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High-frequency shoot multiplication in *Curcuma longa* L. using thidiazuron

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Abstract The effects of plant growth regulators, explant types, and culture regimens were investigated on in vitro shoot proliferation from terminal bud explants of *Curcuma longa*. Each bud was longitudinally divided into four equal pieces, each 1 cm in length, and used as explants. These were then cultured on MS medium supplemented with 18.17 μM thidiazuron for 4 weeks prior to transfer to MS medium without growth regulator for 8 weeks. Under these conditions, a shoot induction rate of 18.22±0.62 shoots/explant was obtained after 12 weeks of cultures. Spontaneous rooting was achieved. The regenerated plants were transferred to soil under greenhouse conditions and subsequently grown successfully in the field.

Keywords *Curcuma longa* · In vitro propagation · Thidiazuron · Turmeric

Abbreviations *BA:* 6-Benzylaminopurine · *2iP:* N^6-(2-Isopentyl)adenine · *Kin:* Kinetin · *NAA:* α-Naphthaleneacetic acid · *PGR:* Plant growth regulator · *TDZ:* Thidiazuron

Introduction

Curcuma longa L. (Zingiberaceae), turmeric, is a tropical herb widely used both in food and traditional medicine. In Thai traditional medicine, turmeric is used as a carminative, for dyspepsia, and also externally for itching and infected wounds (Saralamp et al. 1996). Pharmacological and clinical studies have indicated the efficacy of turmeric for the treatment of dyspepsia (Thamlikitkul et al. 1989), peptic ulcers (Prucksunand et al. 2001), and gastric ulcers (Muderji et al. 1981; Sakai et al. 1989; Rafatullah et al. 1990; Kositchaiwat et al. 1993; Masuda et al. 1993). For medicinal purposes, the quality of turmeric is based on the amounts of curcuminoids and volatile oil present, both pharmacologically active compounds (World Health Organization 1999).

Although the cultivation of medicinal plants has many advantages, little effort has been expended with respect to relevant agronomic research in that direction (Palevitch 1988; Tyler 1988). The plants continue to be cultivated in the same way as they always have been. Most cultivated medicinal plants, excluding *Papaver somniferum*, *P. bracteatum*, *Cinchona* spp., *Digitalis lanata*, *Chamomilla recutita*, and *Mentha piperita*, are still genetically "wild" types (Tyler 1988; Chomchalow 1993). We have found a large variation in curcuminoid and volatile oil contents among turmeric grown in different parts of Thailand (unpublished data). Consequently, we believe that there is a strong need to genetically improve and cultivate turmeric with high and stable contents of curcuminoids and volatile oil.

In vitro propagation offers many advantages over conventional propagation methods. True-to-type multiplication provides uniform plants with genetic identity. Morphological and chemical uniformity among plants regenerated by this technique has been reported in various species of medicinal plants, such as shoot-tip and axillary bud cultures of *Aconitum carmichaeli* (Hatano et al. 1988), shoot-tip cultures of *Atractylodes* spp. (Hatano et al. 1990), node cultures of *Gentiana scabra* (Yamada et al. 1991), and stem-tip cultures of *Stevia rebaudiana* (Tamura et al. 1984).

Our investigations are aimed at applying plant tissue culture techniques to generate high-quality somaclones of turmeric with an increased, constant levels of curcuminoids and volatile oils for phytomedicine production. Although a number of protocols for in vitro propagation

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of *C. longa* have been published using bud or shoot-tip explants (Yasuda et al. 1988; Keshavachandran and Khader 1989; Winnaar 1989; Balachandran et al. 1990; Rajan 1997; Shirgurkar et al. 2001; Sunitibala et al. 2001; Salvi et al. 2002), leaf-base explants (Salvi et al. 2001), and immature inflorescence explants (Salvi et al. 2000), these procedures are inadequate to meet the need in time. We report here a system for high-frequency shoot multiplication of *C. longa* terminal bud explants using TDZ.

