Micropropagation of *Hedychium coronarium* J. Koenig through rhizome bud

Pritam Mohanty · Shashikanta Behera ·
Swasti S. Swain · Durga P. Barik · Soumendra K. Naik

Published online: 16 August 2013
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Abstract An optimized protocol was developed for in vitro plant regeneration of a medicinally important herb *Hedychium coronarium* J. Koenig using sprouted buds of rhizomes. The rhizomes with sprouted bud were inoculated on Murashige and Skoog (Physiol Plant 15:473–497, 1962) medium (MS) supplemented with either N⁶-benzyladenine (BA) alone (1.0–4.0 mg L⁻¹) or in combination with 0.5 mg L⁻¹ naphthalene acetic acid (NAA). Of these combinations, MS supplemented with a combination of 2.0 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA was most effective. In this medium, best shoots (3.6) and roots (4.0) regeneration was observed simultaneously with an average shoot and root length of 4.7 cm and 4.2 cm respectively. Regeneration of shoots and roots in the same medium at the same time (One step shoot and root regeneration) reduced the time for production of in vitro plantlets and eliminates the media cost of rooting. Cent-percent (100 %) success in plant establishment was observed in both gradual acclimatization process as well as when plants were directly transferred to outdoor in clay pots containing a mixture of garden soil and sand (2:1) without any sequential acclimatization stage.

Keywords *Hedychium coronarium* · Micropropagation · Medicinal plant · Rhizome bud

Abbreviations

BA N⁶-Benzyladenine
MS Murashige and Skoog's (1962) medium
NAA Naphthaleneacetic acid

Introduction

Hedychium coronarium J. Koenig (butterfly ginger or butterfly lily) is a perennial ornamental herb of family Zingiberaceae with fragrant flowers. The plant is used in traditional system of medicine. The rhizome is used in the treatment of diabetes (Bhandary et al. 1995), fever (as febrifuge) and headache (Jain et al. 1995), arthritis, inflammation and skin diseases (Kiem et al. 2012). The paste prepared from the rhizome is applied externally in cases of snakebite (Ray et al. 2011). The plant is also useful in the treatment of malaria and stem decoction is used for gargling to relieve tonsillitis (Eusebio and Umali 2004). The flowers and stems are used in the manufacture of perfume and paper respectively (Chadha 2005). Both the flower (Facciola 1990) and rhizomes (Sarangthem et al. 2012) are consumed as vegetables. The plant is considered native to Himalayan region of India and Nepal (Kunnumakkara et al. 2008). The plant is under severe threat in their native habitat of eastern Himalayas and enlisted as vulnerable or near threatened in different states of India including Madhya Pradesh, Odisha, Chhattisgarh, Manipur, Kerala, Karnataka and part of Maharashtra, Uttar Pradesh and Bihar because of restricted distribution, overexploitation, harvest for medicine, habitat destruction and unregulated trade (Molur and Walker 1998; Maiti 2004; Chadha 2005; Biswal and Nair 2008; Verma and Bansal 2012). Thus, the plant needs urgent attention for conservation.

In the last few decades plant tissue culture has played an important role for production of large number of plants, independent of seasons, aiming at reestablishment of these plants in their natural habitat. Besides, they also serve as an alternate source of plant raw materials for commercial utilization, which lessens pressure on the plant population in wild habitat (Krishnan et al. 2011). To date, only a few reports on in vitro plant regeneration of *H. coronarium* through callus mediated somatic embryogenesis from

