

Effects of light and cytokinin on *in vitro* micropropagation and microrrhizome production in turmeric (*Curcuma longa* L.)

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Abstract Using clonal *in vitro* propagated plantlets of *Curcuma longa*, the effects of light and cytokinin on micropropagation and microrrhizome formation were investigated. The results showed the promotive effects of low light intensity for micropropagation and of short day condition for well-developed microrrhizome induction. In the tissue of well-developed microrrhizomes, differentiation of yellowish-pigmented cells similar to the secretory cells of rhizome of the mother plant was observed. Staining of the cells with Nile red suggested the accumulation of essential oil. Rapidly growing callus was isolated from the root tip of micropropagated plantlets which retained totipotency during subculture. High concentration of kinetin induced microrrhizome formation in this callus. Yellow pigmented cells were also observed in the tissue of microrrhizome, although far less frequency.

Key words: Light effects, micropropagation, microrrhizome, secretory cell, turmeric.

About 50 species of the genus *Curcuma* in the Zingiberaceae have been found in the world. *Curcuma* plants, which are distributed in the tropical and subtropical regions of India and south-east Asia, require abundant water and high temperature for growth. In Japan, *Curcuma* is found in Okinawa and southern region of Kyusyu. Throughout Asia, the rhizome of *Curcuma* species is widely used as aromatic stomachic, blood purifier, tonic and antiseptic in traditional medicine and also as spices and sources of yellow dye (Sugaya 1992). *Curcuma longa* commonly known as turmeric is one of the most ancient and important aromatic ingredient for food additives. Recently, the chemical components of the rhizome were analyzed extensively and the pharmaceutical activities of the components were gradually clarified. Chattopadhyay et al. (2004) reviewed the actions and medicinal applications of turmeric and curcumin, a major constituent of rhizome of turmeric. The demand for the rhizome is increasing.

Because flowering is rare and seed set does not occur, turmeric is exclusively propagated through the conventional and time-consuming method of rhizome division. The maximum multiplication that can be

achieved annually is 5–10-fold. Given the limitations of vegetative reproduction, the only method for improvement is the selection of plants with desirable traits. There has long been a need of techniques which allow for the rapid clonal propagation of turmeric plants (Shirgurkar et al. 2001).

The large scale propagation of plants by tissue culture is widely spread and the number of plants species, which can be multiplied by this technique has been steadily increasing (e.g. Bhojwani and Razdan 1983). In *Curcuma*, Mukhri and Yamaguchi (1986) have succeeded in a clonal propagation of two *Curcuma* species, *C. longa* and *C. xanthoria*. Following this initial work, successful propagation has been reported in *C. longa* (Nadgauda et al. 1978; Sato et al. 1987; Salvi et al. 2000; Meenakshi et al. 2001; Sunitibara et al. 2001; Parthanturug et al. 2003), *C. aromatica* (Nayak 2000), *C. zedoaria* (Mello et al. 2000) and *C. amada* (Roy and Raychaudhuri 2004). Regulation of microrrhizome production on the micropropagated plants has been reported in *C. longa* (Shirgurkar et al. 2001; Sunitibara et al. 2001), *C. amada* (Nayak 2002) and *C. zedoaria* and *C. aromatica* (Roy and Raychaudhuri 2004). Detection of secondary metabolites of the mother plant in the

Abbreviations: BA, 6-benzylaminopurine; DFL, diffuse fluorescent light; NAA, 1-naphthaleneacetic acid; NK medium, medium containing 1 mg l⁻¹ of NAA and 0.1 mg l⁻¹ of kinetin

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microrhizome *in vitro* was attempted (Yasuda et al. 1988; Sunitibara et al. 2001), but the results were inconclusive. Differentiation of secretory cells in which the secondary metabolites are accumulated has never been reported.

The use of cultured cells is another approach for the reproduction of regenerated plants and for the regulation of organ formation. Differentiation of shoots in *C. longa* and roots and globular embryoids in *C. xanthorrhiza* were observed, when callus formed on excised buds was recultured (Mukhri and Yamaguchi 1986). Shoot differentiation from successively subcultured callus has also been reported in *C. longa* (Salvi et al. 2001; Shirgurkar et al. 2006).