Materials and methods

Plant materials

Shoots were collected from 2-month-old *Curcuma longa* plants and washed thoroughly in running tap water. Sections of shoots 2 cm long were treated with 70% ethanol for 1 min, rinsed with distilled water, immersed for 20 min in a commercial bleach solution of 1.5% sodium hypochlorite containing 50 μl Tween 80, and finally rinsed three times in sterile distilled water under sterile conditions. Leaf sheaths of the shoots were trimmed off. Only terminal buds were collected and surface sterilized with a commercial bleach solution of 0.75% sodium hypochlorite containing 50 μl Tween 80 for 10 min, then rinsed three times in sterile distilled water. The terminal buds were cultured on MS medium (Murashige and Skoog 1962) supplemented with 13.32 μM BA, 3% sucrose, and 0.55% Agargel for 4 weeks, then transferred to PGR-free MS medium. The plants were subcultured every 4 weeks until the beginning of the experiments.

Explants, media, and culture conditions

The effects of PGRs, explant type, and explant age on shoot regeneration of *C. longa* were studied in three parallel experiments.

1. To study the effect of TDZ alone and in combination with NAA on shoot multiplication, we used both undivided and divided terminal bud explants. For divided terminal bud explants, each bud from in vitro donor plants was longitudinally divided into four equal pieces, each 1 cm in length (Fig 5A). The explants were inoculated vertically on MS medium supplemented with TDZ (0.23–18.17 μM), either alone or in combination with 0.54 μM NAA (Fig 5B). Eight weeks after culture initiation, the explants were transferred to MS medium without PGR (experiment 1).
2. To study the effect of a high concentration of cytokinins on shoot multiplication, we used divided terminal bud explants. The explants were cultured on MS medium supplemented with 35.51 μM BA, 37.17 μM Kin, 39.37 μM 2iP, and 18.17 μM TDZ for 8 weeks (experiment 2A) or 4 weeks (experiment 2B), followed by transfer to MS medium without PGR for 2 weeks.
3. To establish and prove the efficiency of a novel protocol for rapid micropropagation of *C. longa*, we used the divided terminal buds from 1- to 3-month-old donor plants as explants. The explants were cultured on MS medium supplemented with 18.17 μM TDZ for 4 weeks, followed by transfer to MS medium without PGR for 8 weeks (experiment 3).

All media contained 3% sucrose and 0.55% Agargel, and the pH was adjusted to 5.8 before autoclaving. For each treatment, a total of 12 replications each with four explants were inoculated; therefore, 48 explants per treatment were tested. Experiment 3 was repeated five times. Cultures were maintained at 25°C under a 16/8-h (light/dark) photoperiod with a light intensity of 3,000 lux.

Acclimatization and field experiment

After rooting on PGR-free MS medium, regenerated plants were transferred to small pots containing sand and rice shell ash (1:1) under greenhouse conditions for 1 month. The regenerants were then transplanted to the field at the Salaya campus of the Mahidol University, Thailand, and grown for 8 months.

Results and discussion

The incubation of *C. longa* terminal bud explants on MS media supplemented with TDZ (0.23–18.17 μM) for 8 weeks resulted in 3.42–5.08 shoots/explant for undivided explants (Fig. 1A) and 3.47–4.36 shoots/explant for divided explants (Fig. 2A). Balachandran et al. (1990) reported a proliferation rate of 3.43 shoots/bud after growing terminal buds on MS supplemented with

