Indian Journal of Biotechnology
Vol 7, July 2008, pp 407-409

Efficient plant regeneration in small cardamom (*Elettaria cardamomum* Maton.) through somatic embryogenesis

C Manohari [1], S Backiyarani [2], T Jebasingh[1], Archana Somanath [1] and R Usha[1*]

[1]Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai, 625 021
[2]Cardamom Research Station, Kerala Agricultural University, Pampadumpara, Kerala 685 556

Received 20 July 2007; revised 16 November 2007; accepted 5 February 2008

An efficient protocol for the induction of somatic embryogenesis and plant regeneration in small cardamom (*Elettaria cardamomum* Maton.) from the inner core region of rhizome has been established. Calli developed profusely on Murashige and Skoog (MS) medium containing 9.0 μ*M* 2,4 D and 2.3 μ*M* Kn. When the friable calli were cultured in MS medium containing 4.4 or 8.8 μ*M* BAP + 0.5 μ*M* NAA, abundant embryogenic calli were obtained. The highest frequency of embryogenic calli (68%) and plantlets (86%) were obtained from MS medium containing 4.4 μ*M* BAP and 0.5 μ*M* NAA. Further shoot development was observed with 13.2 μ*M* BAP + 0.5 μ*M* NAA. In the same medium, roots also appeared, thereby eliminating an additional step of *in vitro* rooting. The well-developed plants were hardened and transferred to a mist chamber in a greenhouse with 90% survival frequency.

Keywords: Cardamom, somatic embryogenesis, regeneration, *Elettaria cardamomum*.

The production of cardamom in India has been greatly affected by several insect pests and diseases. Chemical control methods are expensive and laborious. Moreover, management of viral diseases through chemicals is not possible in any field crop. Therefore, it becomes imperative to develop plants tolerant to biotic stresses through novel methodologies. A reproducible regeneration procedure is essential for studies involving gene transfer and genetic improvement of crop plants[1]. Ravindran *et al* have clearly outlined the role of biotechnology in the genetic improvement of cardamom[2]. He also emphasized that biotechnological interventions would help in the introduction of genes conferring resistance to viral diseases and insect-pests into elite cardamom lines, thereby producing superior varieties[3]. Somatic embryogenesis forms the key approach for *in vitro* regeneration of crop plants employed in transgenic technology. In this paper, a successful protocol is reported for the regeneration of cardamom through indirect somatic embryogenesis, from the rhizomes of *Green Gold* cultivar, a high-yielding elite variety popularly cultivated in the cardamom-growing tracts of Kerala.

Rhizomes from the *Green Gold* cultivar of cardamom were collected from Cardamom Research Station, Pampadumpara, Kerala and used as source materials for the establishment of multiple shoot formation *via* somatic embryogenesis. Suckers were de-scaled and thoroughly washed with tap water followed by 0.1% Teepol solution and several washes with running tap water. The explants were further treated with 4-5% v/v of liquid commercial bleach for 5 min, carbendazim 0.1% for 20 min and tetracycline antibiotic solution (20 mg/L) for 20 min. After each treatment, the explants were thoroughly washed with distilled water, then surface sterilized with $HgCl_2$ (0.1%) for 15 min followed by 70% ethanol (3-5 seconds) treatment under aseptic conditions. At each step, a minimum of four rinses with sterile distilled water were given. The core region of rhizome was trimmed and sliced to a size of 0.5-1.0 cm and kept in the callus induction Murashige and Skoog (MS) medium supplemented with various combinations of auxins, namely, 2,4-dichlorophenoxyacetic acid (2,4 D, 4.5-18.0 μ*M*) and α-naphthalene acetic acid (NAA, 10-20 μ*M*) as well as cytokinins, 6-benzylaminopurine (BAP, 2.2-4.4 μ*M*) and Kinetin (Kn, 1 μ*M*). All the cultures were maintained under dark conditions at 25±2°C for callus induction. For inducing organogenesis, these calli were transferred to MS medium containing various hormones, viz., 6 (γ,γ dimethyl allyl) amino purine (2ip), BAP and Thidiazuron (TDZ) at various concentrations in all possible combinations. The experiments were conducted in complete block design. Ten culture tubes were used for each experiment and all experiments were repeated three times. The effects of the various treatments were quantified. The data were subjected to

*Author for correspondence:
Tel: 91-452-2458230 Fax: 91-452-2459105
E-mail: usha@mrna.tn.nic.in; abayamba@gmail.com

statistical analysis using ANOVA and the mean values were compared using Duncan's multiple range test (DMRT) at 5% level of significance. The cultures were maintained in a 16/8h light/dark photoperiod at 25±2°C. Fully developed rooted plantlets were removed from the culture tubes, thoroughly rinsed with running water and transferred to cups containing a mixture of sterilized vermiculite and sand (1:1) and maintained in a plant growth chamber for two weeks under controlled temperature (23°C) and 16/8h light/dark photoperiod and 90% relative humidity for hardening. The plants were initially irrigated with ¼ strength of MS medium on alternate days, followed by tap water after 1 wk. Thereafter, the plants were transferred to a mist chamber in a greenhouse.