Although effects of plant growth regulators and sucrose concentrations on micropropagation and *in vitro* formation of microrhizome has been extensively investigated, the impact of environmental factors has scarcely been examined in *C. longa*, except one report (Sunitibara et al. 2001). In this paper, effects of light on the micropropagation and microrhizome formation are investigated and the production of microrhizome from the callus is reported.

Materials and methods

Culture medium

Murashige and Skoog's (MS) medium containing 3% sucrose and combination of various concentrations of NAA and kinetin was used. The medium was solidified with 0.9% agar. After pH was adjusted to 5.8, the medium was autoclaved for 20 min at 120°C.

Plant materials

Rhizome of *Curcuma longa* was purchased from Takii Seed Co., Ltd. Buds (1–2 cm) were excised from fresh rhizome, sterilized with 1/2 commercial hypochlorite solution for 15 min and washed 3 times with sterilized water. The excised buds were placed onto MS medium containing 2 mg l⁻¹ of NAA and 0.2 mg l⁻¹ of kinetin in the dark, occasionally irradiated by diffuse fluorescent light (DFL). The light intensity was 0.067 μmol m⁻² s⁻¹ at the surface of culture box. After 5 weeks at 25°C, the tissues with regenerated shoots and roots (regenerated plantlets) were propagated from the explants. After cutting the leaf at about 2 cm long from the agar surface, the regenerated plantlets were divided into 3–4 pieces and transferred to fresh medium containing 1 mg l⁻¹ of NAA and 0.1 mg l⁻¹ of kinetin, named NK medium. Thereafter, the regenerated plantlets were subcultured to fresh NK medium by the same method every one month. The clonally propagated plantlets were used at one month after subculture.

Experiments for micropropagation and microrhizome formation

Tissue segments with two regenerated plantlets (about 2 cm long shoot) were transferred onto NK medium and cultured under different light conditions as described in "Results and discussion". After the culture period, number of shoots longer than 2 cm and rhizomes longer than 1.5 cm was counted. Five

culture pots were used in each treatment and experiment was repeated at least twice.

Callus culture

The callus was isolated from the root tip of the regenerated plantlets cultured on NK medium and subcultured on the medium of the same composition every 3 weeks. The calluses were used as experimental materials at 3 weeks after subculture.

Observation by microscope

The tissues stored in refrigerator were cut into thin sections by hand or by microtome (VIBRATOME series 1000 sectioning system, Technical Products International, Inc.) The section was placed directly onto a slide glass. After one drop of water or dye solution was dripped, a coverslip was laid on it and the section was observed by fluorescent microscope (Olympus System microscope Model BX50-FLA, Olympus Corporation.).

Results and discussion

Clonal propagation

Under DFL condition, buds excised from the rhizome of *C. longa* were cultured on MS medium containing NAA (0, 0.2, 2 or 20 mg l⁻¹) and kinetin (0, 0.02, 0.2 or 2 mg l⁻¹) in various combinations. After 3 weeks, the basal parts of the excised buds expanded and number of roots developed vigorously, when the medium contained 2 mg l⁻¹ of NAA with 0.2 or 0.02 mg l⁻¹ of kinetin or without kinetin. After 5 weeks, many shoots with roots elongated from the basal expanded tissues of excised buds. Kinetin at the concentration of 0.2 mg l⁻¹ in the presence of 2 mg l⁻¹ of NAA was the most favorable for the formation of shoots. Tissues with regenerated plantlets were successively subcultured on NK medium as described in Materials and methods. The clonally propagated plantlets were used for the following experiments.

Effects of light on micropropagation

When the tissues with regenerated plantlets were cultured on NK medium, it was found that the light intensity strongly affected micropropagation results. Although many plantlets (5 to 7 plantlets/culture pot) developed in the dark at 25°C (Figure 1A), more plantlets (6 to 9 plantlets/culture pot) were regenerated under DFL condition (Figure 1B). In continuous light (about 32 μmol m⁻² s⁻¹) at 25°C, one or two shoots grew vigorously, but the number of the regenerated plantlets was dramatically suppressed (Figure 1C). As shown in Figures 1D and 1E, the similar results were obtained under long day condition (16 h light–8 h dark) and short day condition (10 h light–14 h dark) at 23 or 28°C in the growth chamber (Ikagakukiki Co., Ltd.). Light intensity was about 60 μmol m⁻² s⁻¹ at the surface of culture flask. These results show weak diffuse light is favorable for

micropropagation.

