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Full Length Research Paper

In vitro plantlet regeneration and genetic transformation of sponge gourd (*Luffa cylindrica* L.)

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In vitro **plantlet regeneration via organogenesis was observed in leaf and nodal explants cultured on Murashige and Skoog (MS) medium fortified with various concentrations of thidiazuron (TDZ), benzylaminopurine (BAP) or kinetin (Kn) in sponge gourd (*Luffa cylindrica* L. accession number 1868). TDZ was superior to BAP or Kn in inducing more number of shoots in leaf and nodal explants. Conversely, TDZ was inferior to BAP or Kn in causing shoot elongation. TDZ at 0.5 mg/L induced maximum number of shoots (9.55 ± 0.54 and 5.80 ± 0.45) and caused minimum shoot elongation (1.20 ± 0.25 and 3.05 ± 0.45 cm) in leaf and nodal explants, respectively, after 4 weeks of culture. On the other hand, BAP or Kn (1.5 mg/L) also induced maximum number of shoots (4.55 ± 0.25 and 3.70 ± 0.25) and (3.25 ± 0.27 and 3.10 ± 0.16) maximum shoot elongation (cm) (2.60 ± 0.32 and 5.00 ± 0.27) and (2.20 ± 0.25 and 3.90 ± 0.35) in leaf and nodal explants, respectively, after 4 weeks culture. Micro-shoots transferred to MS + IBA (1.0 mg/L) produced roots in all cultures after two weeks of inoculation. Root tip squashes of the regenerated plantlets was diploid (2n = 26) and did not reveal any chromosomal aberrations. Plantlets were acclimatized in pots for two weeks and then transplanted to research field with 70% survivability. Histochemical assay of nodal explants with callus showed deep blue spots, which confirmed the successful transformation of *gus* gene using particle gun.**

Key words: *Luffa cylindrica* (L.) 1868, plantlets, root tip squash, GUS gene, thidiazuron.

INTRODUCTION

Luffa cylindrica (L.) syn. *Luffa aegyptiaca* Mill., (2n = 26) commonly called sponge gourd, vegetable sponge, bath sponge or dish cloth gourd, is a member of Cucurbitaceae family. It is a wild annual climber, which is monoecious and in the raceme inflorescence of the male flower, one female flower exists, which produces a large green cylindrical fruits called gourds, with spongy endocarp and about 30 flat and round black seeds has been used in the treatment of respiratory disorders (Indumathy et al., 2011). Juice extracted from the stem and the seed has emetic action (Bailey, 1989). Ethanol and aqueous extract of different parts of *L. cylindrica* (L.) syn. *Luffa aegyptiaca* Mill., possess antiinflammatory, analgesic, sedative (Muthumani et al., 2010), antifungal (Parkash et al., 2002), expectorant (Partap et al., 2012) and antimicrobial (Indumathy et al., 2011) properties It has been discovered that sponge gourd can supply some antioxidant constituent to human body (Oboh and

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Abbreviations: MS, Murashige and Skoog; **TDZ,** thidiazuron; **BAP,** benzylaminopurine; **Kn,** kinetin; **IBA,** indole 3-butyric acid; **HCl,** hydrochloric acid; **YEB,** yeast extract broth.

Aluyor, 2009) and is a potential source of vegetable protein in human and animals (Dairo et al., 2007). The seeds are used for extraction of industrial oil (Bal et al., 2004), and its use as biodiesel is now gaining wide acceptance because of low CO_2 emission and other considerations (Ajiwe et al., 2005).

Three new protein synthesis inhibitors have been isolated and purified from *L cylindrica* (L.) *Luffa aegyptiaca* Mill., and they may be involved in regulation of protein synthesis or in defense from some parasite (Keiichi et al., 1990). Two isoforms of ribosome inactivating protein (RIP), luffin-a and luffin-b were extracted from seeds of *L. cylindrica* (L.) syn. *Luffa aegyptiaca* Mill. and the inhibitory activity of luffin-b on protein synthesis in rabbit reticulocyte lysate was approximately ¼ that of luffin-a (Kamenosono et al., 1988). Immature fruits are used as vegetable by diabetes patients (Bal et al., 2004).