P. Mohanty · S. Behera · S. S. Swain · D. P. Barik · S. K. Naik (✉)
Department of Botany, Ravenshaw University, Cuttack 753003,
Odisha, India
e-mail: sknuu@yahoo.com

sheaths and leaves (Huang and Tsai 2002) or rhizome (Verma and Bansal 2012) and axenic shoot tip (Bisht et al. 2012) are available. Besides, one preliminary report of shoot multiplication using zygotic embryo has also been reported in *H. coronarium* (Xiong et al. 2011). In the present study, the sprouted meristematic rhizome buds were chosen as explants as they are less susceptible to genetic instability during in vitro regeneration of plants and thus have advantages over earlier axenic shoot tip explants (Bisht et al. 2012), indirect somatic embryogenesis (Huang and Tsai 2002; Verma and Bansal 2012) or zygotic embryos (Xiong et al. 2011) mediated plant regeneration methods. Recently, a micropropagation protocol of four months duration for *H. coronarium* using axillary bud explants have been published (Parida et al. 2013). In this study, we report a rapid (two and half month's duration) and efficient in vitro plant regeneration protocol for a valuable medicinal plant, *H. coronarium* using rhizome buds.

Materials and methods

Plant material

The plants of *H. coronarium* along with healthy rhizomes were collected from Cuttack, Odisha, India and maintained in the garden of the Department of Botany, Ravenshaw University, Cuttack. Besides, the healthy rhizomes were kept by covering them with sand and soil mixture (2:1) for sprouting. The buds sprouted within 15 days. The rhizomes with sprouted buds (0.5–1.5 cm) were cut into small pieces followed by removal of sheath of the rhizomes and used as explant for the present study. First the explants, sprouted rhizomes, were cleaned thoroughly in tap water to remove the residue of sand and soil. Then they were cut into small pieces and were washed with liquid detergent (Teepol, Reckitt Benckiser Ltd., India) followed by 5–6 rinses with water. The surface sterilization was carried out with 0.1 % (w/v) aqueous solution of mercuric chloride (HgCl_2 ; HiMedia, India) for 15 min. followed by washing with sterile double distilled water for 5–6 times. The surface sterilized explants were used for culture establishment.

Culture media and conditions for culture establishment

Murashige and Skoog's (1962) medium (MS) or MS supplemented with either N^6 -benzyladenine (BA) (1.0–4.0 mg L^{-1}) alone or BA (1.0–2.0 mg L^{-1}) in combination with naphthalene acetic acid (NAA) (0.5 mg L^{-1}) were used. All the media were augmented with 3 % sucrose and 0.8 % agar (HiMedia, India). The pH of the media was adjusted to 5.8 ± 0.1 before autoclaving at 121°C for 17 min. The cultures were kept inside the culture room at $25 \pm 1^\circ\text{C}$, with a

16 h photoperiod of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density provided with cool white fluorescent tubes. The cultures were maintained in the same medium for two months without subculture.

Statistical analysis

Our experiment designed for having three treatments each consisted of 10 replicate flasks and one sprouted rhizome bud in each flask. All the factors including number of shoots per explant, shoot length, number of roots per explant, root length were calculated from the total number explants, from which axenic cultures were established. The contaminated explants were not taken into account. Data were analyzed using ANOVA for a completely randomized design (CRD). Duncan's new multiple range test (DMRT) was used to indicate means with significant effect at $P \leq 0.05$.

Acclimatization

The in vitro regenerated plantlets, with well developed shoots (>4 cm) and root(s) (>3 cm), were taken out of the flask; the adhering agars were removed from the roots by washing with double distilled water. In one set of experiment, the plantlets were transferred to plastic cups containing sterilized garden soil (2): sand (1) and kept covered with transparent polyethylene bags. They were maintained inside the culture room at $25 \pm 2^\circ\text{C}$ and 16 hour photoperiod with $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity for two weeks. After 4 days, holes were punched in the polyethylene bags for gradual adaptation of the in vitro plantlets. The planting media were watered once at an interval of 4 days. After 2 weeks, the plantlets were transferred outside from the culture room and kept under complete shade for 2 days. Subsequently, the plants were transferred to the larger clay pots (20×25 cm) containing a mixture of soil, sand and vermi-compost (3:2:1) and kept outdoors. In the second set of experiment, the plantlets were directly shifted to outdoors by planting in clay pots (20 × 25 cm) containing a mixture of garden soil and sand (2:1).

