EFFICIENT PROTOCOL FOR DIRECT SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN KAEMPFERIA GALANGA L FROM LEAF-SHEATH EXPLANTS

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Abstract: *Kaempferia galanga* Linn. of *Zingiberaceae* is a rhizomatous handsome herb.It is a valuable and highly medicinal plant. It is cultivated for its aromatic rhizomes and is used extensively as spice throughout tropical Asia. Taxonomic and phylogenetic studies of South Indian *Zingiberaceae* revealed that *Kaempferia galanga* has become endangered, due to indiscriminate collection from natural habitat for ayurvedic preparation, deforestation and conversion of forest land to plantation crops. Tissue culture provides efficient techniques for rapid and large scale propagation and conservation of germplasm. The present study intends to establish an efficient protocol for direct somatic embryogenesis and plant regeneration in *Kaempferia galanga.* Leaf-sheath explants produced proliferative burst in the epidermis and the beginning of cellular segregation on 20th day of culture in the medium supplemented with BAP (0.1-2.0 mg/l) and NAA (0.1-2.5 mg/l) . Somatic embryos formed directly at the upper surface of the leaf-sheath when subcultured on the same medium . Highest frequency (80%) with average number of somatic embryos (22.34 ± 1.58) were formed at NAA (2.0 mg/l) and BAP (0.5 mg/l. Matured embryos were transferred to half MS medium containing BAP (1.0 mg/l) and NAA (0.4 mg/l) or KN (1.0 mg/l) and NAA (0.4 mg/l) for germination. From the fourth to fifth week up to 60% of somatic embryos germinated with the emergence of shoot first, then the roots. The germinated plantlets were hardened and transplanted in the soil.

Keywords: *Kaempferia galanga ,* Micropropagation, Somatic embyos

Introduction: *Kampfeia galanga* is used extensively as a spice throughout tropical Asia and has a long history of medicinal use. It is a reputed remedy for respiratory ailments like cough, bronchitis and asthma. The powder extracted from the rhizome is mixed with honey and given for coughs and pectoral affections. The tuber is boiled in oil and applied externally for blocking of nasal tract (Kirtikar and Basu, 1935). The rhizome is chewed and ingested. The rhizomes are considered stimulating, expectorant, carminative and diuretic. They are used in the preparation of gargles and administered with honey in cough and pectoral affections (Wealth of India, 1959, 1992). Rhizome possesses camphoraceous odour, and a decoction of the rhizome is used for dyspepsia, headache and malaria. Rhizome extract is useful to cure skin diseases, wounds and spleen disorders (Kirtikar and Basu, 1997) and is useful to relieve irritation produced by stinging caterpillars (Bhattacharjee, 2000). Roasted rhizomes are applied hot in rheumatism and for festering tumours. Mixed with oil, the rhizomes are used as a cicatrizant (Chithra *et al.,* 2005).

