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REVIEW ARTICLE

OPTIMIZATION OF SOMATIC EMBRYOGENESIS INDUCTION IN WILD SNAKE GOURD (*TRICHOSANTHES CUCUMERINA* L. VAR. *CUCUMERINA*)

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ABSTRACT

In this study, regeneration of *Trichosanthes cucumerina* L. var. *cucumerina* via somatic embryogenesis was investigated. Four different types of explant (Leaf, cotyledon, epicotyl and hypocotyl) from six-eight days old seedlings were used. Growth regulator treatments were one levels of 2,4-dichlorophenoxyacetic acid (0.3 mg/l) and three levels of 6-benzyl aminopurine (0.25, 0.5 and .75 mg/l). After seven weeks, cotyledon explants showed the highest potential in somatic embryo induction and the combination of 0.3 mg/l 2,4-D and 5 mg/l BAP had significant effect on somatic embryogenesis of *Trichosanthes cucumerina* L. var. *cucumerina*.

INTRODUCTION

Trichosanthes cucumerina var. *cucumerina* Linn. belongs to the family Cucurbitaceae and is distributed in throughout India, Bangladesh, Sri Lanka, Burma, Malaysia, Australia (Chakravarty, 1982). It is perennial climber with an attractive white flower. It is highly bitter in taste the bitter taste may supposed to contain medicinal properties (Choudhary, 1967) hence being used in various treatments as a an anti-diabetic (Kiran and Srinivasan, 2008), antimicrobial agent (Devendra *et al*., 2009), in traditional system of medicine (Devendra *et al*., 2008), Anti-inflammatory agent (Devendra *et al*., 2010), Anti-ovulatory agent (Devendra *et al*., 2009), cordiotonic, antipyretic, antiperiodic, useful for intestinal worms and leaf juice rubbed over the liver in remittent fever (Kirtikar and Basu, 2000). Appetizer, laxative, aphrodisiac and blood purifier (Shivarajan, 1994) root is used to cure bronchitis, headache and boils. Leaves, for biliousness, emetic, externally applied over bald patches of alopecia (Annonymus, 1976) to reduce congestion on congestive cardiac failure (Pullaih, 2006). The seed posses anthelmintic and antifibrile properties the seeds are haemo-agglutinating (Chakravarty, 1982) seed is a good source of nutrients (Oloyede *et al.,* 2005). It is used as one of the important ingredient in 16 commercially available herbal products in India (Devendra and Seetharam, 2011).

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The species belongs to *Trichosanthes* are considered as the future plants of Cucurbitaceae (http:// www. pfaf.org/index.html 1996) Trichosanthin is an antiviral protein purified from the root of *T. kirilowii* Maxim. It is an active component of Chinese medicine and is still being used in midterm abortion and to treat carcinoma (Wang, 2000). Trichosanthin shows inhibition of human immunodeficiency virus (HIV) because of its ribosome inactivating activity (Jian-Hua Wang *et al.,* 2003). Karasurin is another new abortifacient protein isolated from root of *T. kirilowii* (Shunsuke, 1991).

Due to large-scale destruction of plant habitats, unscientific manner of harvestment, coupled with limited cultivation and insufficient attempts for its replenishments it is considered as threatened species. Top of that, propagation through seed is unreliable due to poor germination and death of young seedlings under natural conditions the wild stock of this species has been markedly depleted (Devendra *et al*., 2008). The consequence is possible extinction of the species and this provides justification from conservation and propagation of this valuable germplasm. *In vitro* culture technique is an alternative method for conservation and propagation of this species (Devendra *et al*., 2008 and 2015). There is no report on somatic embryogenesis studies on *Trichosanthes cucumerina* var. *cucumerina*. The present investigation describes an efficient protocol for micropropagation of *Trichosanthes cucumerina* var. *cucumerina* via embryogenesis using cotyledon explants.

MATERIALS AND METHODS

Explant preparation

The seeds of wild snake gourd (*T. cucumerina* L. var. *cucumerina*), were obtained from Khanapur forest Karnataka India. These seeds were washed in running tap water for three minutes and then washed repeatedly in double distilled water. Now under aseptic conditions the seeds were surface sterilized with 70% ethanol for one minute followed by 0.1% Mercuric chloride ($HgCl_2$) for five minutes and rinsed five times in sterile distilled water. The sterilized seeds were then placed on MS basal medium (Murashige and Skoog, 1962) solidified with 0.8% bacto agar for germination in 250 ml culture bottles, 20 seeds were cultured per bottle containing 30 ml of medium. This was incubated in dark at 26^{o}C till it germinated and then transferred to cool-white-fluorescent light room and incubated at 24+2^{o}C and allowed to grow. The plant after reaching a height of 6 centimeters was taken in an aseptic condition and Leaf, cotyledon epicotyl and hypocotyl were excised using a sterile scalpel and cut into 6-8 mm sections.