Results and discussion

The establishment of a reliable micropropagation protocol requires the evaluation of different factors for production of large number of clonal progenies in a cost efficient manner within a short span of time. One of the most important factors for the development of such successful micropropagation protocol is, thus, the selection of suitable explants. The source of explant and mode of regeneration influence the genetic stability of the in vitro regenerated plants (Larkin et al. 1989). Explants with organized meristems are preferred

for micropropagation or in vitro clonal propagation as they are less prone to genetic instability than unorganized callus (Shenoy and Vasil 1992) or seedling explants. Thus, in the present study rhizome buds were chosen as explants for micropropagation unlike other recent reports of *H. coronarium*, where shoot tip explants derived from axenic seedling (Bisht et al. 2012) or callus mediated somatic embryogenesis (Huang and Tsai 2002; Verma and Bansal 2012) were used for in vitro regeneration of plants. Besides, the sprouted meristematic rhizome bud is one of the commonly used explants for micropropagation of plants belonging to the family Zingiberaceae.

Establishment of axenic culture in *H. coronarium* was challenging like other plants of Zingiberaceae family including *Curcuma* sp. and *Zigiber officinale* (Salvi et al. 2002; Das et al. 2010). In this study, about 57 % axenic cultures were established with 0.1 % (w/v) HgCl_2 treatment for 15 min. The bud size also played an important role in shoot multiplication of *H. coronarium*. The bud size of 0.5–1.5 cm was found to be optimum for multiple shoot proliferation (Fig. 1a). Rhizome with a sprouted bud size of less than 0.5 cm failed to show any response, while explants having a sprouted bud more than 1.5 cm failed to develop multiple shoots irrespective of the media used (Table 1). The report of Parida et al. (2013) is silent about two vital points, which include the % axenic culture establishment as well as the size of the explants used for culture initiation.

All the explants failed to develop multiple shoots on MS devoid of any growth regulators; each bud developed into a single shoot only. About 46.6 % of the explants showed the development of an average of single shoot with one root

Table 1 Effect of explant size on sprouting of shoots

Growth regulators (mg L^{-1})		Explant size (cm)	% response	Shoots/ explant
BA	NAA			
—	—	<0.5	—	—
		0.5–1.5	46.6 ^{hi}	1.0 ^c
		>1.5	50.0 ^h	1.0 ^c
1.0	—	<0.5	—	—
		0.5–1.5	83.3 ^b	1.3 ^{bc}
		>1.5	70.0 ^e	1.0 ^c
2.0	—	<0.5	—	—
		0.5–1.5	83.3 ^b	1.6 ^{bc}
		>1.5	73.3 ^{de}	1.0 ^c
3.0	—	<0.5	—	—
		0.5–1.5	73.3 ^{de}	1.6 ^{bc}
		>1.5	66.6 ^f	1.0 ^c
4.0	—	<0.5	—	—
		0.5–1.5	66.6 ^f	2.0 ^{bc}
		>1.5	60.0 ^g	1.0 ^c
1.0	0.5	<0.5	—	—
		0.5–1.5	96.6 ^a	2.3 ^b
		>1.5	76.6 ^{cd}	1.0 ^c
2.0	0.5	<0.5	—	—
		0.5–1.5	96.6 ^a	3.6 ^a
		>1.5	80.0 ^{bc}	1.0 ^c

In a column, different letters in superscripts indicate statistically significant difference between the means ($P \leq 0.05$; Duncan's new multiple range test)

