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ORIGINAL ARTICLE

In vitro regeneration from cotyledon explants in figleaf gourd (*Cucurbita ficifolia* Bouché), a rootstock for Cucurbitaceae

Kyung-Min Kim · Chang Kil Kim · Jeung-Sul Han

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Abstract An efficient plant regeneration system has been developed for figleaf gourd (*Cucurbita ficifolia* Bouché), which is exclusively used as a rootstock for cucumber. The protocol is based on results obtained from a series of culture experiments involving different parts of the cotyledons and various media. The culture of cotyledon explants was critical for the enhancement of shoot regeneration frequency. The lower parts of the cotyledon excised at the plumule base were found to display a markedly enhanced production of adventitious shoots compared to other cotyledon regions. Culture in silver nitrate-supplemented Murashige and Skoog (MS) medium was not beneficial for shoot regeneration and suppressed root regeneration. Efficient shoot regeneration was obtained on MS medium containing 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} indole-3-acetic acid. Regenerated shoots successfully elongated and rooted in medium containing 0.1 mg l^{-1} 1-naphthaleneacetic acid after 10–15 days of subculturing. The plantlets were satisfactorily acclimatized in a greenhouse and grew into normal plants without any morphological alterations.

Keywords Cotyledon · Cucurbita · Figleaf gourd · Plant growth regulator · Regeneration · Rootstock · Silver nitrate

Introduction

The figleaf gourd (*Cucurbita ficifolia* Bouché), which is synonymous with Malabar gourd, is a member of the Cucurbitaceae. It most likely originated in Central Mexico, subsequently spreading to South America, with an ecological preference for highland areas (Arriaga et al. 2006; Branca and La Malfa 2008; Roxas 1994). The tender immature fruits and young leaves of figleaf gourd are used as a vegetable food in a similar manner as summer squash or cucumber. Figleaf gourd genotypes are exclusively used as a rootstock for cucumber in Far Eastern and Western countries owing to their high physiological compatibility with cucumber (Lee and Oda 2003; Roxas 1994). While the figleaf gourd is highly tolerant to low temperature and salinity, it is susceptible to biotic stresses, such as nematodes and a *Fusarium* spp. (Davis et al. 2008; Lee and Oda 2003). To date, no breeding programs designed to improve the vigor of *C. ficifolia* have been reported, despite the potential value of this plant as a genetic resource for squash breeding by the use of embryo rescue (Roxas 1994).

The genetic improvement of Cucurbitaceae rootstocks has been achieved by conventional plant breeding methods. Recent techniques in plant genetic engineering in other crops have advanced and opened a new avenue for Cucurbitaceae rootstocks (Gal-On et al. 2005; Han et al. 2004, 2005; Park et al. 2005). A system for plant regeneration from individual cells or explants is essential for the application of genetic engineering. In vitro regeneration occurs across a wide spectrum of Cucurbitaceae crops, including summer squash (*Cucurbita pepo* L.) (Kathiravan et al. 2006), winter squash (*Cucurbita maxima* Duch.) (Lee et al. 2003), and bottle gourd (*Lagenaria siceraria* Standl.) (Han et al. 2004; Saha et al. 2007), all of which have the potential to function as a rootstock.

It is becoming increasingly evident that morphogenesis in cultured cells may be associated with ethylene production. While ethylene stimulates shoot morphogenesis

K.-M. Kim · C. K. Kim · J.-S. Han (✉)
College of Ecology and Environmental Science,
Kyungpook National University,
Sangju 742-711, Republic of Korea
e-mail: peterpan@knu.ac.kr

in rice callus (Adkins et al. 1990) and *Pinus radiata* cotyledons (Kumar et al. 1987), it has the reverse effect in corn (Songstad et al. 1988) and Chinese cabbage (Chi et al. 1991). In bottle gourd, the ethylene inhibitor silver nitrate ($AgNO_3$) promotes shoot regeneration efficiency from cotyledon explants when the latter are cultured solely in the presence of 3.0 mg l^{-1} 6-benzylaminopurine (BA) (Han et al. 2004), while it has no positive effect when interacted with different cytokinins (Saha et al. 2007). These results suggest that ethylene and its inhibitor ($AgNO_3$) may have different modes of action in de novo shoot induction, depending on various in vitro culture factors, such as plant genotype. We have therefore investigated whether $AgNO_3$ affects shoot regeneration from cotyledon explants of figleaf gourd. In bottle gourd and cucumber, the distal part of the cotyledon is known to be less responsive than the proximal part in terms of shoot regeneration efficiency (Han et al. 2004; Mohiuddin et al. 1997). In winter squash, cells in the proximal portion of the cotyledon have the potential for adventitious shoot formation (Lee et al. 2003).