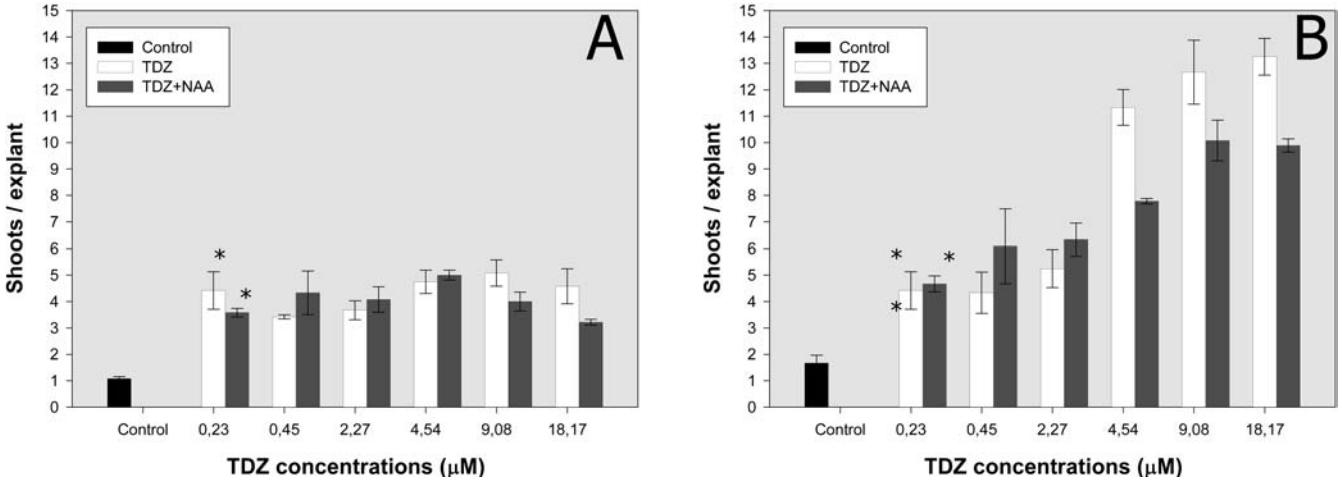

Fig. 1A, B Effect of TDZ alone and in combination with NAA on the shoot induction of undivided terminal bud explants. **A** Eight weeks after inoculation on MS media supplemented with TDZ and NAA, **B** 4 weeks after transfer to PGR-free MS medium. *Vertical lines* Standard error, *single asterisk* non-significant differences, *double asterisk* significant differences (P=0.023) using polynomial regressions

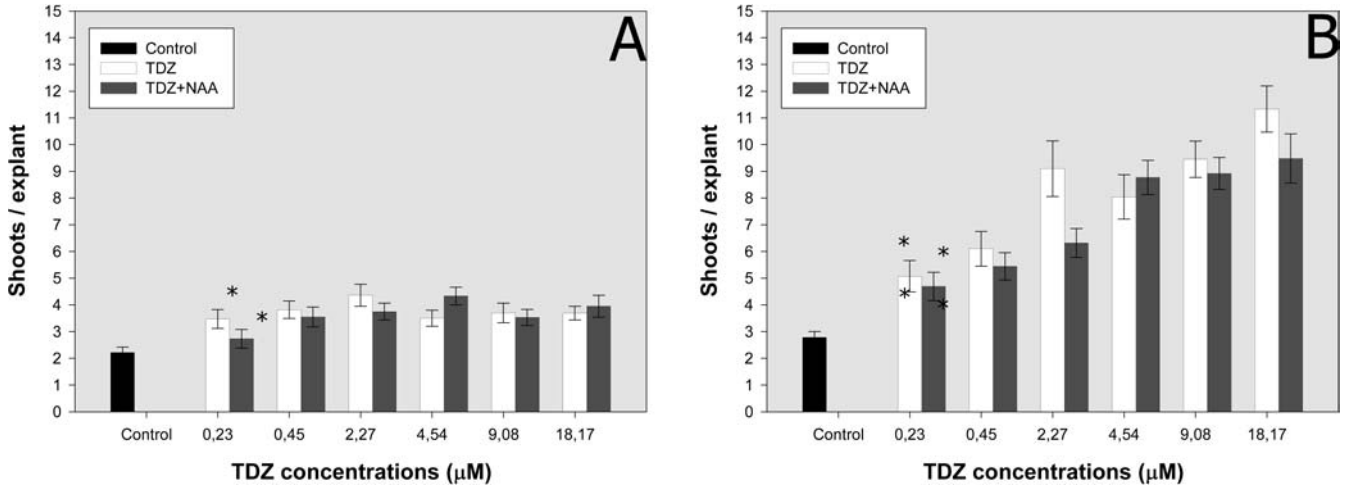

Fig. 2A, B Effect of TDZ alone and in combination with NAA on the shoot induction of divided terminal bud explants. **A** Eight weeks after inoculation on MS media supplemented with TDZ and NAA, **B** 4 weeks after transfer to PGR-free MS medium. *Vertical lines* Standard error, *single asterisk* non-significant differences, *double asterisk* significant differences (P=0.032 and P=0.046 for TDZ treatment and TDZ and NAA treatment, respectively) using polynomial regressions