Data were collected at 45 days of culture

Fig. 1 a. A rhizome bud on a culture medium b. Simultaneous shoot and root regeneration on MS devoid of any growth regulators c. One step shoot and root formation on MS+BA (1.0 mg L^{-1}) d. Multiple bud initiation from rhizome bud explant after 15 days of culture on MS+BA (2.0 mg L^{-1})+NAA (0.5 mg L^{-1}) e. & f. Multiple shoot and root proliferation on MS+BA (2.0 mg L^{-1})+NAA (0.5 mg L^{-1}) after 30 and 55 days of culture g. Acclimatized plant in a clay pot after 40 days of transfer from culture medium

simultaneously on the same medium (Fig. 1b). However, explants cultured on MS supplemented with a cytokinin i.e. BA at different concentration or in combination with an auxin i.e. NAA (0.5 mg L^{-1}) showed varied responses in the regeneration of the number of shoots as well as one step shoot and root regeneration (regeneration of shoots and roots in the same medium at the same time) (Table 2). MS supplemented with either BA (1.0 or 2.0 mg L^{-1}) alone or in combination with NAA (0.5 mg L^{-1}), showed simultaneous production of shoots and roots. However, explants cultured on a comparatively higher concentration of BA (3.0 – 4.0 mg L^{-1}) produced 1.6 – 2.0 shoots only and no simultaneous rooting was observed. This was in accordance with the observation made by Schraudolph and Reinert (1959) that, high concentrations of cytokinin generally inhibit or delay root formation. In MS supplemented with 1.0 – 2.0 mg L^{-1} BA, only fewer shoots (1.3 and 1.6) and roots (1.6 and 2.0) were recorded (Table 2; Fig. 1c). However, the presence of an auxin, NAA, along with BA in the MS medium significantly increases the number of regenerated shoots as well as roots per explant. This may be attributed to the interaction and balance between cytokinin and auxin, which (1) control the various aspects of cellular differentiation and organogenesis and (2) usually require for the formation of adventitious shoot and root meristem in tissue culture (George et al. 2008). A combination of BA and NAA was also found to be optimum in many other species of Zingiberaceae, which includes *Kaempferia galanga* (Shirin et al. 2000) and *Curcuma longa* (Salvi et al. 2002). MS augmented with an optimum concentration of 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA produced highest number of simultaneous shoots (3.6) and roots (4.0) per explant with average shoot and root length of 4.7 cm and 4.2 cm respectively (Table 2; Fig. 1d, e, f). Such simultaneous production of shoots and roots was reported earlier for a number of species of Zingiberaceae such as *Alpinia galanga* (Borthakur et al. 1999), *Kaempferia galanga* (Shirin et al. 2000), *Curcuma ceasia* and *Curcuma zedoaria* (Bharalee et al. 2005) and *Zinger zerumbet* (Faridah et al. 2011). In our study, the occurrence of simultaneous rooting along with shoot proliferation observed on MS medium devoid of any growth regulators as well as in the presence of cytokinin and auxin indicates that, this may be due to the presence of ‘intrinsic’ root inducing factors in the rhizomes of the rhizomatous plants (Agretious et al. 1996) rather than due to the sole influence of plant growth regulators. It has been estimated that the in vitro rooting stage can cost up to 35 – 75% of the total expenditure of the micropropagation protocol (Debergh and Maene 1981). Thus, the in vitro plant regeneration by simultaneous shoot and root formation in this study reduced the additional cost for rooting medium as well as time as it did not require a separate rooting stage. This entire protocol (starting from explant inoculation to

acclimatization) is a rapid one that required about two and a half-months time compared to other reports of *H. coronarium* (ca. 9 months, Verma and Bansal 2012; ca. 5 months, Bisht et al. 2012; ca. 4 months, Parida et al. 2013).