The medium supplemented with higher than 1 mg l^{-1} ($1\text{--}30 \text{ mg l}^{-1}$) of cytokinins was effective for multiple shoot formation in *C. longa* (Sato et al. 1987), *C. longa* and *C. aeruginosa* (Balachandran et al. 1990) and *C. zedoaria* (Yasuda et al. 1987; Mello et al. 2000), or higher ratio of concentration of cytokinin to auxin (Roy and Raychaudhuri 2004). In these experiments, the plant materials *in vitro* were cultured under higher light intensity in growth chamber than the light conditions favorable for micropropagation in our experiment. The higher the light intensity, the higher concentrations of cytokinins may be required for shoot multiplication.

Root induction and development declined with increasing concentrations of cytokinin (Nadgauda et al. 1978). Consequently, an additional step for rooting was needed for clonal propagation. Under our experimental conditions of low light intensity and low concentration of cytokinin, both shoots and roots were induced and the rooting step can be omitted.

Effects of light conditions on microrrhizome formation

When micropropagated plantlets were cultured on NK medium under DFL condition, the microrrhizome formation was observed by chance. Its form was ellipsoid. The color of the surface of microrrhizome was cream yellow and its size was about 2 cm in length and 1 cm in width (Figure 2). The frequency of microrrhizome formation is very low, only one case in more than 150 culture pots during subculture of micropropagated plantlets over 18 months.

In order to determine the exact factor(s) of microrrhizome induction, the effects of light were examined, as the day length has been reported to play important role in *in vitro* induction of other storage organs such as tubers (Gopal et al. 1998; Grewal et al. 1996; Vreugdehil et al. 1998) and bulbs (Slabbert and Niederwieser 1999). The tissues with two clonally propagated plantlets about 2 cm height were transferred to NK medium and cultivated under different light conditions described above, DFL condition, continuous dark, continuous light, short day condition and long day condition. The regenerated plantlets were also transferred to the medium with various combinations of concentrations of NAA (0, 0.01, 0.1 and 1 mg l^{-1}) and kinetin (0, 0.01, 0.1 and 1 mg l^{-1}) and cultured under DFL. The cultures in each of these treatments were subcultured 3 times every month under the same light and temperature conditions.

The microrrhizome was induced only under short day conditions at the rate of 20% (1 in 5 culture pots in each repeated experiment). This low rate is partly due to the fact that only well-developed rhizome (longer than 1.5 cm) were counted in the experiment. While

microrrhizome production *in vitro* has been reported in *C. longa* (Shirgurkar et al. 2001) *C. aromatica* (Nayak 2000) and *C. amada* (Nayak 2002), however, well-developed microrrhizomes *in vitro* have been reported in only one paper (Sunitibara et al. 2001).

In addition to concentrations of cytokinins and sucrose, photoperiod effects were examined in *C. aromatica* and found to be promoted by short days. The medium containing 5 mg l^{-1} BA and 60 g l^{-1} sucrose and grown under an 8 h photoperiod was reported to be optimum for formation of rhizomes *in vitro* (Nayak 2000). Sunitibara et al. (2001) found short day in combination with high concentrations (6–8%) of sucrose were one of important factors for inducing microrrhizome in *C. longa*. The promotive effect of short day was also a case of well-developed rhizome formation in *C. longa* in this paper. In case of *C. amada*, however, the difference in percentage of microrrhizome formation was not conspicuous between 16 and 8 h photoperiod. However, a marked difference was obtained with further reduction to 4 and 0 h (dark) photoperiod (Nayak 2002). The inconsistency between the results in *C. amada* and our results in *C. longa* may be due to the different response to photoperiod between the mother plants. The exact reason was remained to be solved.

Differentiation of yellow pigmented cells

The microrrhizome, stored in the refrigerator, was sliced into thin sections by microtome. The sections were put on water on the slide glass were observed under microscope. The observation revealed that yellow pigmented-cells were dispersed in the parenchyma tissues of microrrhizome (Figure 3A). Magnified figures of the tissue and the cells with yellow pigment are shown in Figures 3B and 3D, respectively. Figure 3C shows the slice of Figure 3B stained with SudanIII. Small red-stained oil droplets were observed in the cells surrounding the yellow cells. The yellow cells were not clearly stained red. When stained with Nile red, a selective fluorescent stain for lipid (Greenspan et al. 1985), and observed by fluorescent microscope, however, the only yellow cells were emitted bright yellowish fluorescence (excitation: 470–490 nm, emission: 510–550 nm) (Figure 3E). The color of the same cells were red under a light microscope. (Figure 3F). Many yellow droplets released from the sections were also observed outside the cells (Figure 3G). These results indicate the cells accumulating both yellow substance(s) and lipid(s) have differentiated in the well-developed rhizome.

