor conversion of *Tagetes erecta* embryos into plantlets.

The estimation of survival rate of the transplanted embryo derived plants is still underway. The plantlets had a fresh look, provided the ambient relative humidity was relatively high. The plants that have been transferred to field appeared normal even after a month. However, only 7-10% of the embryo derived plants fall under this group. The plantlets derived from *de novo* shoots as well as somatic embryos, were transferred to soil:sand:manure mixture (1:1:1). These were hardened by gradual exposition to normal relative humidity. The plantlets followed the same pattern of development as those propagated in soil by seed. The shoots

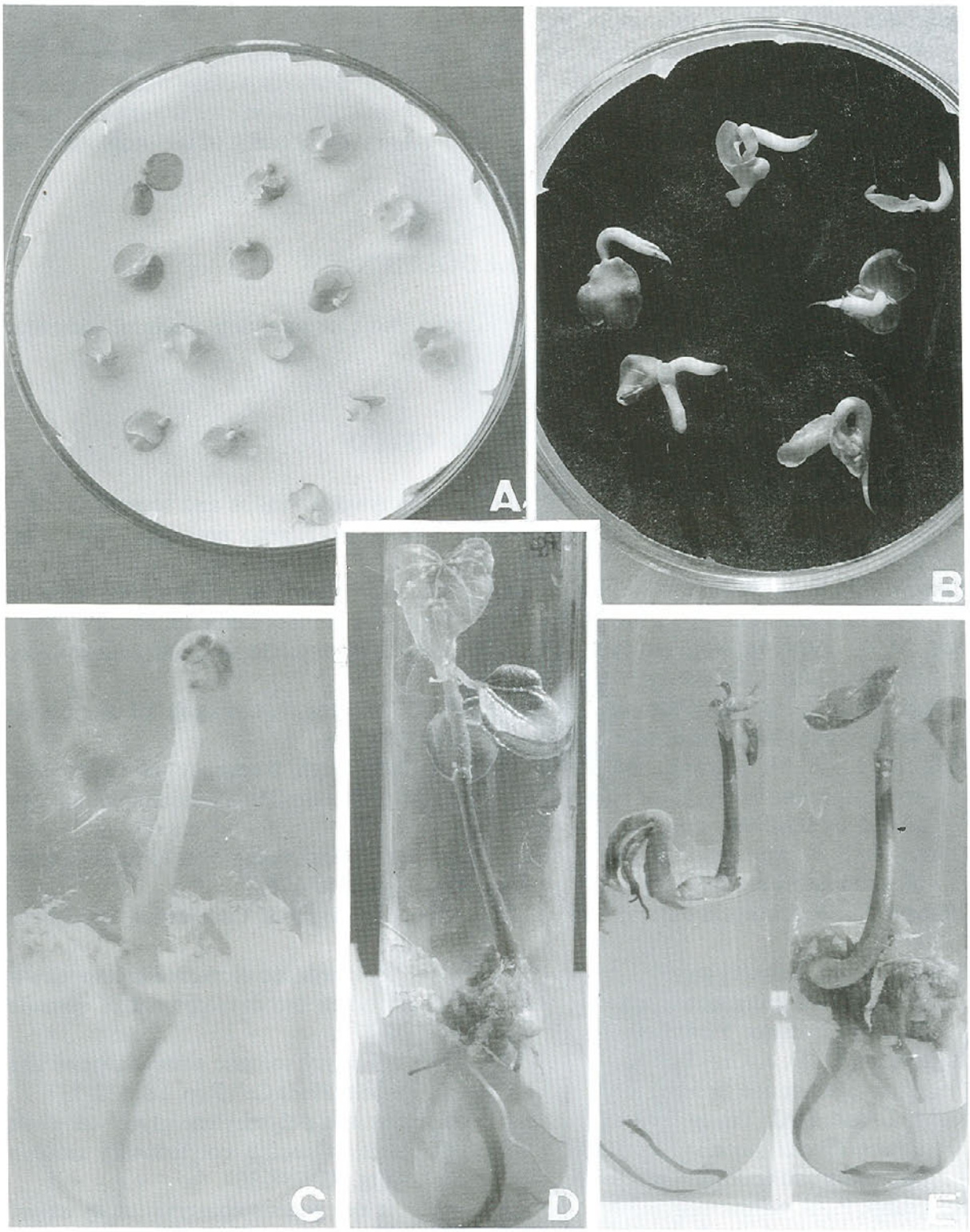

Fig. 3. Somatic embryos of *Hildegardia populifolia*. A. Encapsulated somatic embryos. B. Germinating somatic embryos in half MS + GA_3 (0.5 mgL^{-1}). C. Germinated somatic embryos in half MS + GA_3 (0.5 mgL^{-1}) medium. D. Somatic embryo in half MS + GA_3 (0.5 mgL^{-1}) + Glutamine (500 mgL^{-1}) medium. E. Somatic embryos in Glutamine 250 mg (left) and 1000 mgL^{-1} (right) containing media.

after two weeks showed leaf shedding. Later on i.e. one month after transfer, the shoots generated new leaves and grew normally.

Propagation by somatic embryogenesis has several advantages over organogenesis. The success of regeneration of *de novo* shoots depends on adventitious rooting which may be a constraint in many tree species. On the other hand, the multiplication rate is generally much faster via somatic embryogenesis and this method has proved valuable in the propagation of some forest trees (Gupta *et al.*, 1996).

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荷爾蒙對瀕臨絕種熱帶森林樹種 *Hildegardia populiforia* 的器官發生及體胚發育的影響

T. Anuradha[1] and T. Pullaiah[1, 2]

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摘　　要

從 *Hildegardia populiforia* 幼苗取其節的片段組織培養於含有 2 mgL^{-1} BAP 的 Marashige and Skoog's (MS)培養劑中，經 30 天後即有少數枝條的產生。這些枝條再培養於含有 2 mgL^{-1} NAA 並且已稀釋一倍的 MS 培養劑中，則可長出根來。若把不成熟的接合子胚的片段組織培養於含有 3 mgL^{-1} NAA 及 1000 mgL^{-1} 乳蛋白水解物(CH)的 MS 培養劑中，經 60 天後即可產生體胚。若是把 NAA 及 CH 從培養劑中去除，此體胚則可長成小樹，此小樹再移植於沙/糞肥/土(1:1:1)的混合土壤中生長，並沒有發現其外部型態的特徵有不正常之現象。

關鍵詞：*Hildegardia populiforia*，瀕臨絕種的樹種，體胚發生，節的再生。

1. Sri Krishnadevaraya 大學植物系，Anantapur 515 003，印度。
2.通信連絡員。