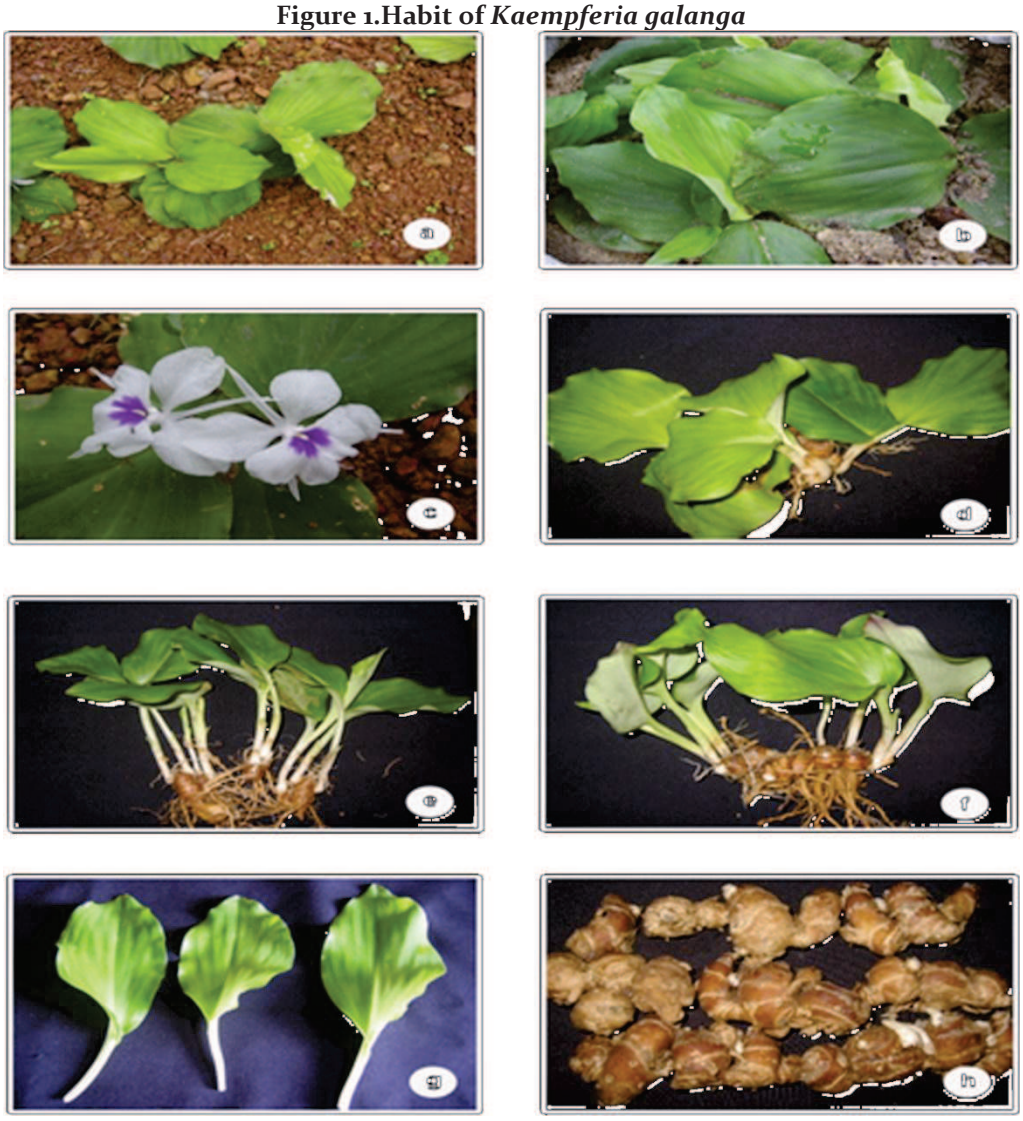
Conventional propagation of this species is through rhizomes and there is no seed setting under natural conditions. Conservation of this medicinal plant and the capability to utilize them in a sustained manner are essential for the well being and continued survival of man. Taxonomic and phylogenetic studies of South Indian *Zingiberaceae* revealed that *Kaempferia galanga* has become endangered, due to indiscriminate collection from natural habitat for ayurvedic preparation, deforestation and conversion of forest land to plantation crops (Sabu, 1991). Amalraj *et al.* (1991) included *K. galanga* under the category endangered species as per IUCN norms since it has been never seen in wild habitat.

To overcome these problems, development of rapid propagation techniques and conservation of germplasm are the most urgent measures to be taken to protect this plant from extinction. Tissue culture provides efficient techniques for rapid and large scale propagation and conservation of germplasm. Tissue culture studies on medicinal plant had already resulted in a spectrum of various technologies ranging from micropropagation and somatic embryogenesis to the production of artificial seeds. The present study intends to establish an efficient protocol for direct somatic embryogenesis and plant regeneration in *Kaempferia galanga.*

Materials and Methods: Healthy plants and fresh rhizomes of *Kaempferia galanga* L. were procured from the herbal garden of Kerala agricultural university, Vellanikkara, Trichur Dt., Kerala. The plants were identified at Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College, Tiruchirappalli, South India. Leaf and leaf-sheath collected from *in vivo* and i*n vitro* raised seedling were used for preset investigation.

The medium consisted of semi-solid MS medium consisting of MS mineral salt, vitamins, 0.6% agar, 30g/l sucrose, auxins and cytokinines. The pH was adjusted to 5.8 before autoclaving at 121^{o}C for 20 min. Leaves were cut into 1.2-1.5 cm squares, leaf-sheath ware cut into 1cm long segments in sterile petri dishes.

Figure 1.Habit of *Kaempferia galanga*



a. *Kaempferia galanga* with smaller leaves
b. *Kaempferia galanga* with larger leaves
c. Flowers
d *Kaempferia galanga* with medium sized leaves
e. & f. *Kaempferia galanga* showing roots, rhizomes and leaves
g. Leaves ,
h. Rhizome

The explants were placed in 25 × 150 mm tubes containing 15 ml semisolid MS medium. The cultures were kept in dark for one week. After two weeks of incubation, the cultures were transferred to fresh media with the same composition.