Callus induction from the inner core region of the rhizome on MS medium supplemented with the plant growth regulators like 9.0 μ*M* 2,4 D + 2.3 μ*M* Kn and additives like 0.1% casein hydrolysate and 0.1% polyvinyl pyrrolidone (PVP) occurred after a period of 30 d. The explants exhibited swelling within four weeks, followed by the development of whitish callus after another six to eight weeks. The high amount of white, soft and friable calli were obtained after 60 d by subculturing twice in the same medium (Fig. 1a). After two subcultures, non-embryogenic, loose calli were obtained. Regunath and Bajaj obtained maximum callus production from the explants of immature inflorescence and immature capsules of cardamom in MS medium supplemented with 21.1 μ*M* NAA and 4.44 μ*M* BAP[4]. However, in the present study, roots directly emerged from the rhizome explants in the above medium. This shows that different explants respond differently to the medium composition. The primary calli were subcultured on the same callus induction medium for inducing somatic embryogenesis. A cross-section of the embryogenic calli (Fig. 1b) confirmed their embryogenic nature. These embryogenic calli were transferred to regeneration medium for the development of somatic embryos as well as plantlet initiation.

The regeneration medium containing 2iP at a range of six concentrations (9.9, 19.8, 29.7, 39.6, and 49.5 μ*M*) with different combinations of Kn (2.3μ*M)* and NAA (0.5 μ*M)* showed no response for embryogenic callus formation (data not shown). At 1 μ*M* concentration of TDZ, the response of embryogenic calli was very low (2%) and at 2-4 μ*M* TDZ in the

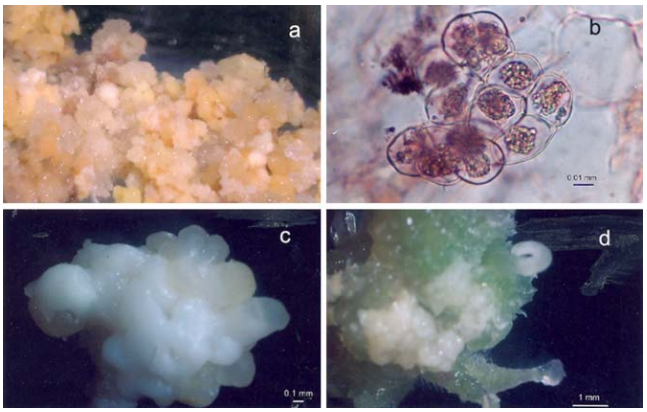

Fig. 1—Calli from rhizome explants of cardamom: a, Cross section of an embryogenic callus under a microscope; b, Mass of somatic embryos under a microscope; & c, Regeneration from callus observed under a microscope.

regeneration medium, all the calli turned black and there was no further proliferation of calli. The friable calli turned into hard green calli and no shoots developed when they were transferred to regeneration medium with 13.2 μ*M* BAP + 1 μ*M* NAA. This is in agreement with the observation that as the cytokinin concentration increases, the probability of obtaining a normal shaped embryo decreases[5]. However, when MS medium was supplemented with 4.4 or 8.8 μ*M* BAP + 0.5 μ*M* NAA, a mass of somatic embryos was produced (Fig. 1c). After subculturing twice in the same regeneration medium (Table 1), plantlet regeneration was observed under a microscope (Fig. 1d). The highest percentage of shooting response (86.5%) was recorded in 4.4 μ*M* BAP + 0.5 μ*M* NAA (Fig. 2a). Thus, the results indicated that BAP (4.4 μ*M*) in combination with NAA (0.5 μ*M*) is the best combination for inducing embryogenic callus and shoot induction. For further shoot development, a combination of 13.2 μ*M* BAP + 0.5 μ*M* NAA was required. This resulted in multiple shoot formation followed by rooting in the same medium without changing the hormone combination (Fig. 2b). This does not coincide with the findings of Lukose *et al*, who had used a separate rooting medium for the initiation of roots in cardamom[6]. These results presumably indicate a threshold level of endogenous hormones in the explants as revealed for *Brassica napus*[7]. After root development, the plantlets with a maximum height of 15 cm were transferred to sterile vermiculite medium and kept in controlled condition for hardening (Fig. 2c). The fully developed plantlets established in the soil and showed 90% survival

Table 1—Effect of BAP and NAA in various combinations on maturation of embryos and germination of somatic embryos derived from cardamom rhizome

BAP (μ*M*)	NAA (μ*M*)	% of calli with embryogenic nature ± SD	Regeneration response (%)± SD	Nature of callus
2.2	0.5	23.3± 0.4	0.0	Friable, slow growth
4.4	0.5	70.0± 0.5	86.6± 0.3	Compact, nodular and embryogenic Organogenic differentiation noticed
8.8	0.5	53.0± 0.5	70.0± 0.5	Compact, nodular and embryogenic Organogenic differentiation noticed.
2.2	1	23.3± 0.4	3.3 ± 0.2	Roots developed.but shoot induction very minimal
4.4	1	40.0± 0.5	53.3± 0.5	Friable, bulky less number of embrygenic calli. And slow growth of plants
8.8	1	20.0± 0.4	36.67± 0.5	Friable, bulky, less number of embrygenic calli and slow growth of plants
13.2	1	0.0	0.0	Hard green mass of callus

Data were analyzed by ANOVA test and means were separated by DMRT; Values represent means ± standard deviation of 10 replicates per treatment in three repeated experiments.

(Fig. 2d). Therefore, the present protocol described for regeneration of plantlets through embryogenic calli could be utilized for generating plants on a large-scale to induce somaclonal variation by *in vitro* mutagenesis and genetic transformation using biolistic and other gene transfer techniques.

Fig. 2—Shooting response from embryogenic callus: a, Multiple shoot and root development from calli; b, Hardening of plants on vermiculite in a growth chamber; & c, Plants transferred to a mist chamber in a greenhouse

Acknowledgement

The authors acknowledge the DBT and CSIR (Government of India), New Delhi for funding and fellowships. We are thankful to Dr P Ravichandran for his helpful suggestions and microscopy. Dr Joseph Rajkumar is acknowledged for support in maintaining the cardamom plants.

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