The results of our study on in vitro regeneration of figleaf gourd demonstrate the effects of cotyledon location and plant growth regulators on shoot organogenesis. We also investigated the effect of $AgNO_3$ on adventitious shoot regeneration from the cotyledon explants of figleaf gourd. To the best of our knowledge, this is the first report on in vitro regeneration of figleaf gourd.

Materials and methods

Plant materials and sowing

The Kurodane figleaf gourd cultivar (Sakata Seed Co, Yokohama, Japan) was used in all experiments. Manually de-coated seeds were sequentially surface-sterilized in 70% (v/v) ethyl alcohol for 2 min, 0.2% (w/v) sodium dodecyl sulphate for 10 min, 1% (v/v) NaOCl for 20 min, and 0.5% (v/v) NaOCl for 10 min. Between each step, the seeds were rinsed three times with sterile distilled water. Most degenerated perisperms, such as a membrane, were removed from the embryos during the sterilization process. The embryos were blot dried on sterile filter paper for about 5 min, and then ten seeds were sown in 15 × 87-mm petri dishes containing 25 ml of hormone-free MS medium (Murashige and Skoog 1962) solidified with 8 g l^{-1} plant agar (Duchefa Biochemie, Haarlem, The Netherlands) and 30 g l^{-1} sucrose (Duchefa Biochemie). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 18 psi for 20 min. The seeds were incubated at 25°C with illumination for 16 h each day (light intensity 50 μmol m^{-2} s^{-1}).

Culture method

Cotyledon explants of seedlings obtained 5 days after in vitro sowing were cultured on MS medium containing different concentrations and combinations of BA, zeatin, indole-3-acetic acid (IAA), and $AgNO_3$ (Sigma–Aldrich, St. Louis, MO). The cotyledon explants were prepared for culture by first removing the radicals with hypocotyls from the seedlings and then carefully splitting open a pair of cotyledons. Each cotyledon was cut into three 1-cm-long explants lacking plumules (Fig. 1a). The M-type explants (Fig. 1a) were used for the experiments assessing the effects of plant growth regulators (PGRs) and $AgNO_3$, while all types were used for the experiment on the positional potential of the cotyledon in shoot organogenesis. About 3–4 mm of the cut edge of each proximal part was embedded in a 20 × 95-mm petri dish containing 30 ml of medium. Adventitious shoots induced from explants were subcultured on half-strength MS medium containing 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA) for root induction. All cultures were incubated at 25°C under a 16/8-h (light/dark) photoperiod with light supplied by cool-white fluorescent lights (intensity 50 μmol m^{-2} s^{-1}). The plantlets from which medium debris was completely washed out using running tap

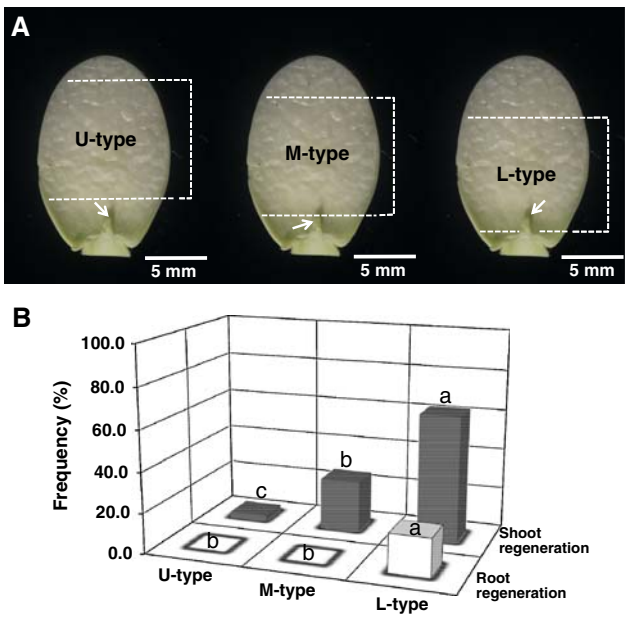

Fig. 1 Diagrams of the cotyledon explant types used in the study and positional effects of cotyledon tissue on shoot and root regeneration. **a** *U-type* Upper part of cotyledon excised at about 3 mm from the top of the plumule (*arrows*), *M-type* middle part of cotyledon excised at the top of the plumule, *L-type* lower part of cotyledon excised at the base of the plumule. None of the cotyledon explants possessed plumules, and the distal parts of the explants were horizontally excised. **b** Shoot and root regeneration frequencies depending on the cotyledon explant types. L-type cotyledon explants had the highest rate of generating adventitious shoots (62.7%)