For embryo development, embryogenic tissues were sub cultured on half strength MS medium containing NAA (0.1 mg/l) + BAP/KN (0.1-2.5 mg/l). Different stages such as globular, club, and banana shaped embryos were observed. Finally mature embryos were washed with hormone free proliferation medium and transferred to semi solid medium. Embryos at different stages of development were separated manually. Mature embryos, which were obtained in 28 days on the semi solid medium and then they were placed in germination medium. Samples were photographed at different stages during growth period.

Mature embryos were placed in culture tubes containing half MS basal medium 3% (w/v) sucrose,

BAP (1.0 mg/l) KN (1.0 mg/l) and GA_3 (0.5 mg/l) individually or in combination with NAA (0.1 - 1.0 mg/l). Embryos were incubated at 25 ± 2°C 80µE m^{-2} S^{-1} light intensity. After root shoot elongation, the plantlets were transferred to plastic cups containing 1:1 mixture of river sand and garden soil and later established in pots.

Results : Plantlets regenerated *in vitro* were found most suitable for explants sources. Among all the explants tested, only leaf-sheath explants produced proliferative burst in the epidermis and the beginning of cellular segregation on 20th day of culture in the medium supplemented with BAP (0.1-2.0 mg/l) and NAA (0.1-2.5 mg/l) (Fig.1. a). Somatic embryos formed directly at the upper surface of the leaf-sheath when subcultured on the same medium (Fig.1. b & c). Highest frequency (80%) with average number of somatic embryos (22.34 ± 1.58) were formed at NAA (2.0 mg/l) and BAP (0.5 mg/l) (Table 1.). Embryos were white or green, small and globular appearing individually or in clusters (Fig.1. d).

Somatic embryos proliferated into larger embryo masses by producing secondary embryos when subcultured in the same fresh medium. Matured embryos were transferred to half MS medium containing BAP (1.0 mg/l) and NAA (0.4 mg/l) or KN (1.0 mg/l) and NAA (0.4 mg/l) for germination. From the fourth to fifth week up to 60% of somatic embryos germinated with the emergence of shoot first, then the roots. Plantlets developed from somatic embryos with shoots and roots were easily separated into individual seedlings (Fig.1. f). The germinated plantlets were hardened and transplanted in the soil (Fig.1.h).

Discussion: Regeneration of *Onidium* through direct somatic embryogenesis has been achieved using young leaf explants (Chen *et al*., 1999). Young leaves have high regeneration capacity and may provide large number of embryos and plantlets in a short period of time. Direct embryo formation on leaf explants was retarded by exogenous auxin, but promoted exogenous cytokinin (Chen and Chang, 2000). In the present investigation leaf-sheath explants produced embryos in the surface of the explants directly and this feature corresponds to previous works.

Induction of somatic embryogenesis was achieved on MS medium supplemented with NAA (5.37 µM) and BA (0.44 µM) from leaf explants of *Ostericum koreanum* (Cho *et al*., 2003). In *Synogonium podophyllum* petiole explants produced somatic embryos when cultured on MS medium containing 2.5 mg/l TDS with 0.5 mg/l NAA (Zhang *et al*., 2006). In *Cicer arietinum* somatic embryos were induced from immature cotyledon on MS medium supplemented with 2, 4, 5 -T or NAA in combination with BA or KN (Kiran *et al*., 2010). In the present investigation NAA (0.5 mg/l) with BAP (2.0 mg/l) induced high frequency of (80.0%) with maximum number of somatic embryos (22.34±1.58) directly from the leaf-sheath explants. This is in agreement with the previous work where the hormone combination induced successful somatic embryos.

In many plant species cytokinins individually or in combination with auxins were used for the maturation of the somatic embryos (Choi *et al*., 1999; Kaur and Kothrari, 2004). Regeneration through the direct somatic embryogenesis could be potential solution to minimize the variation. Somatic embryos that were germinated on half strength MS medium supplemented with 0.01 mg/l BAP and 0.25 mg/l ABA promoted maturation and germination of somatic embryos of *Accacia arabica* (Nanda and Rout, 2003). Similar observations have been made in some crop plants (Das *et al*., 1997 and Ortiz *et al*., 2000). This is in contrast to the present study where best plant conversion frequency (80%) was obtained with cultured embryos on half strength MS solid medium containing BAP (1.0 mg/l) + NAA (0.4 mg/l). These results colloborate the previous fining of Zhang *et al*. (2006) where 85% somatic embryos germinated 5-10 weeks after transferring on to medium containing 2.0 mg/l BAP and 0.2 mg/l NAA. Tiny plantlets were transferred to the greenhouse for further development.