Factors such as high surface area per volume, low specific gravity, strong and durable structure, and reasonable cost makes *Luffa* sponge suitable alternative packaging material (Mazali and Alves, 2005). It has been suggested that the sponge may be used as an immobilization matrix for plant, algal, bacterial and yeast cell (Iqbal and Zafar, 1993, 1994). It has been reported that *Luffa* sponge is an excellent carrier for immobilization of microorganisms, plants and animal cells (Roble et al., 2002; Chen et al., 2003; Ogbonna et al., 2001; Liu et al., 1999). *Luffa* sponge has been extensively used for biosorption of heavy metals (Iqbal and Edyvean, 2004, 2005) and removal of phenolics from olive oil mill waste water and other waste waters (Ahmadi et al., 2006a, b).

Application of plant biotechnology tools for improvement of *L. cylindrica* (L.) is limited. So far, only two papers have reported micropropagation of *L. cylindrica* (L.) but number of plantlets produced was very low (Singh et al., 2011; Nahar et al., 2010). Recently, callus induction in leaf explants on BAP 1.5 mg/L was reported (Shrivastava and Roy, 2012). Preliminary information on *Agrobacterium tumefaciens* mediated genetic transformation of *L. cylindrica* Roem was reported (Oboh and Aluyor, 2009).

The objective of the present study was to develop a rapid *in vitro* clonal propagation from leaf and nodal explants on MS + TDZ, BAP or Kn and genetically transform GUS gene into nodal explant with callus using particle gun in *L. cylindrica* (L.) (accession number 1868), a medicinal cucurbit.

MATERIALS AND METHODS

Sponge gourd (*L. cylindrica* (L.) syn. *Luffa aegyptiaca* Mill.) was collected from wild, Inavolu (Village), Wardhannapet (Mandal), Warangal (District-Geographical location, 18.0° N 79.58° E), Andhra Pradesh (State), India. The seeds in the spongy endocarp were white as opposed to black in *L. cylindrica* (L.) syn. *Luffa aegyptiaca* Mill. The plant material was preserved as herbarium specimen in the Department of Botany, Kakatiya University, Warangal and the authentication the plant material was performed by well known taxonomist, Prof. V. S. Raju, Plant Systematics Laboratory, Department of Botany, Kakatiya University, Warangal, A. P. The plant material was identified as *L. cylindrica* (L.) and an accession number 1868 was given to it.

We planted the seeds of *L. cylindrica* (L.) 1868 during July 2011 and the vine, flower and fruits were morphologically similar to *L. cylindrica* (L.) syn. *Luffa aegyptiaca* Mill. We collected gourds with white seeds in January 2012 and used them for tissue culture, cytological and genetic transformation studies (Figure 1).

Leaf (1 cm ca) and nodal (2 cm long) explants were collected from 3-months old *L. cylindrica* (L.) 1868 and were washed with running tap water for 15 min and surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 3 min, and finally rinsed with several changes of sterile distilled water. Murashige and Skoog (1962) (MS) medium supplemented with 3% sucrose (w/v) was adjusted to pH 5.8 with 1 N NaOH, solidified with 0.8% agar (w/v) and was autoclaved at 15 lbs for 20 min. Leaf and nodal explants were cultured on MS medium fortified with 0.5 to 2.5 mg/L thidiazuron (TDZ), benzylaminopurine (BAP) or kinetin (Kn) for shoot bud induction and individual micro-shoots were cultured on MS medium fortified with 1.0 mg/L indole-3-butyric acid (IBA) for rooting. All cultures were maintained under white fluorescent light (80 µ EM^{-2} S^{-1}) at 25 ± 2°C under 16 h photoperiod. Complete plantlets were transferred to pots containing sterile soil and compost (1:1) for 2 weeks, and then transplanted to research field.

Cytological procedures

Root tips of randomly selected 30 plantlets were fixed in ethanol and acetic acid (3:1). They were placed in I N HCl and 2% aceto-orcein (9:1) for 2 h, then squashed with a drop of 45% acetic acid, covered with a cover slip and observed under a Magnus MLX compound microscope at 60x magnification.

Isolation of plasmid DNA with GUS gene

A single colony of the *A. tumefaciens* LBA 4404 growing in disposable 9 mm Petri dishes were inoculated in 10 mL liquid YEB medium supplemented with 25 mg/L rifampicin, 50 mg/L kanamycin and 75 mg/L chloramphenicol in 100 mL Erlenmeyer flask and grown overnight on a incubator shaker (100 rpm) at 28°C.

