Direct *in vitro* regeneration of *Curculigo orchioides* Gaertn., an endangered anticarcinogenic herb

Curculigo orchioides is an endangered medicinal herb belonging to the family Amayrllidaceae. The rhizome extract shows hypoglycemic, spasmolytic and anticancer activities. Its anticarcinogenic activity was observed against Sarcoma-180 in mouse[1,2]. The tuberous roots are valued in both Indian and Chinese herbal remedies, and used as a tonic for health, vigour and vitality due to the presence of flavnone glycosides. The powdered rhizome applied to cuts is said to stop bleeding and dry up wounds[3].

This monocotyledonous herb is popularly known as 'Kali Musli' in this part of the subcontinent. The total produce per year varies from 400 to 500 g per plant and the cost of produce is around Rs 200 per kg of root[4]. Due to its significant medicinal properties, *C. orchioides* has been overexploited, which in turn has led to its inclusion in the list of threatened plant species[5].

C. orchiodes was collected from the Daangs forest of Waghaai in south Gujarat during the season and grown in the Sardar Patel University Botanical Garden, Vallabh Vidyanagar. The plants were first washed in running tap water to remove soil from the surface. They were then treated with a mixture of 0.1% (w/v) mercuric chloride and 0.1% (w/v) sodium lauryl sulphate for 5 min and rinsed thoroughly with sterile distilled water. Rhizome segments (8 mm) and leaf explants (10 mm) were excised from the young plants. The explants were cut transversely and inoculated on MS medium supplemented with MS vitamins, 0.2% (w/v) activated charcoal (Qualigens), 1.5 and 3.0% (w/v) sucrose (Himedia) and plant growth regulators as required[6]. The cultures were maintained under 16 h photoperiod at 25 ± 0.5°C under cool white fluorescent light (light intensity 50 µmol m^{-2} s^{-1}) and 55–60% relative humidity. After the plantlets regenerated, they were shifted to the greenhouse in small polycups (covered with a transparent plastic bag with holes to maintain humidity) containing cocopeat and organic manure (0.1%). Subsequently, they were transferred to the garden and planted in the field after one month.

For statistical analysis, means were based on 12 replicates for each treatment. Standard error is given to indicate the variation among the means of three experiments repeated in time. Data regarding shoots and roots were collected 70 and 85 days respectively, after the beginning of the experiments. Data were analysed using one-way ANOVA followed by LSD test with a confidence limit of 0.05.

Leaf explants inoculated in different strengths of MS basal media with 1.5% sucrose concentration showed differentiation, whereas those with 3% sucrose concentration showed moderate response (Table 1). Entire plantlets with single shoots and well-developed roots were obtained after nine weeks in culture. The explants in MS basal medium gave direct organogenesis, but only with single shoots. Rarely two shoots were obtained. Augustine and D'souza[5] reported that the explants of *C. orchiodes* cultured on cytokinin-free medium produced entire plantlets directly from the cut end of the midrib.

Among the cytokinins tried, 6-benzylaminopurine (BAP) gave better results compared to kinetin (KN, data not shown). But even here, multiple shoot formation was not observed. Lower concentrations of BAP gave rise to single shoots from the explants. Results obtai-

Table 1. Response of leaf explants of *Curculigo orchioides* to various concentrations of MS medium and BAP

Media combination	Percentage response	Mean no. of shoots	Mean length of shoots (mm)
MS* (3% sucrose)	$60.8 \pm 0.7_y$	1.14 ± 0.6	$56.4 \pm 0.6_x$
MS	$76.9 \pm 0.7_{yz}$	1.08 ± 0.3	$63.7 \pm 0.6_y$
½MS* (3% sucrose)	$29.7 \pm 0.9_x$	1.06 ± 0.8	$58.1 \pm 0.9_x$
½MS	$90.3 \pm 1.0_z$	1.16 ± 0.5	$72.1 \pm 0.4_y$
¼MS* (3% sucrose)	$35.6 \pm 0.6_x$	1.44 ± 0.3	$60.6 \pm 0.3_y$
¼ MS	$63.5 \pm 0.5_y$	1.25 ± 0.4	$87.5 \pm 0.5_z$
MS + 0.2 mg/l BAP	$75.7 \pm 0.4_z$	1.44 ± 0.2	$68.5 \pm 0.8_y$
MS + 0.5 mg/l BAP	$80.1 \pm 0.8_z$	1.27 ± 0.3	$92.7 \pm 0.4_z$
MS + 1.0 mg/l BAP	$63.9 \pm 0.2_y$	1.36 ± 0.2	$78.8 \pm 0.5_{yz}$
MS + 1.5 mg/l BAP[a]	$51.2 \pm 0.5_y$	–	–
MS + 2.0 mg/l BAP[a]	$47.8 \pm 0.8_y$	–	–

Mean values within the column followed by the same letters are not significantly different at $P \leq 0.05$ (LSD test). Observations were made after 54 days.
½MS and ¼ MS mean half strength and one-fourth strength concentration of MS basal salts and vitamins.
*Indicates 3.0% sucrose. The rest are 1.5% sucrose.
[a]Showed traces of callus.