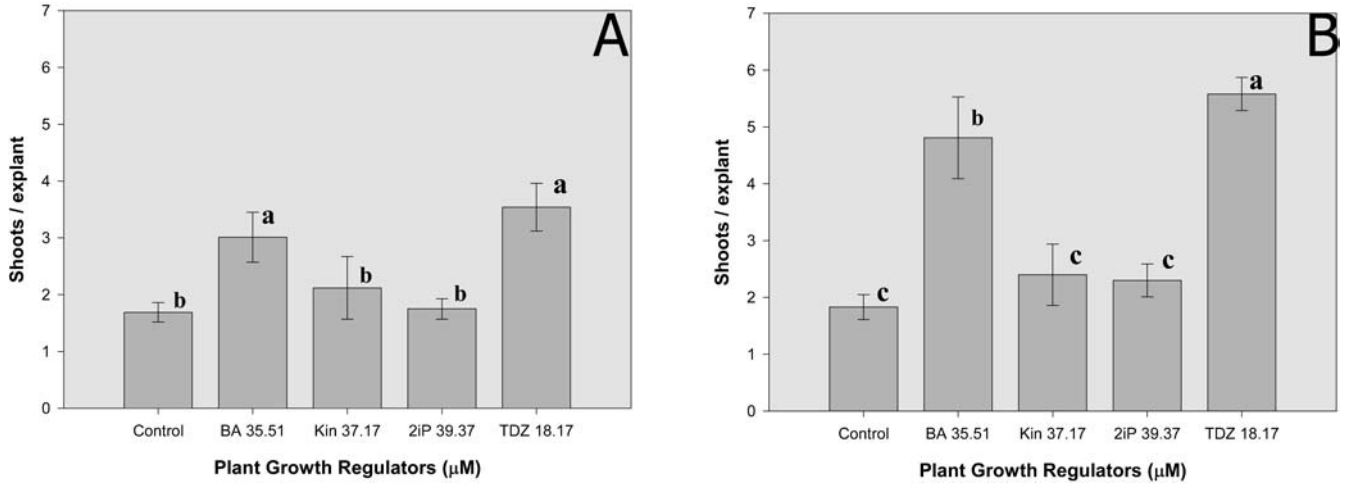

Fig. 3A, B Effect of cytokinins on the shoot induction of divided terminal bud explants. **A** Eight weeks after inoculation on PGR-MS medium, **B** 2 weeks after transfer to PGR-free MS medium. *Vertical lines* Standard error. *Bars* with the *same letter* are not significantly different at the 5% level by the Student-Newman-Keuls test

13.32 μM BA for 4 weeks. Salvi et al. (2002) also reported shoot multiplication rates of 4.2, 3.5, and 6.6 shoots/explant following the culture of shoot-tip explants for 8 weeks in liquid medium supplemented with 1 μM NAA and BA, Kin, or 2iP (10 μM each), respectively. Although we obtained lower shoot induction rates than those reported in previous publications, we were able to demonstrate that the terminal buds of *C. longa* can be divided into four parts and used as explants for in vitro propagation.

Four weeks after the transfer of the explants to MS medium without PGR for 4 weeks, both the shoot induction rate and shoot length had increased in all treatments (Figs. 1B, 2B). In both explant types, the maximum shoot induction rates were obtained at a TDZ concentration of 18.17 μM (13.25±0.70 and 11.33±0.86 shoots/explant for undivided and divided explants, respectively). TDZ, a non-purine cytokinin-like compound, has been shown to exhibit stronger effects than conventional cytokinins over a wide range species. It is effective for axillary shoot proliferation and adventitious shoot organogenesis (Huetteman and Preece 1993). Its mode of action may be attributed to its ability to induce cytokinin accumulation (Victor et al. 1999) and also enhance the accumulation and translocation of auxin within TDZ-exposed tissue (Murch and Saxena 2001).