Particle gun mediated genetic transformation

Plasmid DNA with 500 bp *gus* gene (Figure 2a) was isolated from overnight grown cultures of *A. tumefaciens* LBA 4404 and was coated on to gold particle according to Sanford et al. (1993) method. Nodal explant with callus was bombarded with plasmid DNA with *gus* gene (Bio-Rad PDS- 1000/ He System). The distance between flying disk and target tissue (Nodal explant with callus) was 8 cm and the pressure used for shooting gold particles coated with *gus* gene was adjusted to 900 psi. Four days after incubation, nodal callus was analyzed histochemically (Jefferson et al., 1987).

Data collection and statistical analysis

Data on shoots (number and shoot elongation) was scored after 4 weeks of cultures of 20 replicates and was subjected to statistical analysis including mean and standard error.

RESULTS

Among the three cytokinins TDZ, BAP or Kn tested, TDZ

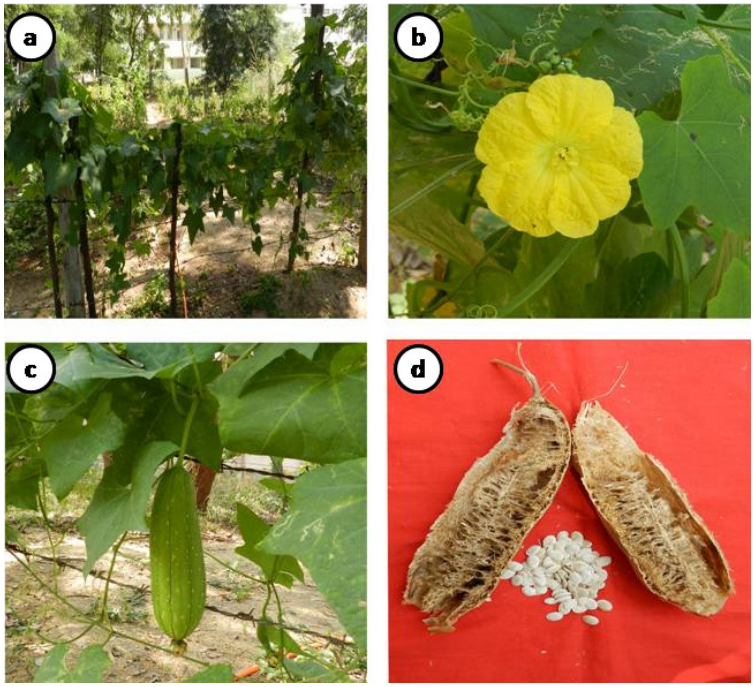


Figure 1. *L cylindrica* (L.) 1868; (a) 3 month old vine grown research field; (b) Female flower; (c) green gourd*;* (d) spongy endocarp with white seeds.

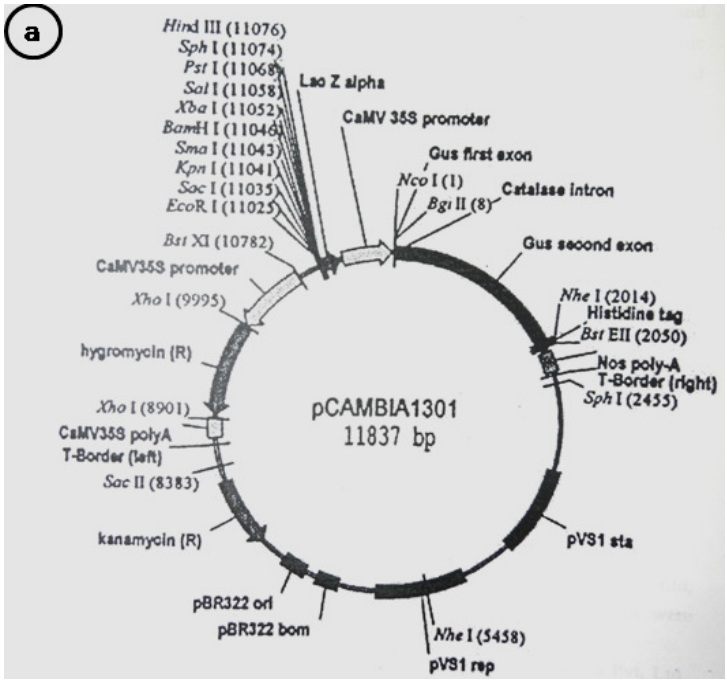


Figure 2. (a) pCAMBIA 1301 showing *gus* gene (500 kb).