Table 2. Effect of various concentrations of 2,4-D as supplement to MS medium on multiple shoot formation and direct organogenesis of *Curculigo orchioides* leaf explants

Concentration (mg/l)	Percentage explants showing response	Mean no. of shoots per explant	Mean length of shoots (cm)	Mean no. of roots	Mean length of roots (cm)
0.5	41.7 ± 0.5	2.6 ± 0.9	5.6 ± 0.2	9.3 ± 1.5	1.95 ± 0.3
1.0	62.5 ± 0.3	3.2 ± 1.3	6.3 ± 0.9	14.5 ± 2.1	2.21 ± 0.1
1.5	77.6 ± 0.6	3.7 ± 0.7	6.7 ± 0.7	19.7 ± 1.0	2.02 ± 0.2
2.0	83.4 ± 0.2	5.4 ± 0.2	10.3 ± 0.5	11.7 ± 0.5	2.15 ± 0.4
2.5	68.9 ± 0.4	4.6 ± 1.0	8.2 ± 0.6	10.3 ± 2.2	1.88 ± 0.5

Values are mean ± SD of three independent experiments each with 12 replicates.
Observations were made after 70 and 85 days for shooting and rooting respectively.

Figure 1. ***a***, Initiation of shoot formation from rhizome explant on MS basal medium; ***b***, Leaf explant of *C. orchioides* showing direct organogenesis in 2,4-D containing medium; ***c***, Fully-grown *in vitro* plant of *C. orchioides* showing multiple shoots and profuse rooting; ***d***, Plantlets undergoing acclimatization process in moist cocopeat and manure.

ned with the leaf explants in MS medium supplemented with 0.2, 0.5 and 1.0 mg/l BAP showed single shoot regeneration with rooting. In MS medium augmented with 1.5 and 2.0 mg/l BAP, leaf explants with midrib initially showed callus formation. However, further growth did not occur even after sixty days and the callus gradually turned black and died.

The rhizome explants, except for a few, did not show any response with the combinations tried. In a few explants, half strength of MS medium showed whole plantlet regeneration with single shoot – 20.1 and 37.6% response in full and half strength sucrose respectively (Figure 1 *a*). Factorial combinations of KN formed meagre amount of callus, which later became stunted and gradually turned black, although the response was very low. For different concentrations of KN, i.e. 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l, the percentage response was low, viz. 13.6, 20.1, 19.3, 21.0 and 18.7 respectively.

Leaf explants inoculated in MS medium augmented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) ranging from 0.5 to 2.5 mg/l showed differentiation of multiple shoots (Table 2). The number of leaf explants showing differentiation of multiple shoots increased with increasing concentration of 2,4-D. Leaf explants of *C. orchioides* with midrib gave rise to multiple shoots along with rhizogenesis after a period of 60 days in 2,4-D-containing medium (Figure 1 *b* and *c*). The plantlets obtained contained an average of 6–8 leaves and 15–20 roots. The average length of leaves obtained was 10–12 cm and average length of roots was around 2 cm, after 21 days of culture. The increasing concentration of 2,4-D gave rise to increased number of multiple shoots along with profuse rooting through direct organogenesis, without intervening callus formation. The induction of plantlets directly from the parent tissue without intervening callus is of potential value for the *in vitro* multiplication and storage of a given genotype[7].

Interestingly, we found that MS medium containing 2,4-D induced direct organogenesis by giving rise to shoots. The multiple shoots elongated and rhizogenesis occurred in the 2,4-D-containing medium itself. Transfer of the rooted shoots in the same combination of MS supplemented with 2,4-D resulted in the formation of more multiple shoots and rooting.

One of the major problems during culture establishment in higher plants is the exudation of phenolics from the cut ends of the explants in the culture medium. Addition of activated charcoal (AC) to the culture medium has been found to be useful to overcome the problem of phenolic exudation[8]. It was also observed that tubes inoculated with rhizome explants showed fungal contamination and exuded abundant phenolics in the medium. Addition of AC was found to be almost indispensable during the first phase of the cultures. Better growth of the shoots and roots occurred in tubes containing AC com-

pared to those without AC. The cultures containing 0.3% polyvinylpyrollidone showed no improvement in response.

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in the soil[9]. The regenerated plantlets of *C. orchioides* were transferred to thermocol cups containing moist cocopeat and manure for acclimatization at 25 ± 2°C for ten days during which elongation and growth of leaves was observed (Figure 1 *d*). Later, these plantlets were transferred to the greenhouse, kept for ten days and then transferred to pots containing autoclaved garden soil. The pots containing the *in vitro* plantlets were finally transferred to the garden plot after one week. The *in vitro*-grown plants exhibited survival rate of 96.27 and 82.57% in polyhouse and field conditions respectively. The high survival rate indicates that this protocol could be easily adopted for large-scale cultivation of *C. orchioides*.