Our results indicate that exposure of the explants to a high concentration of cytokinins prior to transfer to PGR-free MS medium led to the increased shoot proliferation of *C. longa*. To determine the optimum period that *C.*

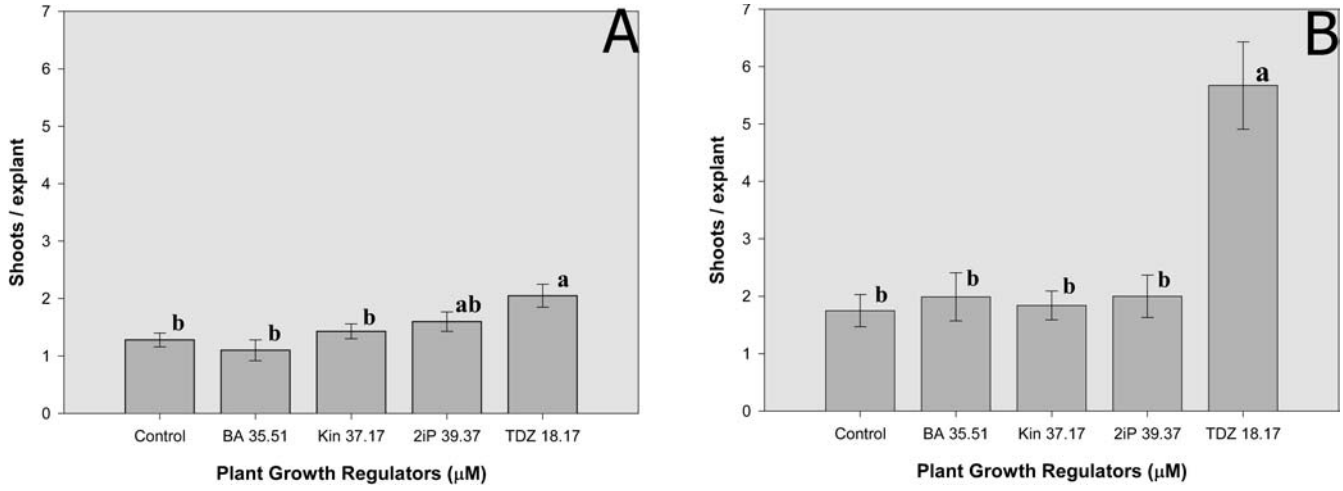

Fig. 4A, B Effect of cytokinins on the shoot induction of divided terminal bud explants. **A** Four weeks after inoculation on PGR-MS medium, **B** 2 weeks after transfer to PGR-free MS medium. *Vertical lines* Standard error. *Bars* with the *same letter* are not significantly different at the 5% level by the Student-Newman-Keuls test

longa cultures should be exposed to cytokinins, we carried out experiments 2A and 2B. The average frequency of explants responding in the control, BA, Kin, 2iP, and TDZ treatments were 68%, 51%, 58%, 64%, and 74%, respectively, calculated across both experiments. In experiment 2A, after the divided explants were cultured on MS medium supplemented with 35.51 μM BA, 37.17 μM Kin, 39.37 μM 2iP, or 18.17 μM TDZ for 8 weeks, the highest shoot multiplications were obtained in the TDZ and BA treatments [3.54±0.42 and 3.01±0.44 shoots/explant, respectively (Fig. 3A)]. When the explants were transferred to MS medium without PGR for 2 weeks, the number of induced shoot per explant increased markedly in both the TDZ and BA treatments [5.58±0.29 and 4.81±0.72 shoots/explant, respectively (Fig. 3B)].