Conclusion: Direct somatic embryogenesis avoids the passage through callus and thus avoids the genetic instability often associated with somatic embryos obtained indirectly from callus. In the present study, a protocol was developed for induction of somatic embryogenesis directly from leaf-sheath and indirectly using rhizome bud explants. Successful regeneration of plants from leaf-sheath, *via*. direct somatic embryogenesis has been reported for the first time.

Table - 1

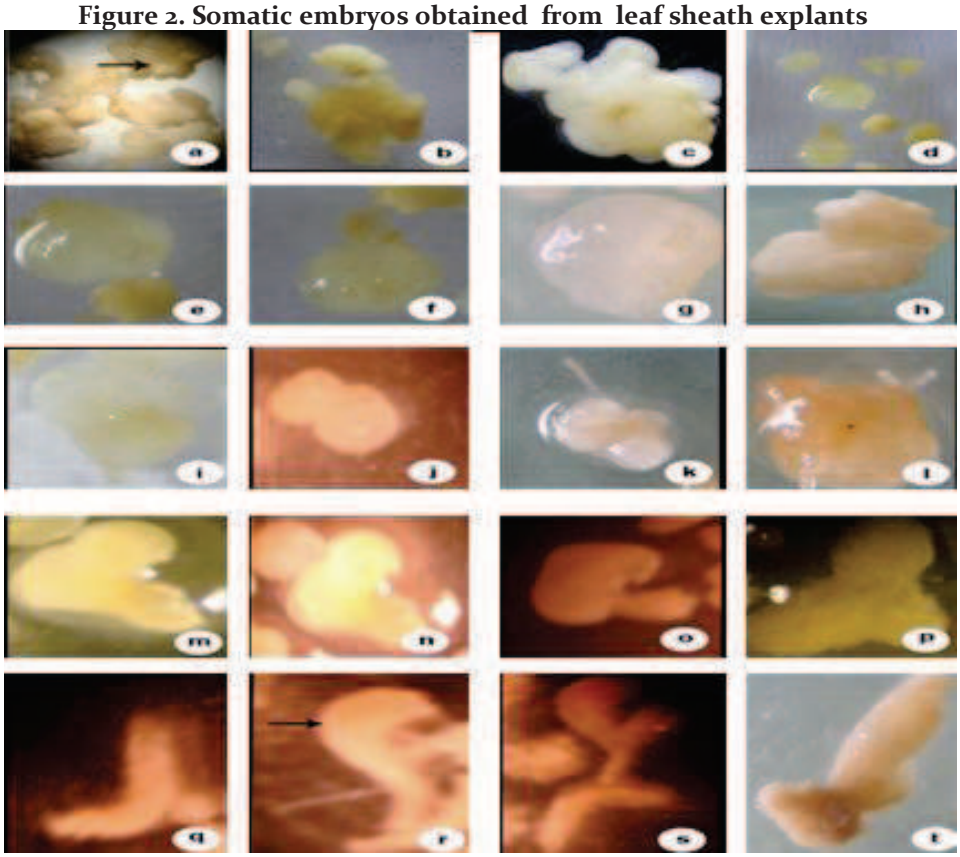
Influence of different concentrations of auxin (NAA) alone in combination with cytokinins (BAP or KN) on induction of direct somatic embryogenesis from leaf sheath explants of *Kaempferia galanga*

Total number of explants taken for observation =35 (each treatment consists of at least 7 explants and the experiments were repeated five times).

Mean value within column having the same alphabet are not statistically significant (P=0.05) according to New Duncan's Multiple Range Test.

