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DIRECT AND INDIRECT SOMATIC EMBRYOGENESIS FROM LEAF AND LEAF DERIVED CALLUS OF *MOMORDICA DIOICA* ROXB.

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ABSTRACT
An efficient protocol has been developed for both direct and indirect somatic embryogenesis from leaf explants and leaf derived callus of *Momordica dioica*. Direct somatic embryogenesis was achieved from leaf explants after 7 weeks of culture on MS medium supplemented with 2.0mg/l TDZ+2.0mg/l L-Glutamic acid and influenced by other factors. Indirect somatic embryogenesis was also achieved on MS medium supplemented with 2.0mg/l TDZ +1.0mg/l NAA from leaf derived callus in 2nd subculture. The embryogenic callus was different in color and morphology. These somatic embryos were increased in size and developed into torpedo, heart and bipolar shaped on the same medium after 2nd subculture. Such somatic embryos developed into new plants. Such plants were rooted on MS medium supplemented with 3.0 mg/l IBA, after roots, the plantlets were transferred to for acclimatization.

KEYWORDS: Acclimatization; Callus; Leaf explant; Plantlets; Sub Culture; Somatic embryos.

INTRODUCTION

Teasle gourd (*Momordica dioica*) is locally called "Kakrol," belongs to the family Cucurbitaceae. Kakrol is an important vegetable crop during summer and monsoon in India. Among the summer vegetables it has high food value, as it is rich in protein, carotene, carbohydrates, vitamins (vitamin C) and minerals. Its cultivation is gaining popularity for its high economic return as compared to that of cereals. It has better shelf life and being exported to Middle East, UK and other countries thereby earning foreign exchange. The propagation becomes difficult because of dioecious nature of the plant, the propagation entirely depends on underground tuberous roots (root stocks), and fruit quality deteriorates due to presence of large number of seeds. Ayurvedic practitioners prescribe this vegetable and its fruits to diabetic patients. It is used as an astringent, febrifuge, antiseptic, anthelmintic spermicide. It possesses antioxidant, hepatoprotective, antibacterial, anti-inflammatory, hypoglycemic and analgesic properties (Bawara *et al.*, 2010). Somatic embryogenesis is a process whereby embryos develop from somatic cells or tissues in tissue culture achieve maturity and can develop and subsequently germinate, forming normal plants (Witjaksono, 1997). Somatic embryogenesis may be induced directly or indirectly. In direct somatic embryogenesis they developed directly on the surface of organized tissue or explant in the absence of conspicuous callus proliferation (Aly *et al.*, 2002) and in indirect somatic embryogenesis they developed from callus. Here in our investigation direct and indirect somatic embryogenesis is achieved from leaf explants and leaf derived callus by reducing the incubation time by using MS medium supplemented different concentrations of TDZ +L-Glutamic acid and TDZ+NAA combinations.

MATERIALS AND METHODS

The wild plants of *Momordica dioca* were collected from different areas of Warangal, and Khammam Districts of Telangana state. These plants were planted in the Research field, Department of Botany, Kakatiya University. The leaf explants were collected and thoroughly washed under running tap water for 10minutes and surface sterilized with 0.1% $Hgcl_2$ for 7-8 minutes and rinsed 3-4 times with sterilized distilled water. The sterilized leaves were cut into small pieces with size of 0.5 -2.0 cm aseptically by using sterile forcep and scalpel. These explants were inoculated on MS medium supplemented with various concentrations of TDZ+ L-Glutamic acid and TDZ+NAA combinations. Medium pH was adjusted to 5.8 before sterilization at 121^0C temperature, and 15 lbs pressure for 20minutes. After inoculation, the culture tubes were incubated at 25 ± 2^oC temperature and 2000 lux light with a photoperiod of 16 hrs. After successive sub cultures on the above media, small somatic embryos were proliferated from the explants and callus.

