Research Paper | *The Asian Journal of Horticulture,* (June, 2010) Vol. 5 No. 1 : 76-79

In vitro culture establishment techniques from field-grown *Heliconia* plants

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Accepted : *March, 2010*

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ABSTRACT

An experiment was conducted to establish *in vitro* cultures from field grown plants of *Heliconia*. The most responsive explant was shoot tip. For surface sterilization of shoot tip explants, double sterilization with 0.10 per cent mercuric chloride for 10 minutes followed by dipping in 0.05 per cent mercuric chloride for 5 minutes after trimming, gave the best results. Media supplementation with 0.05 per cent activated charcoal resulted in earlier shoot initiation and better survival percentage. Among the different injury treatments tried, longitudinal cutting of the shoot tip with apical dome into two halves yielded the highest number of axillary buds.

Key words : Heliconia, Culture establishment, Shoot tip explant, Surface sterilization, Physical injury treatments, Activated charcoal

Heliconias (*Heliconia* spp.) are attractive tropical plants with banana-like leaves and beautiful, long lasting inflorescences. They possess subterraneous rhizomes, commonly used for propagation from which new buds are developed. This system of vegetative propagation, produces a reduced number of plants and is also prone to serious risks of bacterial disease dissemination. Techniques to mass multiply the planting material through micropropagation serve as a means to bring down the cost of cultivation of elite varieties. However, special precautions need to be taken when explants are derived from field grown materials. Since the shoot tips emerge from below the ground level, they accumulate lot of soil and dirt. The presence of endophytic microorganisms such as *Ralstonia* sp. presents an obstacle even for tissue culture. Hence, thorough and effective surface sterilization is inevitable.

Success of *in vitro* propagation primarily depends on the proper selection of explants. Shiau *et al*. (1999) used terminal shoot tip explants for *in vitro* propagation of *H. psittacorum* cv. RHIZOMATOSA. When shoot buds were used as explants, Talukdar *et al*. (2002) obtained shooting and rooting in *H. psittacorum*. Babu (2005) tried shoot apex, rhizome bits, leaf segments and root segments for culture establishment in *H. psittacorum* cv. DEEP ORANGE and shoot apex was found to be most effective.

The technique of splitting banana shoot tips longitudinally through their apex in order to induce multiple shoot formation was first described by De Guzman *et al*. (1980). Dividing the apical dome into two halves and culturing each half separately was found to be the best one in enhancing the release of axillary buds. By longitudinally splitting the shoot through the apex, individual shoots of banana were induced to form multiple shoot clusters by several workers like Cronauer and Krikorian (1984) and Bhaskar (1991).

In *H. psittacorum* cv. DEEP ORANGE, the highest survival percentage (45.00) was recorded with absolute alcohol one minute wash + treatment with mercuric chloride (0.10 per cent) for 10 minutes, followed by the treatment with 0.05 per cent mercuric chloride for 10 minutes after trimming of the explants (Babu, 2005). In gladiolus, Misra and Singh (1999) recommended treatment with 0.10 per cent mercuric chloride for ten minutes for surface sterilization. In *Polianthes tuberosa*, Krishnamurthy *et al*. (2001) reported the effectiveness of 0.10 per cent mercuric chloride.

Media browning and explant blackening were severe during the culture establishment phase of *Heliconia*. Hosni (2001) which suggested the application of 1.00 per cent activated charcoal to the culture medium for controlling oxidative browning in *Strelitzia reginae*. The decreased oxidation level of banana (cv. GRAND NAIN) shoots in the presence of 0.30 per cent activated charcoal, reported by Costa *et al*. (2006)

In this study an attempt has been made to initiate *in vitro* culture from field-grown *Heliconia* plants belonging to three varieties belonging to three distinct groups *viz*., St. Vincent Red (*Heliconia psittacorum*), Golden Torch Adrian (*Heliconia psittacorum* x *Heliconia spathocircinata*) and Sexy Pink (*Heliconia chartacea*).

MATERIALS AND METHODS

Two types of explants *viz*., shoot tips and rhizome bits were tried for *in vitro* establishment. Shoot tips were collected from young actively growing plants. The selected plants were dug out, detopped and the shoot tips were reduced to a length of about 2 cm using surgical

blade, retaining the apical dome (1 cm). In the case of rhizome bits, the plants were dug out and the rhizomes were collected. After washing thoroughly in running tap water, the rhizomes were kept in gunny bags for 24-48 hours to activate the dormant buds and then cut into small pieces of 1.5-2.0 cm length using surgical blade.