Yasuda et al. (1988) reported that in the parenchyma tissue of the sliced rhizome of mother plants of *C. zedoaria*, there are yellowish cells stained red by Sudan III, which has been considered as the essential oil secretory cells. Therefore it is reasonable to suppose

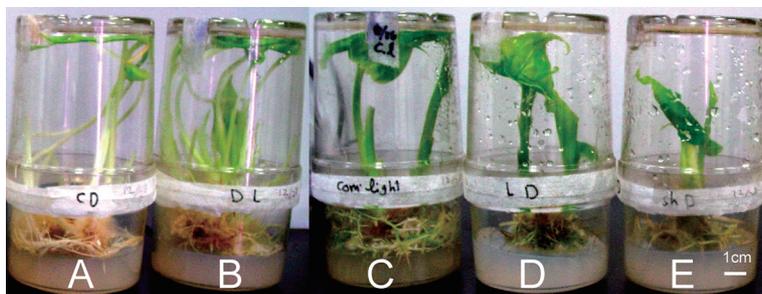


Figure 1. Propagation of plantlets under different light conditions. (A) Dark (B) DFL (C) Continuous light (D) Long day (E) Short day.



Figure 2. Induction of well-developed microrhizome *in vitro*.

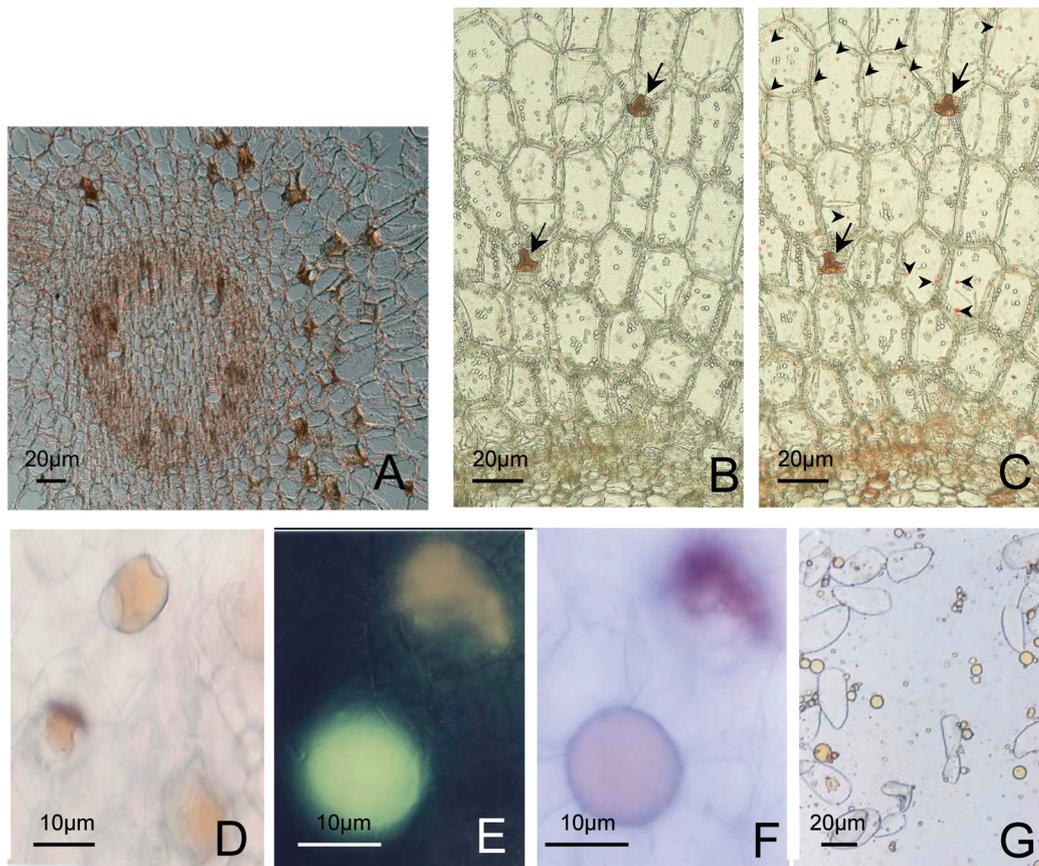


Figure 3. Differentiation of yellowish-pigmented cells in the well-developed microrhizome. (A) Differentiation and distribution of yellow cells in parenchyma tissue of the well-developed microrhizome. (B) Magnified horizontal section of well-developed microrhizome. Arrows indicate the yellow cells. (C) The section of (B) stained with SudanIII. Arrows and arrowheads indicate the yellow cells and the small red-stained oil droplets, respectively. (D) Magnified yellow cells. (E) Yellow cells stained with Nile red and observed by fluorescent microscope (excitation: 470–490 nm, emission: 510–550). (F) The cells in (E) observed by light microscope. (G) Yellow droplets released from well-developed microrhizome cells.

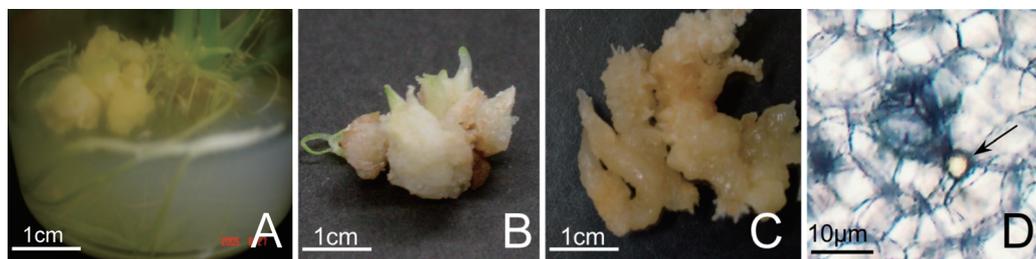


Figure 4. Isolation and differentiation of the callus. (A) Callus formation on the root tip of propagated plantlet *in vitro*. (B) Differentiation of shoots and roots from the callus. (C) Microrhizome induction by kinetin in the callus. (D) Yellow cell in the microrhizome induced in the callus. Arrow indicates the yellow cell.

the cells in the well-developed microrhizome in our experiments contain the same essential oil and curcumin as those in the mother plants, *C. longa*. The small amount of plant material available for analysis made identification of these substances impossible. An increase in the rate of rhizome production is required to solve this problem.

Totipotency of the isolated callus