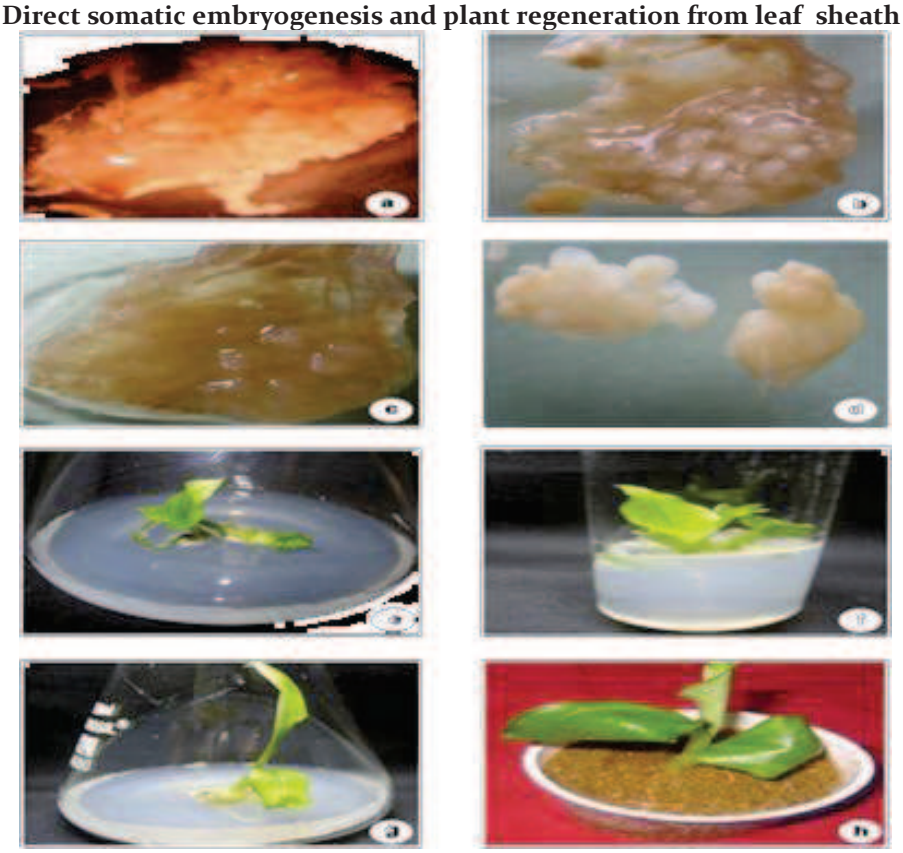
Plant Growth Regulators (mg/l)	No. of explants responded	Percentage of culture responded	Mean no. of somatic embryos harvested per explants
NAA			
0.1	16	45.7^{ef}	9.57±0.61^{ef}
0.5	18	51.4^{de}	10.67±1.14^{e}
1.0	20	57.1^{bc}	13.72±0.98^{cd}
1.5	21	60.0^{b}	14.08±1.15^{c}
2.0	**27**	**77.1^{a}**	**19.24±0.87^{a}**
2.5	19	54.2^{cd}	16.71±2.81^{b}
NAA+BAP			
2.0+0.1	14	40.0^{de}	11.48±1.18^{d}
2.0+0.2	17	48.5^{bc}	21.57±1.81^{ab}
2.0+0.5	**28**	**80.0^{a}**	**22.34±1.58^{a}**
2.0+1.0	19	54.2^{b}	14.08±2.15^{c}
2.0+1.5	15	42.8^{cd}	10.88±0.60^{de}
2.0+2.0	13	37.1^{ef}	10.35±0.40^{ef}
NAA+KN			
2.0+0.1	19	54.2^{b}	9.42±0.82^{d}
2.0+0.2	**23**	**65.7^{a}**	**15.17±1.88^{a}**
2.0+0.5	15	42.8^{c}	13.14±1.13^{b}
2.0+1.0	13	37.1^{cd}	12.14±1.20^{bc}
2.0+1.5	12	34.2^{de}	8.42±0.94^{de}
2.0+2.0	11	31.4^{ef}	7.87±0.77^{ef}

Figure 2. Somatic embryos obtained from leaf sheath explants



A-H	**:Small, round globular somatic embryos directly produced from leaf sheath surface.**
I, J&K	**:Mature embryos, dividing stage**
M,N,O,&P	**:Scutellar shaped somatic embryos established in cell suspension culture**
Q,R,S&T	**:Banana and club shaped somatic embryos.**

Figure 3. Direct somatic embryogenesis and plant regeneration from leaf sheath explants.



A: Early globular embryos formed at cut ends of the leaf sheath explant in 20 days on induction medium (BAP 0.5 mg/l and NAA 1.0 mg/l).

B, C &D: Matured globular embryos on MS medium with BAP 0.5 mg/l and NAA 1.0 mg/l .

E, F &G: *In vitro* germination of somatic embryos on germination medium.

H: Regenerated plantlet acclimatized to green house conditions with river sand, garden soil and farmyard manure in the ratio of 1:2:1, after 6 weeks.

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