Plant Cell Tiss Organ Cult (2005) 81:119–122
DOI 10.1007/s11240-004-3308-2

Research note

Micropropagation of zedoary (*Curcuma zedoaria* Roscoe) – a valuable medicinal plant

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Received 29 July 2003; accepted in revised form 11 September 2004

Key words: *Curcuma zedoaria*, micropropagation, multiple shoots, plantlets, rhizome, *Zingiberaceae*

Abstract

Tissue culture propagation system was developed for zedoary (*Curcuma zedoaria* Roscoe), a valuable medicinal plant, using rhizome sprout cultures. Shoots were induced from rhizomes on basal MS medium containing 20 g l^{-1} sucrose and 5 g l^{-1} agar, supplemented with 20% (v/v) coconut water (CW) and benzylaminopurine (BA) concentrations from 0.5 to 5.0 m g l^{-1}. The excised shoots were subcultured on Murashige-Skoog (MS) medium with 20% (v/v) CW and different concentrations of BA and kinetin (Kin), either alone or in combination with indolebutyric acid (IBA) or naphthaleneacetic acid (NAA). MS medium with 20% (v/v) CW, 3 mg l^{-1} BA, and 0.5 mg l^{-1} IBA resulted in a multiplication rate per shoot; 5.6 shoots per explant were obtained on average after 30 days of culture. Well-developed shoots (30–40 mm in length) were rooted on MS medium containing 20 g l^{-1} sucrose and 8 g l^{-1} agar, supplemented with 20% (v/v) CW and 2 mg l^{-1} NAA. More than 95% of the rooted plants were established in pots after hardening.

Abbreviations: AC – activated charcoal; BA – 6-benzylaminopurine; CW – coconut water; IBA – indolebutyric acid; Kin – kinetin; $M \pm$ SE – mean ± standard error; MS – Murashige and Skoog (1962); NAA – α-naphthaleneacetic acid

Zedoary (*Curcuma zedoaria* Roscoe) is a valuable medicinal plant; the essential oil obtained from its rhizomes is reported to have antimicrobial activity. It is also used clinically in the treatment of cervical cancer, as the aqueous extract of zedoary has antimutagenic activity (Syu et al., 1998). In traditional Asian medicine, zedoary (*Zedoariae rhizoma*) is also used for treatment of stomach diseases, hepato-protection (Yoshioka et al., 1998), the treatment of blood stagnation, and to promote menstruation (Matsuda et al., 1998). Furthermore, zedoary has anti-inflammatory potency related to its antioxidant effect (Yoshioka et al., 1998).

In vitro culture methods have been reported for some species of *Curcuma*. *viz* and *C. xanthorhiza* (Mukhri and Yamaguchi, 1986), *C.* spp. (Balachandran et al., 1990), *C. amada* (Borthakur and Bordoloi, 1992), *C. alismatifolia* (Wannakrairoj, 1997), *C. aromatica* (Nayak, 2000), and *C. longa* (syn. *C. domestica*) (Nadgauda et al., 1978; Mukhri and Yamaguchi, 1986; Salvi et al., 2002; Prathanturarug et al., 2003).

Generally, zedoary cannot be improved by breeding because it flowers rarely and seed set does not occur. It is propagated vegetatively through underground rhizomes at a very low rate. Consequently, a tissue culture technique would play an important role in studies of this plant. Here, we report the *in vitro* multiplication of this pharmaceutically valuable plant through shoot proliferation from underground rhizomes followed by the

successful outdoor establishment of regenerated plants.

Mature rhizomes of zedoary (*C. zedoaria* Roscoe) collected from healthy, disease-free plants were planted in soil to initiate sprouting. The sprouted rhizomes were removed from the soil and pieces of the rhizomes with sprouts were washed thoroughly in running water. Then, they were rinsed with 70% (v/v) ethanol for 1 min and subsequently surface sterilized with 0.2% (w/v) $HgCl_2$ solution for 20 min. After rinsing with sterile distilled water five times, the outer leaves were removed from the sprout, and explants (10–15 mm in length) consisting of a shoot tip with a small portion of the rhizome were used for culture initiation.

The basal medium used in this study was MS medium (Murashige and Skoog, 1962) containing 20 g l^{-1} sucrose as a carbon source. Explants were cultured on medium supplemented with 20% (v/v) CW and different concentrations (0.5, 1, 2, 3, 4, and 5 mg l^{-1}) of BA for shoot induction. The pH of the medium was adjusted to 5.8 before gelling with 5 g l^{-1} agar and autoclaving at 121 °C and 104 kPa for 20 min. The cultures were maintained at 25 ± 1 °C with 10 h of light at an intensity of 3000–4000 lux. After 4 weeks, multiple shoots from culture initiation were removed and individual shoots were excised aseptically. These shoots were subcultured on fresh MS medium supplemented with 20% (v/v) CW and different concentrations of BA (0.5–5 mg l^{-1}/L) and Kin (0.5–5 mg l^{-1}), either individually or in combination with IBA (0.5 mg l^{-1}) or NAA (0.5 mg l^{-1}) for shoot multiplication.