water were transferred to plastic plug trays (32 holes, hole size 6 × 6 cm) filled with commercial compost (Plant World; Nongwoo Bio Co, Suwon, South Korea) and then put into a 15 × 40 × 60 cm plastic container for 2 weeks. The container was covered with polyethylene bags that were opened at increasing intervals to help the plantlets adapt to the lower humidity of the greenhouse (Han et al. 2004).

Microscopic observation by scanning electron microscopy

Explants were fixed in buffered 4% glutaraldehyde solution (0.1 M, pH 7.0, sodium cacodylate trihydrate), dehydrated in 100% ethanol, critical point dried, and fixed onto metal plates prior to sputter coating with gold to a thickness of 20 nm. Explants were viewed using S-2460 N scanning electron microscopy (SEM; Hitachi, Japan) at 25× magnification.

Data collection and statistical analysis

The responses of cotyledon explants in each treatment were averaged over two or three petri dishes (seven explants per dish), and each experiment as a replicate was repeated five to eight times. The data were subjected to analysis of variance (ANOVA), and mean values were evaluated at the $p < 0.05$ level of significance using Duncan's multiple range test.

Results and discussion

Responses of cotyledon explants to PGRs and $AgNO_3$ in figleaf gourd

The responses of the cotyledon explants to the cytokinins BA and zeatin were investigated. In this experiment, M-type cotyledons were prepared, as shown in Fig. 1a, to avoid an incorrect estimation of regeneration competence caused by spontaneous elongation of the plumules. Few de novo shoots were observed in the treatments with 5.0 mg l^{-1} BA, and 1.0–5.0 mg l^{-1} zeatin. The cytokinins markedly suppressed root organogenesis while promoting callus induction and proliferation (Table 1). The supplementation of cytokinins alone to culture media has been reported to be crucial for adventitious shoot regeneration and/or in vitro micropropagation in numerous Cucurbitaceae cultigens, such as bottle gourd, squashes, and a *Cucurbita* interspecific hybrid (Ananthakrishnan et al. 2003; Han et al. 2004; Lee et al. 2003; Saha et al. 2007; Sarowar et al. 2003). The shoot regeneration frequency of 8.6% obtained in our study is much lower than values previously reported for other Cucurbitaceae species, even though the explants of figleaf gourd were under similar exogenous hormonal regulation. Indeed, the addition of IAA with high concentrations of BA (5.0 or 7.0 mg l^{-1}) is known to improve shoot regeneration efficiency in watermelon (Dong and Jia 1991), and the frequency of shoot regeneration is maximized using the border region near the apical bud in *Cucurbita pepo* (Ananthakrishnan et al. 2003). These results from earlier studies were the basis for further experiments on the effects of additional substances, such as auxins, and the positional effect of cotyledons. Cotyledon explants of figleaf gourd displayed a general pattern of cytokinin-mediated suppression of root organogenesis during root morphogenesis (Table 1). Cytokinins usually promote cell division, especially if added together with an auxin. At higher concentrations (1–10 mg l^{-1}), they can induce adventitious shoot formation, but root formation is generally inhibited (Pierik 1987). Interestingly, the extent of callus proliferation from the cotyledon explants of figleaf gourd observed in our investigation depended on the concentration of the cytokinin (Table 1, Fig. 2a). Taken together, these data suggest the possibility that the cotyledon explants of figleaf gourd naturally contain an endogenous hormone composition with a high auxin/cytokinin ratio. Consistent with this suggestion, Pierik (1987) found that the exogenous PGR requirement (i.e., type of PGR, concentration, auxin/cytokinin ratio) for callus formation strongly depends on the genotype and endogenous hormone content.