In experiment 2B, the length of the incubation period of the divided explants on the PGR-supplemented MS media was reduced to 4 weeks. The shoot multiplication rates were between 1.11 shoots and 2.05 shoots per explant (Fig 4A). When the explants were transferred to PGR-free MS medium for 2 weeks, only the TDZ treatment demonstrated the dramatically increased shoot induction rate to 5.67±0.76 shoots/explant (Fig. 4B).

After transfer of the explants to PGR-free MS medium, the maximum shoot induction rates were essentially the same for those cultured 8 weeks or 4 weeks in TDZ-supplemented MS medium (5.58±0.29 and 5.67±0.76 shoots/explant, respectively). Although the cluster of shoots induced by TDZ were about 1 cm in length, they elongated to almost 3 cm following their transfer to PGR-free MS medium. In comparison with TDZ, only BA could increase shoot induction rate when the explants were incubated 8 weeks. No increased shoot proliferation effect was observed after the Kin and 2iP treatment of 4 weeks or 8 weeks.

In general, a low range of concentrations of TDZ—from 1 nM to 10 μM—has been recommended for shoot proliferation (Huetteman and Preece 1993). High levels of TDZ cause abnormalities or are toxic (Kim et al. 1997; Chand et al. 1999). Our results indicate that an optimum exposure time of explants in TDZ-supplemented medium followed by the withdrawal of PGR effectively triggered shoot multiplication in *C. longa*. The PGR may be needed for initiating the multiplication of bud meristems. Subsequently, incubation on PGR-free MS medium led the explants to elongation of regenerated shoots as well as induction of more shoot initials.

In experiment 3, we established an improved protocol for the micropropagation of *C. longa*. The divided terminal bud explants were cultured on MS medium supplemented with 18.17 μM TDZ for 4 weeks prior to their transfer to MS medium without PGR for 8 weeks. After 4 weeks of incubation on TDZ, the shoot induction rate was 2.22±0.14 (Fig. 5C), and this increased to 7.12±0.52 and 18.22±0.62 shoots/explant at the 8th week and 12th week, respectively (Fig. 5D). The average shoot length was 3.85 cm after 12 weeks of culture. Rooting was spontaneously achieved (Fig 5E), with an average frequency of 88.4±2.6% (Table 1). The induced shoots that did not produce any roots were transferred to MS medium without PGR for another 4 weeks; every one of those shoots subsequently produced roots. The regenerated plants were transferred to soil under greenhouse conditions and successfully grown under the field conditions (Fig 5F–H). They did not show any detectable variation in morphology or growth characteristics compared to their donor plant.

Although, we used explants derived from 1 to 3-month-old donor plants, no significant difference among shoot induction rates from explants of different ages was found at week 12. Our results demonstrate that the age of terminal bud explants does not affect the shoot induction