In vitro shoot explants (30–40 mm in length) derived from the shoot multiplication cultures were inoculated onto MS medium containing 20 g l^{-1} sucrose and 8 g l^{-1} agar, supplemented with 20% (v/v) CW and different concentrations of either IBA (0.5–2.0 mg l^{-1}) or NAA (0.5–2.0 mg l^{-1}) for root induction. The effects of AC on root induction were studied using MS culture medium (containing 20% (v/v) CW, 2 mg l^{-1} IBA, 0.8% agar) supplemented with AC at concentrations of 0.5–2 g l^{-1}.

Plantlets with well-developed shoots (60–80 mm in length) with roots (10–20 mm in length) were removed from the flasks and washed thoroughly in running tap water. They were then transferred to pots containing a mixture of soil and rice husk ash (3:1 ratio) and covered with polythene bags for 4–5 days to prevent desiccation. After transfer, the plants were placed in a greenhouse for acclimatization for two weeks before being planted in the field directly. All experiments were conducted with a minimum of three replicates consisting of four explants per flask. The experiments were repeated three times. The results are expressed as the means ± SE of three experiments. Statistical comparisons were performed using Duncan's test ($p < 0.05$).

Freshly sprouted shoots excised at the base from the rhizomes of field-grown plants of zedoary were cultured on MS basal medium supplemented with 20% (v/v) CW and different concentrations of BA to induce shoots. More than 40% of the explant buds were discarded after 10 days of culture because of bacterial or fungal contamination. The medium supplemented with 3 mg l^{-1} BA was the most effective for multiple shoot formation, producing an average of 2.3 ± 0.5 shoots per culture. Shoots attained a length of 15–35 mm within 30 days (Table 1).

In vitro single shoots from culture initiation were then subcultured on MS medium containing different concentrations of BA and Kin, either individually or in combination with IBA or NAA for 30 days. The maximum number of shoots per explant was obtained on MS medium supplemented with 3 mg l^{-1} BA and 0.5 mg l^{-1} IBA, and exceeded the numbers obtained with all other media (Table 2). All of the excised shoots proliferated new shoots (data not shown). In the present study, BA played a significant role in shoot formation in zedoary. BA at a concentration of 3 mg l^{-1}, alone or in combination with IBA (mg l^{-1}), NAA (0.5 mg l^{-1}), promoted shoot multiplication strongly. This

Table 1. Effects of BA on *in vitro* shoot induction from rhizomes of *Curcuma zedoaria* on MS medium

BA(mg l^{-1})	No. shoots/explant (mean ± SE) [a]	Shoot length (mm) (mean ± SE) [a]
–	1.1 ± 0.3	15.1 ± 0.7
0.5	1.2 ± 0.3	19.3 ± 0.9
1	1.5 ± 0.5	24.8 ± 0.6
2	1.7 ± 0.6	27.9 ± 0.8
3	2.3 ± 0.5	34.2 ± 1.2
4	2.1 ± 0.4	35.1 ± 1.0
5	2.2 ± 0.5	34.6 ± 0.8
$LSD_{0.05}$	1.33	2.65

[a] Mean of three repeat experiments; each experiment consisted of 12 explants.

Table 2. Effects of BA, kinetin, IBA, and NAA on *in vitro* shoot multiplication of *Curcuma zedoaria* on MS medium

Concentration of plant growth regulators (mg l^{-1})				Number of shoots/ explant[A,B]	Shoot length (mm)[A,B]
BA	Kin	IBA	NAA		
–	–	–	–	3.1[de]	39.1[d]
0.5	–	–	–	3.4[d]	46.8[cd]
1	–	–	–	3.8[c]	49.2[c]
3	–	–	–	5.4[a]	62.3[ab]
5	–	–	–	5.3[a]	68.4[a]
–	0.5	–	–	3.7[c]	67.5[a]
–	1	–	–	3.8[c]	61.6[ab]
–	3	–	–	3.3[d]	51.2[c]
–	5	–	–	3.1[de]	41.1[d]
1	–	0.5	–	3.2[de]	37.7[de]
3	–	0.5	–	5.6[a]	61.4[ab]
–	0.5	0.5	–	3.6[cd]	47.3[cd]
–	1	0.5	–	4.3[b]	51.8[c]
1	–	–	0.5	3.0[de]	42.4[d]
3	–	–	0.5	5.3[a]	59.1[b]
–	0.5	–	0.5	3.2[de]	42.2[d]
–	1	–	0.5	5.1[a]	64.3[ab]

[A]Mean of three repeat experiments; each experiment consisted of 12 explants.
[B] Different letters indicate significantly different means using Duncan's test ($p < 0.05$).

experiment showed that the presence of cytokinin in the culture medium positively influenced shoot multiplication in zedoary. The high number of adventitious buds produced in the presence of cytokinin is consistent with the observation that cytokinins overcome apical dominance, release lateral buds from dormancy, and promote shoot formation. Combinations of cytokinins and auxins stimulate the *in vitro* multiplication and growth of shoots of several plant species. In this experiment, the addition of 0.5 mg l^{-1} of either NAA or IBA to the multiplication medium (MS with 3 mg l^{-1} BA or 1 mg l^{-1} Kin) significantly increased the number of shoots per explant and reduced shoot length.