RESULTS

Direct somatic embryogenesis

Direct somatic embryogenesis was achieved from leaf explants of *M. dioica* on MS medium fortified with TDZ and L-Glutamic acid combinations. Green colored bipolar embryos were developed from the margin of the leaf explants and little amount of callus was developed on lower side of the explant on MS medium fortified with 2.0mg/l TDZ and 2.0 mg/l L-Glutamic acid after 28 days of culture (Table:1). Higher the concentration of TDZ promoted few shoots and callus growth. Higher the concentration of L-Glutamic acid promoted the formation of green and friable callus. When the cluster of torpedo shaped embryos of *M. dioica* were transferred to same medium, developed into shoot buds and later regenerated into plantlets. This is the first report of somatic

embryogenesis directly from the leaf explants in *M. dioica*. Leaf explant of *M. dioica* proliferated into green patches on MS medium supplemented with 0.5 mg/l TDZ + 0.5 mg/l L-glutamic acid (Table-1; Fig. 1A). Moderate number of somatic embryos (18.40 ±1.29) were observed on MS medium fortified with 2.0 mg/l TDZ + 0.5 mg/l L-glutamic acid (Table-1; Fig. 1B).The high frequency of somatic embryos (46.16 ±1.39 were induced on MS medium fortified with 2.0 mg/l TDZ +2.0ml/l L-glutamic acid (Table-1; Fig.1C) with 87% of cultures response after second subculture. These cultures were allowed to grow on the same medium for 3rd sub culture. This combination was proved to be the best for the induction of the high frequency of somatic embryogenesis in *M. dioica*. Greening of callus was observed on MS medium supplemented with higher and lower concentrations of both TDZ and L-glutamic acid (Table-1: Fig. 1D). However further increase in concentration of TDZ also did not favour the induction of embryos, but promoted callus growth. Bipolar embryos were regenerated into plantlets on MS basal medium devoid of hormones.

TABLE 1: Direct somatic embryogenesis from leaf explants of *Momordica dioica* on MS medium supplemented with various concentrations of TDZ+L-Glutamic acid (mg/l).

S.No.	Hormone concentration TDZ +L-Glutamic acid mg/l	Percentage of response	No. of somatic embryos (mean ±S.E)
1	0.5+0.5	47.12	7.30±1.14
2	1.0+0.5	51.01	10.36±1.14
3	1.5+0.5	54.12	12.50±2.26
4	2.0+0.5	59.45	18.40± 1.29
5	2.5+0.5	60.00	20.37 ±1.40
6	3.0+0.5	60.54	28.55± 1.37
7	0.5+1.0	62.03	28.18 ±1.13
8	10.+1.0	63.45	32.00 ±1.41
9	1.5+1.0	61.00	36.12± 1.27
10	2.0+1.0	66.84	36.16± 1.17
11	2.5+1.0	68.42	38.25± 3.01
12	3.0+1.0	71.42	40.12±1.28
13	1.0+2.0	74.21	40.56± 2.06
14	1.5+2.0	82.56	43.65±0.89
15	2.0+2.0	87.00	46.16± 1.39
16	2.5+2.0	82.12	42.30± 1.28
17	3.0+2.0	69.32	38.60± 1.30