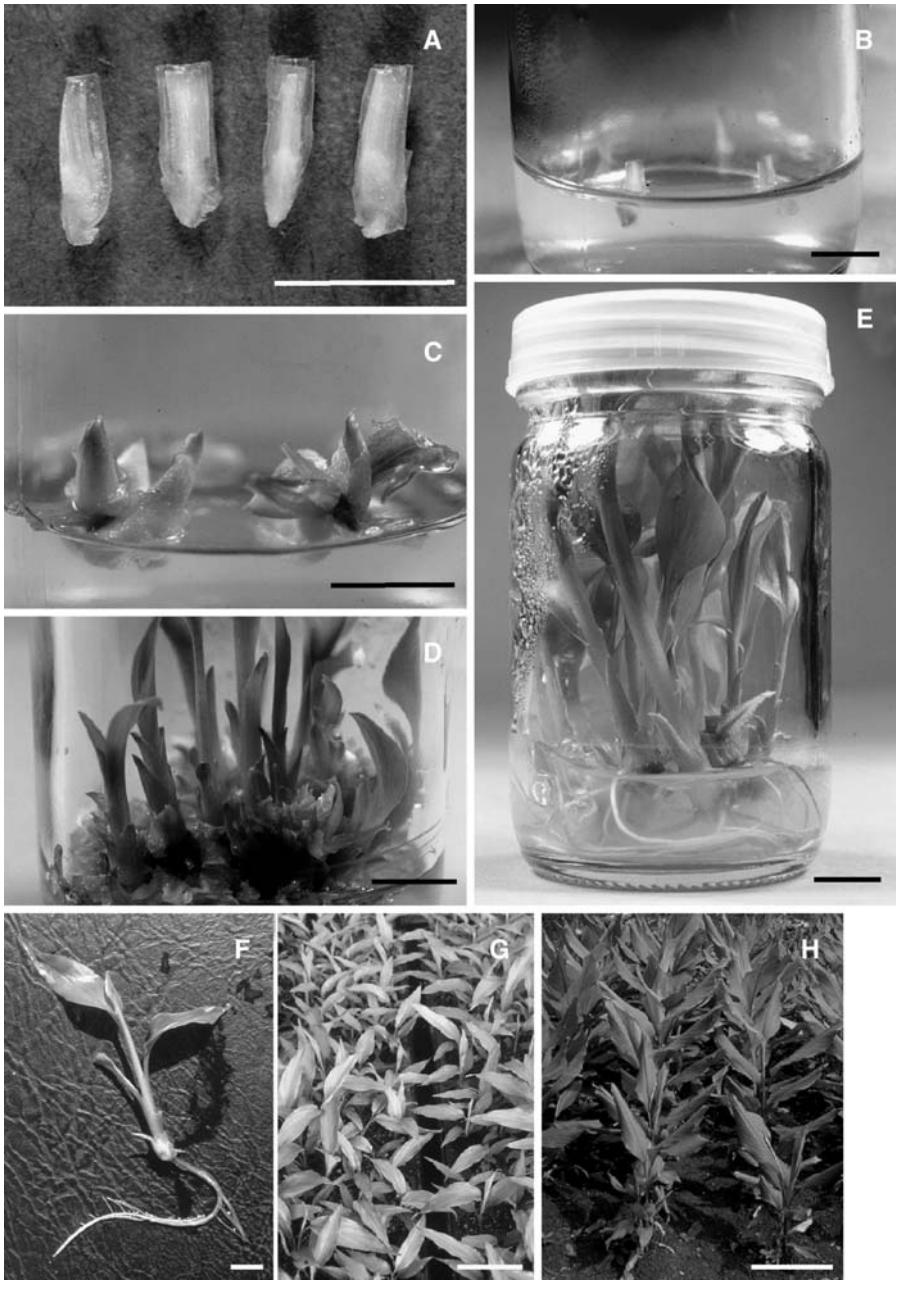

Fig. 5A–H A protocol developed for rapid micropropagation of *Curcuma longa*. **A** Divided terminal bud explant, **B** inoculated explant, **C** explants with newly developed shoots, **D** a cluster of shoots after transfer to PGR-free medium, **E**, **F** rooted plantlets, **G** *C. longa* plantlets after acclimatization, **H** micropropagated *C. longa* in the field. *Bars*: **A–F** 1 cm, **G** 5 cm, **H** 50 cm

Table 1 The frequency of response of explants, shoot formation, and the frequency of rooting of the explants. Data (mean ± S.E.) were collected every 4 weeks from five experiments, each with 12 replicas

Duration of cultures	Percentage of regenerating explants	Shoot formation		Percentage rooting
		Shoots/explant	Shoot length (cm)	
4 weeks	75.4±3.2	2.22±0.14	1.18±0.11	9.8±1.9
8 weeks	86.6±3.1	7.12±0.52	1.51±0.21	38.4±6.5
12 weeks	90.2±3.0	18.22±0.62	3.85±0.31	88.4±2.6

rate of *C. longa*, which is contrary to those effects reported in woody plants such as *Corylus avellana* (Betulaceae) (Messeguer and Mele 1987), *Wrightia tomentosa* (Apocynaceae) (Purohit et al. 1994), *Maytenus ilicifolia* (Celastraceae) (Pereira et al. 1995), and *Morus* (Moraceae) (Pattnaik and Chand 1997).

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