at 0.5 mg/L induced maximum number of shoots (9.55 ± 0.54 and 5.80 ± 0.45) and minimum shoot elongation (cm) (1.20 ± 0.25 and 3.05 ± 0.45) in leaf and nodal explants, respectively, after four weeks of culture (Table 2). BAP or Kn also induced maximum number of shoots (4.55 ± 0.25 and 3.70 ± 0.25) and (3.25 ± 0.27 and 3.10 ± 0.16) (Table 1) and maximum shoot elongation (cm) (2.60 ± 0.32 and 5.00 ± 0.27) and (2.20 ± 0.25 and 3.90 ± 0.35)

Table 1. Effect of BAP and Kn alone or in combination with 2,4-D on induction of multiple shoots from leaf and nodal explants of *L. cylindrica* after 4 weeks of culture.

Hormone concentration (µM/L)		Leaf		
		Percentage of cultures responding	Number of shoots/explants (X±S.E)	Shoot length (cm) (X±S.E)
TDZ	0.5	55	3.65 ± 0.53	0.50 ± 0.15
	1.0	68	6.40 ± 0.35	0.80 ± 0.20
	1.5	75	9.55 ± 0.54	1.20 ± 0.25
	2.0	70	5.45 ± 0.41	0.90 ± 0.12
	2.5	62	2.80 ± 0.30	0.45 ± 0.25
BAP	0.5	40	2.15 ± 0.24	0.80 ± 0.24
	1.0	46	3.40 ± 0.23	1.10 ± 0.15
	1.5	52	4.55 ± 0.25	2.60 ± 0.32
	2.0	55	2.65 ± 0.30	2.20 ± 0.46
	2.5	50	1.85 ± 0.35	1.70 ± 0.20
Kn	0.5	45	1.90 ± 0.29	0.40 ± 0.16
	1.0	48	2.10 ± 0.37	1.80 ± 0.28
	1.5	50	3.25 ± 0.27	2.20 ± 0.25
	2.0	55	2.00 ± 0.72	1.80 ± 0.15
	2.5	52	1.45 ± 0.35	0.95 ± 0.10

Values are mean of 20 explants ±S.E. and each experiment was repeated twice; Mean followed by the same super script in a column is not significantly different at 0.05.

Table 2. Effect of TDZ, BAP and Kn on adventitious shoot bud induction in nodal explants of *L. cylindrica* after 4 weeks of culture.

Hormone concentration (µM/L)		Node		
		Percentage of cultures responding	Number of shoots/explants (X±S.E)	Shoot length (cm) (X±S.E)
TDZ	0.5	60	2.20 ± 0.35	1.55 ± 0.32
	1.0	65	4.65 ± 0.26	2.60 ± 0.22
	1.5	72	5.80 ± 0.45	3.05 ± 0.45
	2.0	70	3.65 ± 0.35	2.15 ± 0.11
	2.5	62	1.55 ± 0.16	1.20 ± 0.25
BAP	0.5	52	1.95 ± 0.21	3.90 ± 0.16
	1.0	60	2.10 ± 0.31	4.60 ± 0.31
	1.5	65	3.70 ± 0.25	5.00 ± 0.27
	2.0	62	2.85 ± 0.21	3.80 ± 0.31
	2.5	58	2.65 ± 0.24	2.90 ± 0.22
Kn	0.5	50	1.55 ± 0.16	2.20 ± 0.29
	1.0	55	1.80 ± 0.24	3.50 ± 0.19
	1.5	62	3.10 ± 0.16	3.90 ± 0.35
	2.0	65	2.15 ± 0.22	2.50 ± 0.40
	2.5	60	2.10 ± 0.19	2.30 ± 0.32

Values are mean of 20 explants ±S.E. and each experiment was repeated twice; Mean followed by the same super script in a column is not significantly different at 0.05.

in leaf and nodal explants, respectively, but the treatment regimen was 1.5 mg/L (Figure 3a to f). On concentrations greater than 0.5 mg/L, TDZ induced less number of shoots in leaf and nodal explants and on concentration