*Data was collected after four weeks of 2rd sub culture

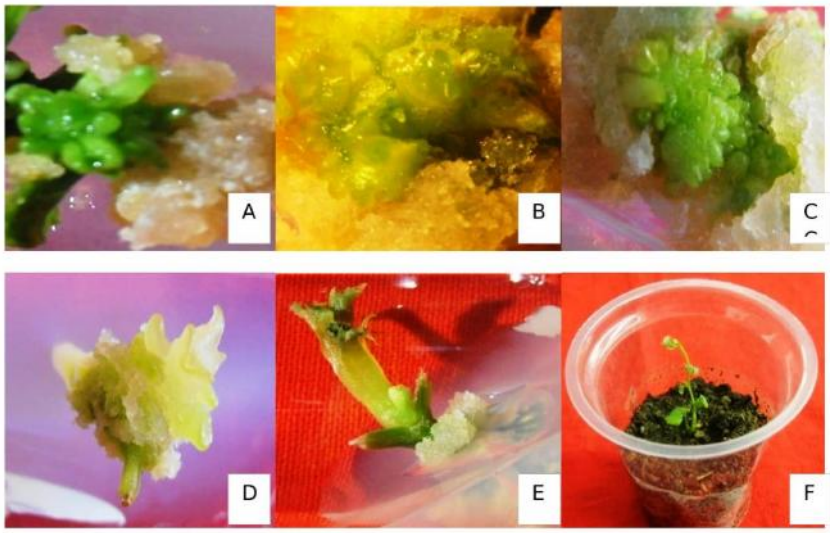

FIGURE 1: Direct somatic embryogenesis from leaf of *Momordica dioica*

A) Somatic embryos formation and callus growth on MS with 0.5 mg/l TDZ + 0.5mg/l L- Glutamic acid. B) Somatic embryos formation on MS + 2.0mg/l TDZ + 1.0mg/l L-Glutamic acid. C) Somatic embryos and callus growth on MS + 2.0mg/l TDZ + 0.5mg/l Glutamic acid. D) Somatic embryos formation on MS + 3.0mg/l TDZ + 1.0mg/l L- Glutamic acid. E) Small shoot buds formation from somatic embryos MS + 1.0mg/l Kn + 0.5mg/12,4D. F) *In vitro* plants established in poly cups.

Indirect somatic embryogenesis

Green compact leaf derived callus (40 days old) of *M. dioica* proliferated into green patches with less number of somatic embryos on MS medium fortified with 0.5 mg/l TDZ and 0.5 mg/l NAA (Table-2; Fig. 2A). By increasing the concentration of TDZ from 0.5 mg/l to 2.0 mg/l along with 1.0 mg/l NAA, induced more number of somatic embryos within 3 weeks of inoculation (Table-2; Fig. 2B). Higher and lower concentrations of TDZ did not favour the induction of somatic embryogenesis but callus growth was achieved (Table-2; Fig. 2C, D). Among all the tested hormonal concentrations, MS medium fortified with 2.0

mg/l TDZ + 1.0 mg/l NAA combination was suitable for the induction of high frequency of somatic embryogenesis (48.41 ±1.32) after second sub culture. However, the percentage of response was almost similar both in MS medium supplemented with 2.0 mg/l TDZ + 2.0 mg/l L-Glutamic acid and 2.0 mg/l TDZ + 1.0 mg/l NAA. All the somatic embryos were developed into plantlets on MS medium without hormones. The obtained plantlets on both the media were rooted on MS medium supplemented with 3.0 mg/l IBA. Later these plantlets were transferred to greenhouse for hardening and acclimatization then to the field.

TABLE 2: Indirect somatic embryogenesis from leaf derived callus of *Momordica dioica* on MS medium supplemented with various concentrations of TDZ+NAA (mg/l).

S.NO	Hormone concentration TDZ +NAA mg/l	Percentage of response	NO. of somatic embryos (Mean ±S.E)
1	0.5+0.5	32.22	8.42± 1.60
2	1.0+0.5	38.10	10.31 ±1.12
3	1.5+0.5	40.23	14.43± 1.29
4	2.0+0.5	43.00	17.00± 1.00
5	2.5+0.5	45.00	21.75± 1.57
6	3.0+0.5	55.62	28.12± 1.00
7	1.0+1.0	63.01	30.42± 1.82
8	1.5+1.0	74.20	36.13± 1.25
9	2.0+1.0	85.21	48.41 ±1.32
10	2.5+1.0	78.10	38.00± 1.57
11	3.0+1.0	68.12	33.10 ±1.48