Acclimatization of in vitro regenerated plants with low mortality is vital to development of a successful micropropagation protocol. In our first set of experiment, with gradual acclimatization of plantlets in plastic cups containing sterilized garden soil : sand ($2:1$) inside the controlled environment of a culture room followed by placing them under complete shade and finally transferring to outdoor in larger clay pots containing a mixture of soil, sand and vermi-compost ($3:2:1$), we could achieve 100% acclimatization of in vitro regenerated plantlets. Cent percent acclimatization prompted us to carry out the second set of experiment on acclimatization, where we planted the in vitro regenerated plantlets in clay pots containing a mixture of garden soil and sand ($2:1$) and directly shifted them to outdoors (without any acclimatization stages of controlled temperature, light and humidity of a culture room or plant growth chamber). In this second experiment also 100% plant survival was observed, which indicated that no gradual acclimatization stages were needed for in vitro regenerated plants of *H. coronarium* from rhizome buds (Fig. 1g). It is a well known fact that in a number of cases hydraulic connectivity between shoot (stem) and roots is usually low or abnormal in the plantlets regenerated by sequential development of shoots and roots (Fila et al. 1998; Hazarika 2003). This limits the water uptake from the root into shoot (Hazarika 2003), which is one of the causes of high mortality of plantlets during acclimatization (Pospisilova et al. 1999). The micropropagated plantlets, thus, require a period of gradual acclimatization for vascular connections to

Table 2 Effect of various growth regulators on in vitro shoot and plant regeneration

Growth regulators (mg L^{-1})		% response	Shoots/ explant	Shoot length (cm)	Roots/ explant	Root length (cm)
BA	NAA					
-	-	46.6 ^e	1.0 ^c	2.7 ^{bc}	1.0 ^c	2.0 ^b
1.0	-	83.3 ^b	1.3 ^{bc}	3.6 ^{abc}	1.6 ^{bc}	2.2 ^b
2.0	-	83.3 ^b	1.6 ^{bc}	2.6 ^c	2.0 ^{bc}	2.1 ^b
3.0	-	73.3 ^c	1.6 ^{bc}	2.4 ^c	-	-
4.0	-	66.6 ^d	2.0 ^{bc}	2.2 ^c	-	-
1.0	0.5	96.6 ^a	2.3 ^b	4.5 ^{ab}	2.6 ^b	3.7 ^{ab}
2.0	0.5	96.6 ^a	3.6 ^a	4.7 ^a	4.0 ^a	4.2 ^a

In a column, different letters in superscripts indicate statistically significant difference between the means ($P \leq 0.05$; Duncan's new multiple range test)

Data were collected at 45 days of culture

be proper or substantial (Grout and Aston 1977; Hazarika 2003). In the present study, as the plantlets have been regenerated by simultaneous shoot and root regeneration, it is quite possible that a proper anatomical connection between the shoot and root of the plantlets has been established in vitro, much before acclimatization. This may help the plantlets overcome the water limitation and account for zero mortality. Thus the high success rate of acclimatization in this study may be attributed to simultaneous regeneration of shoots and roots. However, further study is necessary to substantiate this assumption. In this study, we could achieve 100 % outdoor plant establishment in clay pots without any acclimatization stage, which is better compared to other reports of acclimatization for *H. coronarium* (65 %, Bisht et al. 2012), (95 %, Verma and Bansal 2012) and (80 %, Parida et al. 2013).

In conclusion, we have developed a micropropagation protocol for *H. coronarium* using rhizome buds, where shoot proliferation has been achieved with simultaneous rooting of the shoots and the regenerated plants can be transferred directly to the mixture of garden soil and sand with 100 % success. This rapid and cost-effective in vitro plant regeneration protocol of *H. coronarium* can be utilized in pharmaceutical, paper and perfume industries without disturbing the plants in their natural habitat as well as for conservation by reintroduction of this plant species in their wild habitat.

Acknowledgment We thank the anonymous reviewers for their valuable comments to improve the manuscript. Our thanks are also to Dr. Subhra Prakash Das, Reader in English, Ravenshaw University, Cuttack, India for going through the manuscript for improvement of the English. Fund support under FIST program by Department of Science and Technology (DST), Government of India to Department of Botany, Ravenshaw University, Cuttack-753 003, India is gratefully acknowledged (SR/FST/LSI-017-2010).

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