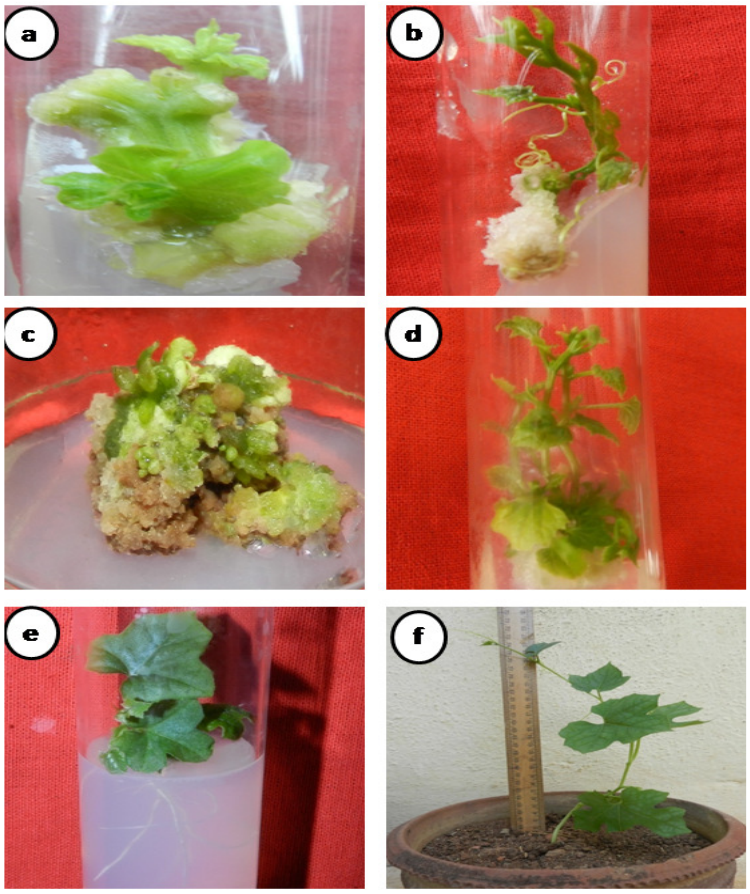


Figure 3. *In vitro* plantlet regeneration in *L. cylindrica* (L.) 1868; (a and b) Induction of multiple shoots form nodal explants on MS + TDZ (0.5 mg/L)*;* (c and d) Induction of multiple shoots in leaf explants on MS + TDZ (0.5 mg/L)*;* (e) Rooting of microshoot on MS + IBA (1.0 mg/L); (f) Complete plantlet growing in pots.

greater than 2.0 mg/L, BAP or Kn, only single shoot was induced in nodal explant, with variable amount of callus at the cut end (Figures 4a, b, and 5a, b).

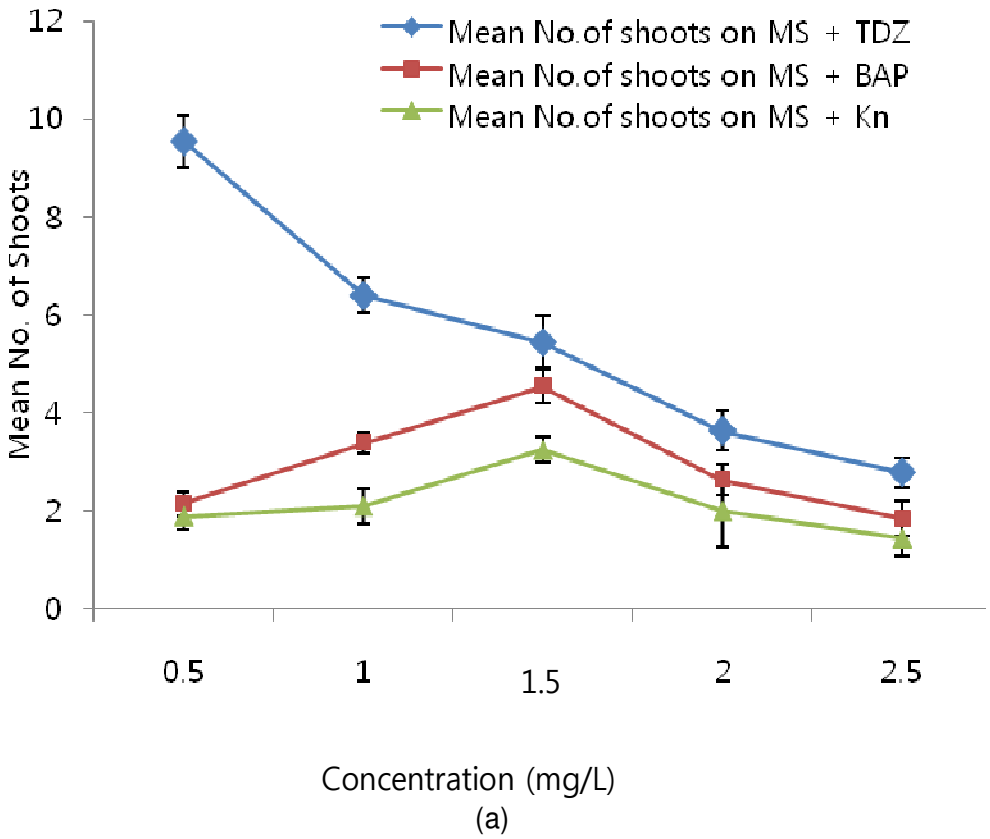
Individual micro-shoots produced roots on MS + IBA (1.0 mg/L) in 100% cultures. Root tip squashes of plantlets regenerated from leaf and nodal explants were normal diploid (2n = 26) and did not show any chromosomal aberrations. Plantlets were acclimatized in pots for two weeks and then transplanted to research field with 70% survivability.