Table 1 Effect of BA and zeatin on adventitious shoot and root regeneration, and callus proliferation from cotyledon explants of figleaf gourd

BA	Zeatin	Shoot regeneration frequency (%)	Root regeneration frequency (%)	Callus proliferation[a]
–	–	0.0 ± 0.0 b	77.1 ± 3.5 a	–
0.1	–	0.0 ± 0.0 b	16.3 ± 5.8 b	–
1.0	–	0.0 ± 0.0 b	0.0 ± 0.0 c	+
3.0	–	0.0 ± 0.0 b	0.0 ± 0.0 c	++
5.0	–	4.1 ± 2.6 a,b	2.0 ± 2.0 c	+++
–	0.1	0.0 ± 0.0 b	0.0 ± 0.0 c	+
–	1.0	5.7 ± 3.5 a,b	0.0 ± 0.0 c	++
–	3.0	8.6 ± 5.7 a	0.0 ± 0.0 c	+++
–	5.0	8.6 ± 3.5 a	0.0 ± 0.0 c	+++

M-type cotyledon explants horizontally excised at the top position of the plumule were used

Data are given as the mean value (±SE) of five replicates. Values in a column followed by the same lower case letter are not different at the 5% level (Duncan's multiple range test)

BA, 6-Benzylaminopurine, *SE* standard error

[a] Levels of callus proliferation: –, none; +, poor; ++, moderate; +++, abundant

Fig. 2 Plant regeneration from cotyledon explants of figleaf gourd. **a** Callus proliferation (*arrows*) in medium supplemented only with 5.0 mg l^{-1} zeatin. High concentrations of cytokinins alone promoted callus initiation and further proliferation. **b** Vigorous root growth in medium supplemented with the combination of 1.0 mg l^{-1} zeatin and 1.0 mg l^{-1} 1-naphthaleneacetic acid (NAA). NAA strongly induced root organogenesis from the cotyledon explants. **c** Adventitious shoot regeneration from M-type cotyledon explants in medium supplemented with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} indole-3-acetic acid (IAA). **d** Adventitious shoot regeneration from L-type cotyledon explants in medium supplemented with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA. This treatment achieved the highest shoot regeneration frequency—62.7%. **e** Scanning electron microscopy observation of adventitious shoot regeneration from the proximal part of cotyledon explants. The shoot primordia were generated from the adaxial region of the cotyledon adjacent to the plumule. The *white letters ab* and *ad* indicate the abaxial and adaxial sides, respectively. **f** Elongated and rooted plantlets in medium supplemented with 0.1 mg l^{-1} NAA. **g** Transplanted plantlets in a plug tray for acclimatization. **h** Plants growing and developing in a greenhouse. No plant displayed any morphological alterations

We applied cytokinins alone to induce de novo shoots from the cotyledon explants of figleaf gourd. Cytokinins occasionally increase ethylene production in some plant species, at least partially through increased l-aminocyclopropane-1-carboxylate synthase activity (Abeles et al. 1992; Saha et al. 2007), with ethylene assuming a contrasting role in in vitro regeneration. Ethylene has been reported to have either no effect or a positive influence on organogenesis (Huxter et al. 1981; Kumar et al. 1987), and even to suppress in vitro shoot organogenesis (Chi et al. 1991; Songstad et al. 1988). The latter can be prevented by ethylene inhibitors (Pua 1993; Saha et al. 2007). $AgNO_3$ inhibits the action of ethylene (Beyer 1979), which has beneficial effects on shoot organogenesis in a wide spectrum of plant species, including Cucurbitaceae bottle gourd (Burgos and Alburquerque 2003; Burnett et al. 1994; Han et al. 2004; Songstad et al. 1988; Mohiuddin et al. 1997). In our culture system, the application of $AgNO_3$ to figleaf gourd was not beneficial in all experiments involving high concentrations of cytokinins, rather, it was somewhat suppressive on shoot regeneration (Table 2). In addition, callus induction and proliferation were markedly reduced by high levels of $AgNO_3$ (Tables 1, 2). These observations and those of others (Dong and Jia 1991) indicate that an exogenous cytokinin alone is not crucial for in vitro regeneration of all Cucurbitaceae species. We therefore omitted $AgNO_3$ from the culture media in subsequent experiments.