Medium and culture condition

All media were fortified with 30 g/L sucrose, gelled with 0.8% agar and the pH was adjusted to 5.7 after adding the growth regulators. The media was sterilized under 15 psi pressure and 121^{o}C temperature for 15 minutes. All the cultures were grown at 25 ±2^{o}C under 16 hours photoperiod supplied by luminal T5 28 W fluorescent tubes

Experimental design

A. Induction of somatic embryos

Leaf, cotyledon epicotyl and hypocotyl explants were cultured on nutrient medium with salts and vitamins of MS supplemented with 3% (w/v) sucrose and varied concentrations of BAP, Kn (0.25-0.75 mg^{-l}) lone or in combination of Kn (0.25-0.75 mg^{-l}) and NAA and 2,4-D (0.1-0.3 mg^{-l}) to check their embryogenic efficiency.

B. Development and maturation of somatic embryos

Embryos at globular stage were transferred to MS medium having different concentrations of Kn, BAP and ABA for maturation. The cultures were initiated by transferring 30 days old cultures onto half-strength MS medium containing various concentrations of Kn (0.25-1.0 mg^{-l}) + BAP (0.5 mg^{-l}) + ABA (0.25 mg^{-l}).

C. Maturation and germination of somatic embryos

Embryos at globular stage were transferred to MS medium having different concentrations of Kn, BAP ABA for maturation. Mature embryos were matured embryos were germinated in MS medium supplemented with Kn (0.25-1.0 mg^{-l}) + BAP (0.5 mg^{-l}) + ABA (0.25 mg^{-l}) in combinations. Medium which promotes the initiation of both root and shoot primordial was identified as best germination medium.

D. Plantlet formation

Germinated embryos with well developed root and shoot systems were developed into plantlets on same medium. The ratio of plantlet formed to the total number of mature embryo was calculated as conversion percentage.

RESULTS AND DISCUSSION

Effect of 2,4-D and cytokinins on induction of somatic embryos from cotyledon explants of *T. cucumerina*L. var. *cucumerina*

Among four explants used for somatic embryogenesis, cotyledon shown highest response than other. The auxins and cytokinins play a vital role in somatic embryo induction, maturation and germination. The cotyledon explant of *T. cucumerina* L. var. *cucumerina* was assessed within 13-15 days, globular embryos formed on the explants directly without a callus phase (PFig-1, a). The frequency of cotyledon explants was about 50 per cent (Table-1).

Table 1. Frequency of somatic embryos differentiated from cotyledon explant of *T. cucumerina* L. *var. cucumerina* on MS medium supplemented with different concentrations of auxins

Concentraton of growth regulators (mg^{-l})	Cotyledon explant	
	Frequency (%)	No. embryos/culture
2,4-D		
0.1	20	2.2±0.07
0.2	20	6.1±0.50
0.3	50	16.8±0.65
0.4	-	callus
NAA		
0.1	-	callus
0.2	-	callus
0.3	-	callus
0.4	-	Rhizogenesis

Data represents average of three replicates, each consists of 10 cultures. Mean ± Standard error.

The number of embryos varied from 10-25 per explant. It was possible to induce embryos with 0.1-0.3 mg^{-l} concentrations of 2,4-D with different cytokinins. The maximum number of embryo induction from cotyledon explants was obtained on MS with 2,4-D (0.3mg^{-l}) + BAP (0.5mg^{-l}). Globular and heart shaped structures appeared on the explants after 15-20 days of cultivation (Fig-1, c) and there after embryos reaching torpedo stage within 25 days (Table-2) (Fig-1, b & c).

Table 2. Frequency of somatic embryos differentiated from cotyledon explant of *T. cucumerina* L. var. *cucumerina* on MS medium supplemented with 2,4-D and different concentrations of cytokinins

Concentration of growth regulators (mg^{-l})	Cotyledon explant	
	Frequency (%)	No. embryos/culture
2,4-D + BAP		
0.3 + 0.25	60	24.6±0.49
0.3 + 0.5	70	49.3±0.44
0.3 + 0.75	50	32.8±0.46
2,4-D + Kn		
0.3 + 0.25	30	18.0±0.74
0.3 + 0.5	30	26.5±0.28
0.3 + 0.75	20	11.6±0.16