Histochemical assay of nodal explants with callus showed deep blue spots, which confirmed successful transformation of *gus* gene (Figure 6a and b).

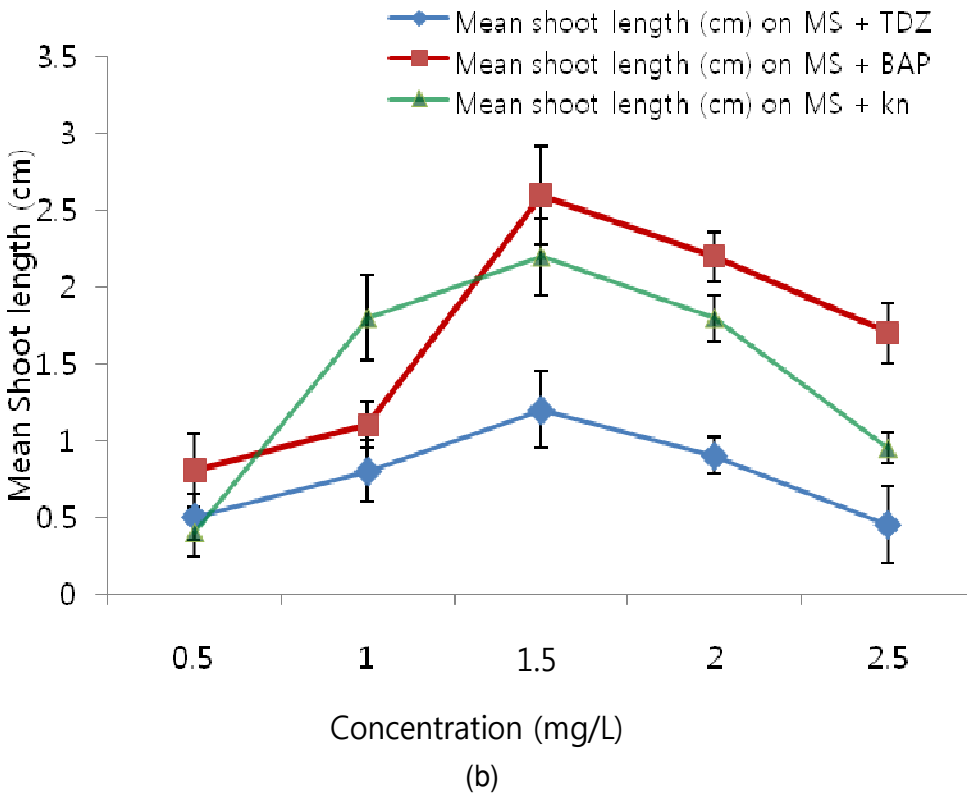
DISCUSSION

In our study, TDZ induced more shoots (number) in leaf and nodal explants as compared to BAP or Kn. Thidiazuron (TDZ), a substitute of phenyl urea (N-phenyl-1,2,3–thidiazol-5-ylurea) is a potent cytokinin used in *in vitro* shoot induction experiments and its efficiency in inducing more number of *in vitro* shoots than Kn or BAP has been reported in other cucurbits like *Cucurbita pepo* (Pal et al., 2007), *Melothria maderaspatana* (Baskaran et al., 2009) and *Citrullus colosynthis* (Savitha et al., 2010). Its mode of action maybe to counter the action of cytokinin oxidase, which in turn may