1. Dhar, M. L., Dhar, M. M., Dhawan, B. N., Mehrotra, B. N. and Ray, C., *Indian J. Exp. Biol.*, 1968, **6**, 232–247.
2. Bishit, B. S. and Nayar, S. L., *J. Sci. Indian Res.*, 1960, **19C**, 25–27.
3. *Dictionary of Chinese Traditional Medicine*, Jiyangsu College of New Medicine, People's Press, Shanghai, 1979, p. 1363.
4. Shah, G. L., *Flora of Gujarat State–II*, Sardar Patel University Press, Vallabh Vidyanagar, 1978.
5. Augustine, C. A. and D'souza, L., *In vitro Cell Dev. Biol.–Plant*, 1997, **33**, 111–113.
6. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–497.
7. Hussey, G., *J. Exp. Biol.*, 1975, **26**, 253–262.
8. George, M. M., Ph D thesis, Sardar Patel University, Vallabh Vidyanagar, 1999.
9. Saxena, S. and Dhavan, V., *Plant Cell Rep.*, 1999, **18**, 438–443.

ACKNOWLEDGEMENT. We are grateful to Mr Rama Rao for photography.

Received 10 December 2002; revised accepted 3 February 2003

HIREN A. PRAJAPATI
DARSHAN H. PATEL
SAURABH R. MEHTA
R. B. SUBRAMANIAN*

Department of Biosciences,
Sardar Patel University,
Vallabh Vidyanagar 388 120, India
**For correspondence*
e-mail: bagsubbs@yahoo.com

Larvicidal properties of a perennial herb *Solanum xanthocarpum* against vectors of malaria and dengue/DHF

The use of different parts of locally available plants and their various products in the control of mosquitoes has been well established[1]. The use of chrysanthemum flower heads and tobacco leaf smoke was known to people since ancient times. A number of unsaturated *N*-(2-methyl propyl) amides with larvicidal activities have been reported from the plants of families Compositae, Piperaceae and Rutaceae[2]. Studies on azadirachtin-rich fractions from neem[3] and water extracts of de-oiled neem kernel[4,5] reveal the larvicidal properties of different fractions of these plants. The larvicidal properties of indigenous plants have also been documented in many parts of our country[6,7], along with the repellant and anti-juvenile hormone activities[8,9]. *Solanum xanthocarpum*, the Indian nightshade, commonly known as 'baigan kateli', is found throughout the country, but more abundantly in arid areas. The plant is known to have multiple medicinal properties[10–12] and the extracts of various parts have been used against agricultural pests as repellant[13] and contact poison[14], and as molluscicide[15] in public health. However, studies against the pests of public-health importance are totally lacking. In the present study, the extracts of different parts of the plant have been evaluated against the larvae of important vectors of malaria and dengue, and the findings are summarized here.

For evaluating the larvicidal activity of *S. xanthocarpum*, the crude extracts of fruits and roots from the fresh plants were collected from the fields. For obtaining the fruit extract, the unripe berries of the plant were used. For extracting the fruit extract the pulp of the berries was weighed, blended and finally centrifuged. The supernatant so obtained was used as stock solution, from which the serial dilutions according to requirements, were prepared in distilled water for experimentation. The root extract was also prepared following a similar method. Concentrations between 0.001 and 10.0% were tried initially, but after subsequent experiments the final concentrations of the extracts of different parts were determined to obtain graded-mortalities of tested mosquito species. The concentrations of fruit extract between 0.01 and 0.6% against the larvae of *Anopheles culicifacies*, between 0.005 and 1.0% against *Anopheles stephensi*, and between 0.005 and 1.0% against *Aedes aegypti* were finally considered for evaluating the efficacy. In case of root extract, concentrations between 0.1 and 6.0% were used against all the three mosquito species.

The experiments were conducted according to WHO methods[16,17]. The larvae used in the experiments were laboratory-reared. Tests were conducted during the month of September. In the experiments, the late third and early fourth instar larvae of *An. culicifacies*, *An. stephensi* and *Ae. aegypti* were used. Observations were made after 24 h. In case of individual species, three to four replicates were performed for each concentration. For each species, against a particular extract, six concentrations were used to obtain concentration–mortality data, for determining the lethal concentrations at LC_{50} and LC_{90} levels by log-probit analysis[18]. Laboratory temperature and relative humidity during the experiments were recorded as 27 ± 2°C and 60 ± 5% respectively.

The results of the tests conducted for evaluating the larvicidal efficacy of fruit extract of *S. xanthocarpum* revealed that the extract has larvicidal activity against two tested anopheline species, viz. *An.*