*Data was collected after four weeks of 2nd sub culture

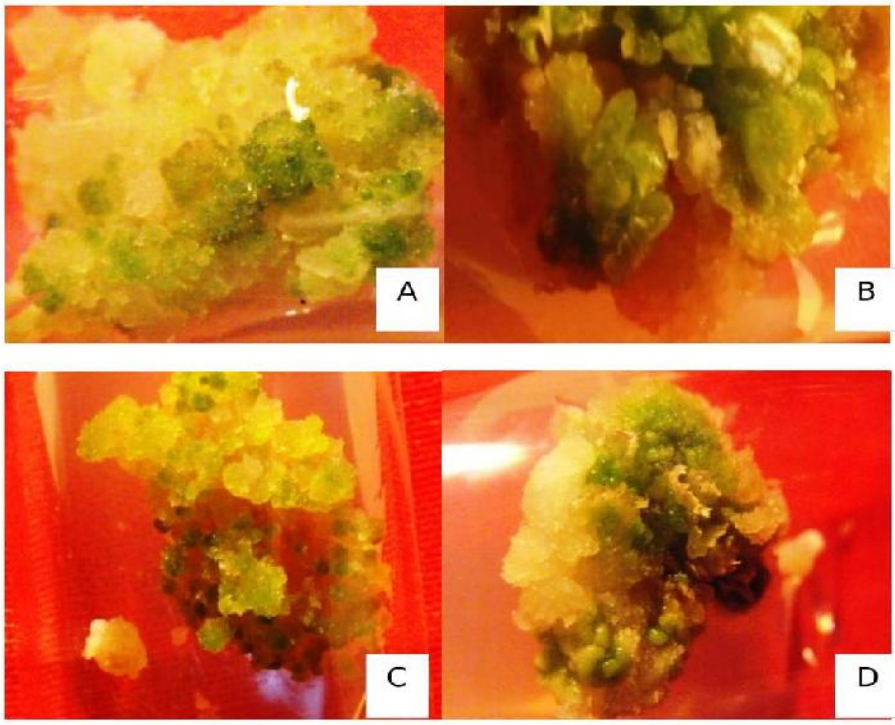

FIGURE 2: Indirect somatic embryogenesis from leaf derived callus of *Momordica dioica*

A) Proliferation of green patches on MS medium supplemented with 0.5 mg/l TDZ + 0.5 mg/l NAA**. B)** Fprmation of moderate number of somatic embryos on medium supplemented with 2.0 mg/l TDZ + 0.5 mg/l NAA. **C)** High frequency of somatic embryos on MS medium supplemented with 2.0 mg/l TDZ + 1.0mg/l NAA. **D)** Callus growth and fewer numbers of embryos on embryoid formations on medium supplemented with lower and higher concentration of TDZ and NAA.

DISCUSSION

In the present investigation, the MS medium fortified with 2.0 mg/l TDZ and 2.0 mg/l L-glutamic acid combination was proved to be the best for somatic embryogenesis. High frequency of direct somatic embryogenesis was achieved on this medium after 2rd sub culture. We have used a novel combination of TDZ with an effective aminoacid (L-Glutamic acid) for obtaining regeneration results. The previous reports related to the amino acids like L-glutamic acid, urea and alanine served as reliable substitutes for the induction of somatic embryogenesis (Wetherell and Dougall., 1976). Amino acid like proline is known to enhance somatic embryogenesis in maize (Armstrong and Green., 1985), while tryptophan was found to favour somatic embryogenesis in some cultures of rice (Siriwardana and Nabors., 1983). Addition of glutamine (Nitsch, 1974; Xu *et al.,* 1981) and serine

(Nitsch, 1974) promoted pollen embryogenesis in many crop plants.

The requirement of TDZ in tissue culture of dicotyledonous plants for the direct differentiation of somatic embryos is gaining importance (Gill and Saxena., 1992). In general, TDZ at very low levels induced direct somatic embryogenesis in *Geranium* (Qureshi and Saxena., 1992), water melon (Compton and Gray., 1993) and muskmelon (Gray *et al.,* 1992). The association between TDZ and L-glutamic acid induced response and endogenous plant growth regulators of dicotyledonous plants has been documented by mean of the quantification of their profiles. TDZ promotes the synthesis and accumulation of purines (Capelle *et al.,* 1983) and also alters cytokinin metabolism (Mok *et al.,* 1982). Since TDZ is involved in cytokinin metabolism, it was decided to explore its effect on direct somatic embryogenesis in *Coffea* (Giridhar *et al.,* 2004) and the results of present study also support their observations.