modulate the level of endogenous cytokinin (Hare and Van Staden, 1994) or varied translocation rates to the meristematic region and metabolic processes, in which cytokinin may be degraded or get conjugated with sugars or amino acids to form biologically inert compounds (Kaminek, 1992). Another observation for TDZ was, the length of shoot was lesser than those induced by BAP or Kn. Inclusion of TDZ reduced shoot length resulting to miniature shoots in red ginger (Hamirah et al., 2010) and Korarima (Tefera and Wannakrairoj, 2006).

For the first time, we used TDZ in tissue culture of *L. cylindrica* and observed that it is more efficient than BAP or Kn in inducing more number of shoots. TDZ 0.5 mg/l induced 10 and 6 shoots in leaf and nodal explants,

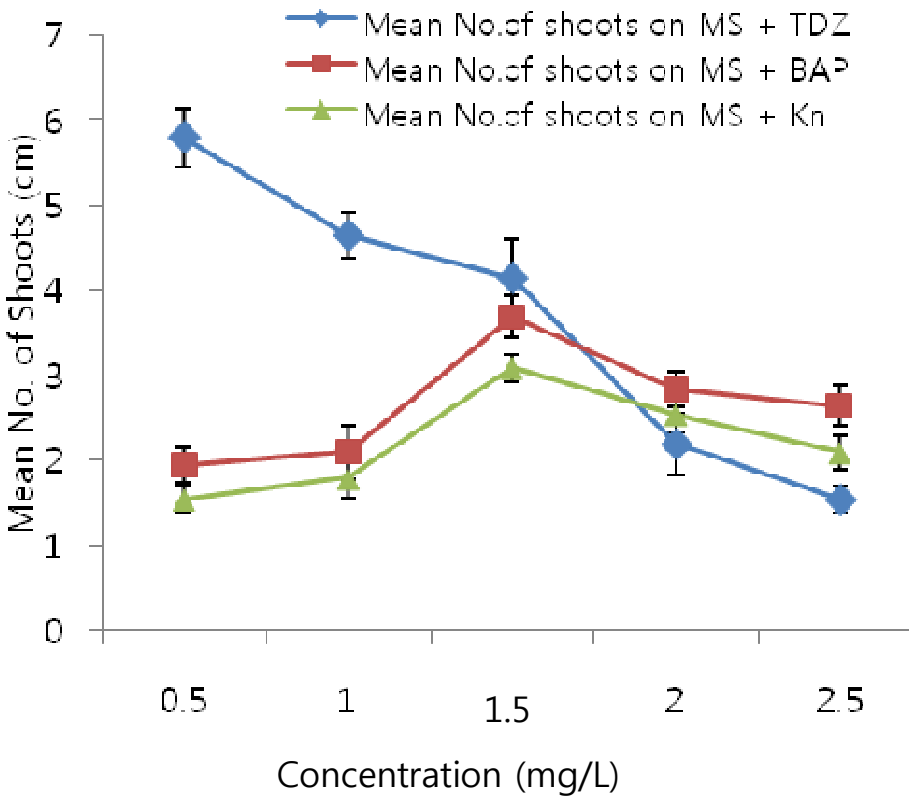


(a)