The *in vitro* shoots were separated from multiple shoots in the subculture to develop whole plants on root-induction media (MS + 8 g l^{-1} agar + 20% (v/v) CW + 0.5–2 mg l^{-1} IBA or 0.5–2 mg l^{-1} NAA). The NAA and IBA concentrations generally affected the number and length of roots developing on subcultured plantlets (Figure 1A,B). All the excised shoots developed roots within 30 days of culture (data not shown). The root number increased

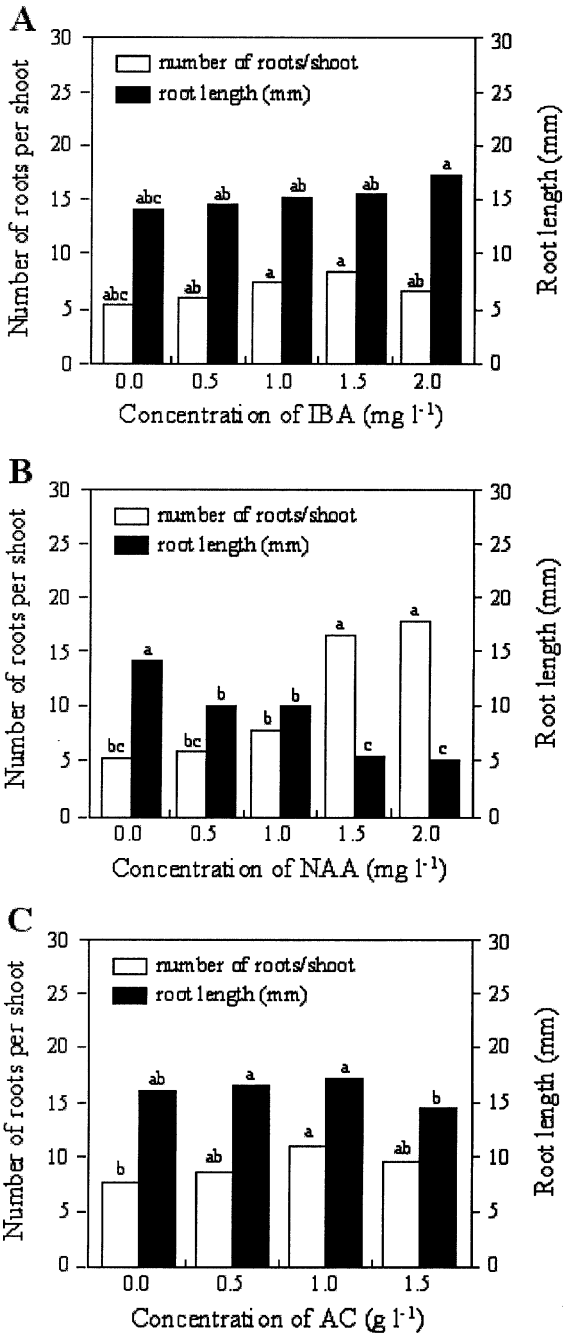

Figure 1. Effects of auxins and activated charcoal on root induction from *in vitro* shoots of *C. zedoaria*. The results are expressed as the means of three repeat experiments; each experiment consisted of 12 explants. Different letters indicate significantly different means using Duncan's test ($p < 0.05$).

with the NAA concentration up to an optimum, and the root length diminished with rising NAA concentration. MS medium with 2 mg l^{-1} NAA formed

18.5 ± 4.8 adventitious roots of 5.1 mm in length within 4 weeks of culture. In rooting media supplemented with IBA, the root number increased with IBA concentration from 0.5 to 1.5 mg l^{-1}, and then decreased at 2 mg l^{-1}, while root length increased with increasing IBA concentration. AC was useful for regenerating *C. zedoaria* (Figure 1C). All the explants placed in medium with 0.5–2.0 g l^{-1} AC formed roots. AC enhanced the shoot explant response in all three treatments (0.5, 1.0, and 1.5 g l^{-1}). In the medium with 1 g l^{-1} AC, the explants reacted well, producing adventitious roots at the highest rate of 10.8 ± 1.6 roots/shoot. For acclimatization, *C. zedoaria* p1antlets derived *in vitro* with well-developed root and shoot systems were transferred to pots containing a mixture of soil and rice husk ashes (3:1 ratio), and healthy roots appeared after 2 weeks. The clone survival rate was about 95%. The plants were hardened for 4–5 days before being transferred to a greenhouse.

Based on these observations, we conclude that our *in vitro* technique is useful for the rapid production of zedoary with a multiplication rate of about 5.6 clones per bud in 30 days. The rooted plants were transferred to soil, and exhibited normal development.

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