The significance of this study is the appearance of rapid repetitive somatic embryogenesis by using TDZ directly from leaf explants, indirectly from leaf derived callus of *M. dioica*. And more over this is the first report of direct *in vitro* somatic embryogenesis from leaf explants of *M. dioica* regenerated plantlets. Use of other hormonal regimes resulted in callusing from cut portions (margin) and eventually used for indirect somatic embryogenesis to form plantlets. Earlier investigations have suggested the process of high frequency of somatic embryos in both *Coffea arabica* and *Coffea canephora* (Hatanaka *et al.,* 1991, 1995; Etienne-Barry *et al.,* 1999).

In the present investigations, healthy regenerates were obtained from leaf explants on MS medium supplemented with TDZ, through morphogenetic routes including direct & indirect somatic embryogenesis. Addition of amino acid improved both the mean number of somatic embryogenesis and plantlets formed in the MS medium containing 4.50µM maltose. It has been shown to possess a stimulatory effect during embryogenesis and to confer osmotolerance (Bela and Shetty., 1999; Hita *et al.,* 2003). Other reports have also stated that the vital role of L-proline on somatic embryo maturation in callus cultures of corn (Armstrong and Green, 1985) and somatic embryogenesis in long term callus cultures of barley (Rengel and Jelaska, 1986). It has been suggested that the presence of amino acids in the culture medium is important because they partially replace the NH_4^+. Amino acids can also increase the levels of reduced nitrogen, which stimulates the development of somatic embryogenesis in several species (George, 1993). The highest number of somatic embryogenesis and plantlets were obtained in the maturation medium containing 4.50 µM TDZ, 120 Mm maltose and 200µM L-proline. Addition of these compounds to the maturation medium offers a rapid and efficient multiplication of kodo millet via somatic embryogenesis. MS medium fortified with 2.0 mg/l TDZ + 1.0 mg/l NAA also favoured the induction of somatic embryogenesis from leaf derived callus, which is lower than TDZ + L-glutamic acid combination. It was evident that calli influenced on the formation of somatic embryos and organogenesis in plants. However, the embryos were green in color and smaller in size but other combinations did not promote positive results. BAP has been used for shoot induction of melon (Dirks and Buggenum, 1989). The effect of BAP on somatic embryogenesis was tested by Oridate and Oosawa (1986) and the most efficient embryo formation was obtained with 0.1mg/l BAP. The adventitious shoot formation and somatic embryogenesis in melon can be controlled by the ratio of auxin and cytokinin in the medium. Matsuoka and Hinata (1979) found the effect of NAA concentration on organogenesis and embryogenesis in egg plant. They cultured hypocotyl tissue of egg plant in media with 8.0 mg/l NAA and obtained somatic embryos. Indirect somatic embryogenesis from callus cultures derived from immature zygotic embryos has been reported (Barwale *et al.,* 1986). In these works using different plant species, a similar effect of auxin concentration on morphogenesis of cultured tissues was found. When callus was initiated on a medium containing 10.0 mg/l 2, 4-D and was transferred on to the medium with low concentration of BA, somatic embryos were formed (Marsolais *et al*., 1991). Somatic embryo formation was observed in spine gourd when calli were exposed to medium containing 1.0 mg/l NAA (Karim and Ahmed, 2010). Explants from garlic plantlets taken 15 to 18 days after sprouting showed 95% shoots from somatic embryos, when cultured on MS medium supplemented with1.0 mg/l NAA and 10.0 mg/l BAP (Houque *et al.,* 1998). Somatic embryos formation was observed when callus was exposed to medium containing l.0 mg/1 BAP and 0.1mg/l NAA. *Brassica napus* showed the highest rate of mature embryo formation when it was exposed to higher concentration of ABA for 30 days. In our investigation 3.0 mg/l IBA was so effective for the induction roots to the maximum extent. *Ex vitro* rooting is more beneficial as it reduces one step of hardening and acclimatization (Rathore *et al*., 2007; 2010). The combination of IBA and way of its application also has a significant effect on the root induction (Van der Krieken *et al*., 1993).

Acclimatization of *in vitro* regenerated plants has been established in *M. dioica* for the first time. The potting mixtures containing red + vermicompost (1:1) showed better results 75% survival. About 75% of plantlets were hardened successfully and efficient protocol for shoot regeneration was also reported from other medicinally important plants (Balaraju *et al*., 2008)

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