To ascertain whether the additional supplementation of auxins (IAA and NAA) with cytokinins could positively

Table 2 Effect of plant growth regulators and $AgNO_3$ on adventitious shoot regeneration and callus proliferation from cotyledon explants of figleaf gourd using different cytokinins and different concentration backgrounds

Plant growth regulators (mg l^{-1})		$AgNO_3$ (mg l^{-1})	Shoot regeneration frequency (%)	Callus proliferation[a]
BA	Zeatin			
3.0	–	0.1	0.0	++
3.0	–	0.5	0.0	+
3.0	–	1.0	0.0	+
3.0	–	3.0	0.0	–
3.0	–	10.0	2.9	–
5.0	–	0.1	2.9	++
5.0	–	0.5	0.0	+
5.0	–	1.0	0.0	+
5.0	–	3.0	0.0	+
5.0	–	10.0	0.0	–
–	3.0	0.1	2.0	++
–	3.0	0.5	2.0	+
–	3.0	1.0	2.0	+
–	3.0	3.0	2.0	+
–	3.0	10.0	0.0	–
–	5.0	0.1	2.0	++
–	5.0	0.5	0.0	+
–	5.0	1.0	0.0	+
–	5.0	3.0	2.0	+
–	5.0	10.0	0.0	–

Data are given as the mean value (±SE) of seven replicates

M-type cotyledon explants horizontally excised at the top of the plumule were used

[a] Levels of callus proliferation: –, none; +, poor; ++, moderate; +++, abundant

affect figleaf gourd culture, we tested various combinations of auxins and cytokinins. In 27 of the combinations, adventitious shoots were observed from the proximal ends of cotyledon explants (Table 3). The highest frequency of adventitious shoot regeneration (25.7%) was obtained in the medium supplemented with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA (Table 3, Fig. 2c). When compared to the results in the independent experiment on the supplementation of cytokinins alone (Table 1), auxin augmentation, especially with IAA, positively influenced adventitious shoot regeneration under low concentrations of cytokinins. Both auxins strongly induced root regeneration (Table 3, Fig. 2b), although the regeneration frequencies were reduced in those treatments with comparatively high frequencies of shoot regeneration. These results indicate that the hormonal regulation spectrum for shoot regeneration from cotyledon explants of figleaf gourd is narrow and that NAA may be useful to induce the roots from a regenerated shoot of this species. However, the highest shoot regeneration frequency we observed (25.7%) is still not enough to integrate this protocol into an efficient genetic transformation system.

Efficient type of cotyledon explant for shoot regeneration in figleaf gourd

The size and detached position of cotyledon explants are important factors that influence the induction of de novo shoots in Cucurbitaceae (Ananthakrishnan et al. 2003; Han et al. 2004; Lee et al. 2003).

In the search for a promising cotyledon explant type, we investigated three 1-cm-long cotyledon types in a culture system supplemented with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA. The highest shoot regeneration frequency was obtaining using L-type cotyledon explants (Fig. 2d)—62.7%; in sharp contrast, shoot regeneration frequencies of only 25.7 and 2.9% were obtained using M- and U-type cotyledons, respectively (Fig. 1b). In addition, 19.1% of L-type explants, including the regenerative ones, also spontaneously rooted in the same medium. We also observed adventitious shoots initiating from the adaxial regions of cotyledons adjacent to the plumule (Fig. 2e).

Our results suggest that explants possessing the region immediately adjacent to the plumule are likely to generate adventitious shoots. Ananthakrishnan et al. (2003) clearly demonstrated that the initiation of shoot formation is strictly restricted to the region within the junction of the hypocotyl and cotyledon in a summer squash. Their finding may be in accordance with our result using another Cucurbitaceae species, figleaf gourd. Lee et al. (2003) obtained different shoot regeneration efficacies depending on the explant size of the cotyledon in a winter squash. We also investigated whether horizontal thin layers about 1 mm long excised from a variety of cotyledon positions could generate adventitious shoots, but no adventitious shoots were obtained (data not shown). This negative result may have been caused by an adverse endogenous condition, especially in terms of nutrient and/or hormone composition. Regenerated shoots successfully elongated and rooted in the MS medium supplemented with 0.1 mg l^{-1} NAA after 10–15 days of subculturing (Fig. 2f). The plantlets were satisfactorily acclimatized (Fig. 2g) and grew into normal individuals without any morphological alterations in a greenhouse (Fig. 2h).