The callus which grew rapidly was isolated from the root tip of the regenerated plantlet cultured under continuous light, when the root tip attached to the surface of NK medium (Figure 4A). This callus was white in color and somewhat dry in texture. Green spots sometimes appeared on the surface of callus as well as plantlets with differentiated shoots and roots. Apparently this callus retained its totipotency (Figure 4B). Although there are many reports of callus formation from explants in various *Curcuma* species (reviewed by Sugaya 1992), the regeneration of plantlets from an established cell line of *Curcuma* species was described in only two papers (Salvi et al. 2001; Shirgurkar et al. 2006).

Effects of kinetin on microrhizome differentiation in the callus

The callus cultured on NK medium for 3 weeks was transferred to the fresh medium containing various concentrations of NAA (0, 0.1 and 1 mg l⁻¹) and kinetin (0, 0.01, 0.1 and 1 mg l⁻¹) at various combinations. Although no promoting effects were observed on shoot and root differentiation, after 1 month a compact structure was found in the callus tissues cultured on the medium containing 1 mg l⁻¹ of kinetin regardless of NAA concentrations. In the absence of NAA, however, the compact structure microrhizome-like was induced most frequently, although its structure was shorter and thinner than well-developed microrhizomes from the regenerated plantlets described above. Figure 4C shows the morphology after more friable part was removed. The structure looks similar to the microrhizome on the micropropagated plants previously reported in *C. longa* (Shirgurkar et al. 2001) and *C. aromatica* (Nayak 2000). The finding in this paper is the first report on the differentiation of microrhizome from successively subcultured callus.

Microscopic observation revealed that yellowish pigmented cells differentiated in the compact structure (Figure 4D). The frequency was far less than in the well-developed microrhizomes on the regenerated plantlets. Attempts to detect curcumin which is a yellow major secondary component of *C. longa* mother plant have yet to produce definitive results.

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