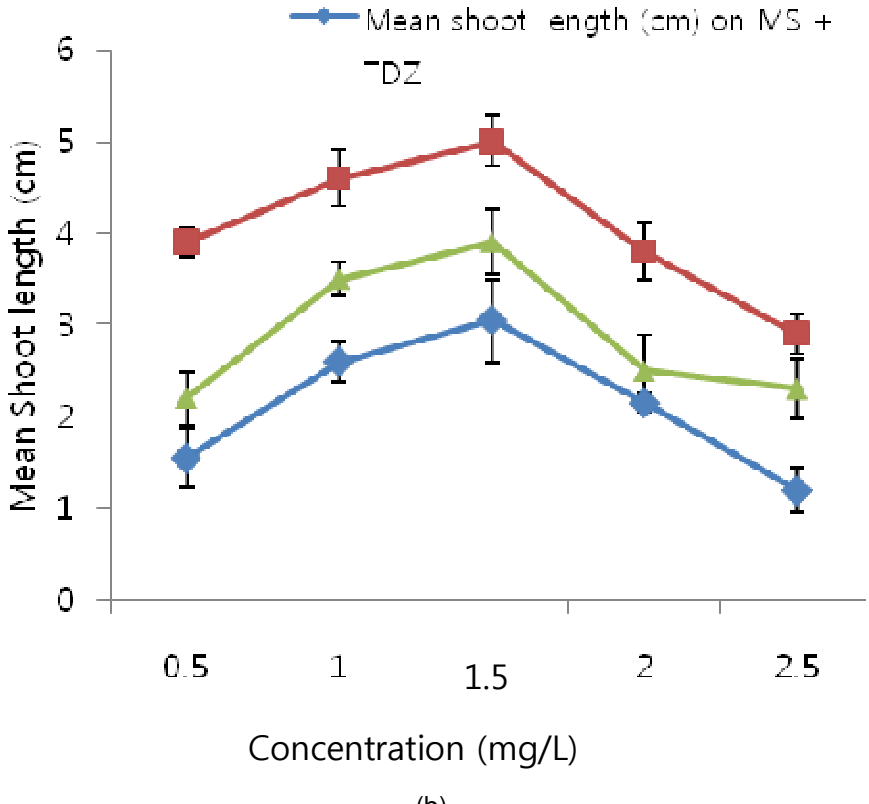


(b)

Figure 4. Effect of TDZ, BAP or Kn on (a) multiple shoot (b) shoot elongation (cm) induction in leaf explants of *L. cylindrica* (L.) 1868.



(a)



(b)

Figure 5. Effect of TDZ, BAP or Kn on (a) multiple shoot (b) shoot elongation (cm) induction in nodal explants of *L. cylindrica* (L.) 1868.

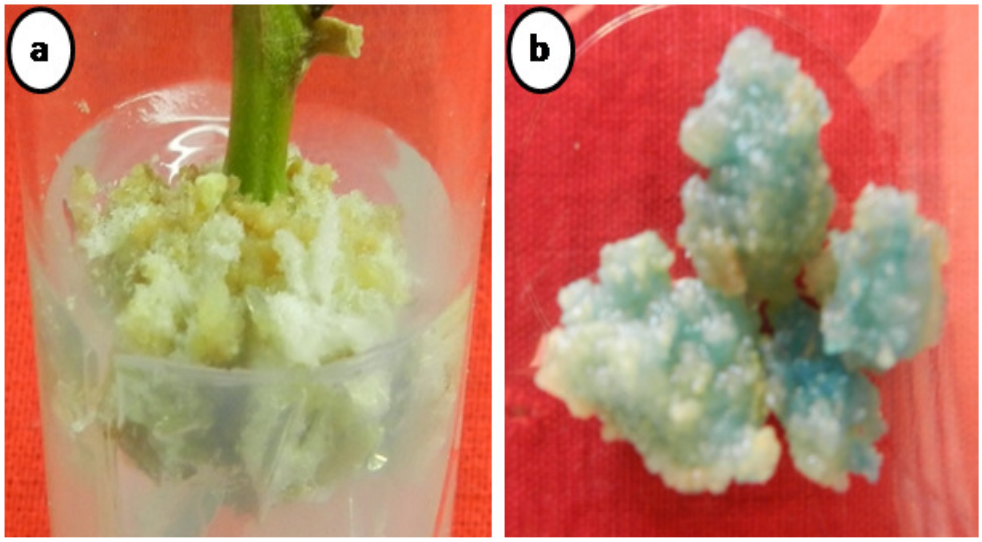


Figure 6. Nodal explant with callus of *L. cylindrica* (L.) 1868; (a) Untransformed nodal explant with callus; (b) transformed explant with callus showing GUS gene expression (blue color).

respectively as compared to 5 shoots on BAP 1.5 mg/L (Nahar et al., 2010). The regenerated plantlets did not show chromosomal aberrations in its root tip squashes, therefore, they may be clonal in origin. Moreover, a reliable procedure of particle gun mediated genetic transformation of *gus* gene into nodal explant with callus was developed, which can be used for genetic engineering studies of *L. cylindrica* L.

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