While different concentrations of 2,4-D and 2,4-D + Kn display moderate and poor induction response. From the data it is clear that 2, 4-D (0.3mg^{-l}) + BAP (0.5mg^{-l}) was found to be the most suitable combination for obtaining maximum number of somatic embryos. Efficacy of BAP into the induction of somatic

embryos was also been demonstrated in other members as *Cantaloupcharantais* (Debeaujon and Brachard, 1998), cucumber calli (Panja *et al.*, 1990a) and *Tylophoraindica* (Manjula *et al.*, 2000). Thus in all the above attempts none of the methods employed was able to bring about the development of embryos beyond the globular like stage and auxin is the most important for the induction and progression of embryogenesis. In other words, auxin is necessary for competent cells to express totipotency. Lower levels of NAA (0.1-0.3mg^{-1}) gave callus. But higher concentrations of NAA roots were formed. Embryogenesis was never observed in the absence of exogenous auxin and low levels of NAA + Kn.

Effect of BAP and Kn on maturation and conversion of plantlets

The explants with globular somatic embryos were transferred on to MS medium containing BAP (0.5mg^{-1}) + ABA (0.25mgl^{-1}) different concentrations of + Kn (0.25 -0.75mg^{-1}) favor the formation of embryo maturation, germination and conversion and the responses are summarized in Table-3.

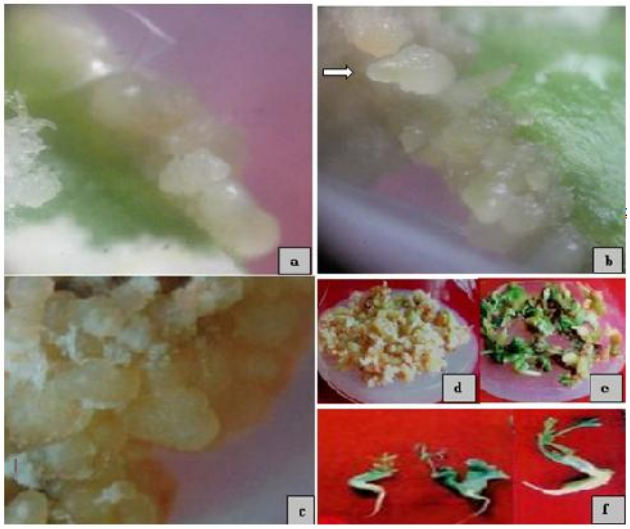
Table 3. Effect of cytokinins and abscissic acid on germination of somatic embryos of *T. cucumerina* L. var. *cucumerina*

Concentration of growth regulators (mg^{-1}) BAP + ABA + Kn	Cotyledon explant	
	No of embryos/ culture	No. embryos germinated
0.5 + 0.25 + 0.25	25	24.6±0.76
0.5 + 0.25 + 0.50	25	26.2 ± 0.34
0.5 + 0.25 + 0.75	25	11.2±0.43
0.5 + 0.25 + 1.0	25	3.6±0.24

The highest frequency of somatic embryo development from the initial globular stage to fully developed cotyledonary stage occurred in the presence of BAP 0.5mg^{-1} + ABA 0.25mg^{-1} and Kn 0.5mg^{-1} from cotyledon explants, within 20 days and the extent of growth was observed for 30 days. The frequency of reaching cotyledonary stage was 70 per cent. All the stages of embryo development i.e., globular to cotyledonary stages were observed (Fig-1, d) (Table-3). Development of somatic embryos appeared asynchrony with wide range of varied sizes and structures within same explant (Fig-1, e). Somatic embryos maturation was commonly accomplished with growth regulator free media, although with cytokinins (Chee, 1991; Kageyama, *et al.*, 1990) or rarly ABA (Ladyman and Girard, 1992) or Gibberlin (Tabei *et al.*, 1991). Germination is characterized by cotyledonary expansion and chlorophyll formation, followed by radical, hypocotyl elongation with subsequent leaf formation. In addition single typical embryos of normal morphology, groups of single trumped shaped embryos are multiple embryos with fused cotyledon were observed (Fig-1, f).

Figure 1. Somatic embryogenesis from leaf explants of *T. cucumerina* L. var. *cucumerina*

a. Leaf explant with embryogenic clumps.
b. Torpedo shaped embryo.
c. Cotyledonary shaped embryos.
d. Asynchronous development of different stages of somatic embryos *i.e.*globular to cotyledonary.
e. Germinated embryos with shoot and root



The germinated embryos were transferred on to half strength MS basal medium for further development and developed into regenerated plant lets with (35%) frequency. The well developed roots obtained within 4 weeks of culture. The germinated embryos are thus developed into plantlets and transfer to the field after acclimatization.

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