To summarize, we report a plant regeneration protocol for figleaf gourd, which is a valuable rootstock for Cucurbitaceae crops, especially cucumber. The highest shoot regeneration frequency (62.7%) was obtained by culturing proximal cotyledon explants obtained near the plumule in medium supplemented with 1.0 mg l^{-1} zeatin and 0.1 mg l^{-1} IAA. We recently determined appropriate antibiotics and their concentrations for the selection of

Table 3 Combination effect of plant growth regulators on adventitious shoot and root regeneration from cotyledon explants in figleaf gourd

Plant growth regulators (mg l^{-1})			Shoot regeneration frequency (%)	Root regeneration frequency (%)	Plant growth regulators (mg l^{-1})			Shoot regeneration frequency (%)	Root regeneration frequency (%)
BA	IAA	NAA			Zeatin	IAA	NAA		
0.1	0.1	–	5.7 ± 5.7 b,c,d	80.0 ± 5.7 a,b,c	0.1	0.1	–	14.3 ± 4.5 b,c	54.3 ± 8.3 e
0.1	1.0	–	5.7 ± 3.5 b,c,d	82.8 ± 7.0 a,b	0.1	1.0	–	14.3 ± 4.5 b,c	77.1 ± 3.5 c,d
0.1	3.0	–	2.9 ± ± 2.9 c,d	94.3 ± 3.5 a	0.1	3.0	–	8.6 ± 3.5 c,d	82.8 ± 5.4 c
0.1	–	0.1	2.9 ± 2.9 c,d	94.3 ± 3.5 a	0.1	–	0.1	0.0 ± 0.0 d	82.8 ± 5.4 c
0.1	–	1.0	0.0 ± 0.0 d	77.1 ± 7.3 b,c	0.1	–	1.0	0.0 ± 0.0 d	85.7 ± 4.5 b,c
0.1	–	3.0	0.0 ± 0.0 d	54.3 ± 5.3 d	0.1	–	3.0	0.0 ± 0.0 d	68.5 ± 5.4 d
1.0	0.1	–	20.0 ± 5.7 a	5.7 ± 3.5 f	1.0	0.1	–	25.7 ± 5.4 a	2.9 ± 2.9 g
1.0	1.0	–	17.1 ± 2.9 a,b	34.3 ± 5.7 e	1.0	1.0	–	20.0 ± 3.5 a,b	17.2 ± 2.9 f
1.0	3.0	–	11.4 ± 7.0 a,b,c,d	42.9 ± 7.8 d,e	1.0	3.0	–	17.2 ± 5.4 a,b,c	20.0 ± 3.5 f
1.0	–	0.1	14.3 ± 6.4 a,b,c	85.7 ± 6.4 a,b	1.0	–	0.1	14.3 ± 4.5 b,c	80.0 ± 7.3 c,d
1.0	–	1.0	2.9 ± 2.9 c,d	85.7 ± 7.8 a,b	1.0	–	1.0	5.7 ± 3.5 c,d	100.0 ± 0.0 a
1.0	–	3.0	2.9 ± 2.9 c,d	68.6 ± 7.0 c	1.0	–	3.0	0.0 ± 0.0 d	97.1 ± 2.9 a,b
3.0	0.1	–	1.8 ± 1.8 d	0.0 ± 0.0 f	3.0	0.1	–	17.2 ± 5.4 a,b,c	0.0 ± 0.0 g
3.0	1.0	–	3.6 ± 2.3 c,d	1.8 ± 1.8 f	3.0	1.0	–	14.3 ± 4.5 b,c	2.9 ± 2.9 g
3.0	3.0	–	3.6 ± 3.6 c,d	1.8 ± 1.8 f	3.0	3.0	–	5.7 ± 3.5 c,d	2.9 ± 2.9 g
5.0	0.1	–	5.4 ± 2.6 c,d	0.0 ± 0.0 f	5.0	0.1	–	0.0 ± 0.0 d	0.0 ± 0.0 g
5.0	1.0	–	3.6 ± 2.3 c,d	1.8 ± 1.8 f	5.0	1.0	–	0.0 ± 0.0 d	2.9 ± 2.9 g
5.0	3.0	–	7.2 ± 3.8 b,c,d	8.9 ± 5.4 f	5.0	3.0	–	0.0 ± 0.0 d	8.6 ± 5.7 f,g

Data are the mean value (±SE) of five to eight replicates. Values in a column followed by the same lower case letter are not different at the 5% level (Duncan's multiple range test)

M-type cotyledon explants horizontally excised at the top position of plumule were used

IAA Indole-3-acetic acid, *NAA* naphthaleneacetic acid

transgenic shoots from the L-type cotyledon explants (Fig. 2a) using *Agrobacterium tumefaciens* C58C1, which is a null strain without any vectors for plant transformation (data not shown). We believe that the regeneration protocol for figleaf gourd that we report here will aid in the development of a *Agrobacterium*-mediated genetic transformation system in this rootstock.

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