The collected shoot tips were washed thoroughly in running tap water to remove all the dirt and soil particles adhering to them. Thereafter, they were kept immersed in water with a few drops of wetting agent, labolene for half an hour followed by rinsing in distilled water to remove traces of labolene. The shoot tips were transferred to sterile conical flasks and subjected to surface sterilization using chemicals inside laminar air flow chamber. The shoot tip explants after surface sterilization were rinsed five times with sterile distilled water. For explant standardization, shoot tips and rhizome bits were treated with 0.10 per cent mercuric chloride for 10 minutes before inoculation. Surface sterilization treatments were also standardized and the treatments tried are furnished in Table 1.

Table 1 : Treatments tried for surface sterilization of shoot tip explants in *Heliconia*

Treatments	Name of sterilant, concentration and duration of treatment
HS_1	Mercuric chloride 0.08% for 5 minutes
HS_2	Mercuric chloride 0.08% for 10 minutes
HS_3	Mercuric chloride 0.10% for 5 minutes
HS_4	Mercuric chloride 0.10% for 8 minutes
HS_5	Mercuric chloride 0.10% for 10 minutes
HS_6	Mercuric chloride 0.10% for 12 minutes
HS_7	Absolute alcohol (1 minute dip) + Mercuric chloride 0.10% for 10 minutes
HS_8	Mercuric chloride 0.10% for 10 minutes + Mercuric chloride 0.10% for 2 minutes (after trimming)
HS_9	Mercuric chloride 0.10% for 10 minutes + Mercuric chloride 0.05% for 10 minutes (after trimming)
HS_{10}	Mercuric chloride 0.10% for 10 minutes + Mercuric chloride 0.05% for 5 minutes (after trimming)

(Medium – MS + inositol 100 mg l^{-1} + sucrose 30.00 g l^{-1} + agar 6.30 g l^{-1})

Observations on the percentage of cultures contaminated, percentage of cultures blackened and percentage of cultures survived were made on six explants per treatment after four weeks of culturing. The presence of bacterial exudates in the shoot apex and/or bacterial and fungal growth were characterized as contamination, while the gradual darkening of explants until death was characterized as blackening.

The sterilized shoot tips were further reduced in size by trimming down the cut surface and removing the outer layer of apical dome by using sterilized forceps and blade.

To study the effect of physical injury made to shoot tips on culture establishment and shoot proliferation, the following treatments (Table 2) were tried.

Table 2 : Treatments tried to study the effect of physical injury on culture establishment of shoot tip explants in *Heliconia*

Treatments	Physical injury treatments
HD_1	Apical dome intact
HD_2	Apical dome split longitudinally
HD_3	Apical dome removed
HD_4	Shoot tip with apical dome split longitudinally into half

(Medium – MS + inositol 100 mg l^{-1} + sucrose 30.00 g l^{-1} + agar 6.30 g l^{-1} + BA 5.00 mg l^{-1})

In order to study the effect of activated charcoal on culture establishment of *Heliconia* varieties, treatments involving different concentrations of activated charcoal (Table 3) were tried.

Table 3 : Treatments tried to study the effect of activated charcoal on culture establishment in *Heliconia*

Treatments	Concentration of activated charcoal (%)
EAC_1	0.05
EAC_2	0.10

(Medium – MS + inositol 100 mg l^{-1} + agar 6.30 g l^{-1} + sucrose 30.00 g l^{-1} + BA 5.00 mg l^{-1})

RESULTS AND DISCUSSION

Explant selection is a critical step in micropropagation. In the varieties St. Vincent Red and Golden Torch Adrian, both shoot tips and rhizome bits were tried for culture establishment (Table 4).

The data represents the mean value of 10 replications.

In the present study, shoot tips were found to be more effective than rhizome bits in establishing *in vitro* cultures with respect to shoot initiation as well as survival rates. The rhizome bits exhibited only greening. The difference in the performance of the explants can be attributed to the difference in the level of endogenous hormones, nutrients and also due to various physiological factors. Since shoot tip performed better, it was selected as the explant for *in vitro* studies. Shoot tip explants have

Table 4 : Effect of explants on *in vitro* culture establishment in *Heliconia*

Varieties	Explants	Shoot initiation or greening (%)	Survival (%) after four weeks
St. Vincent	Shoot tips	50.00	40.00
Red	Rhizome bits	30.00	33.33
Golden	Shoot tips	70.00	57.14
Torch	Rhizome bits	30.00	33.33
Adrian			
Sexy Pink	Shoot tips	70.00	57.14

The data represents the mean value of 10 replications.
(Medium – MS + inositol 100 mg l^{-1} + sucrose 30.00 g l^{-1} + agar 6.30 g l^{-1} + BA 5.00 mg l^{-1})

been successfully used earlier by Shiau *et al.* (1999) for culture establishment in *H. psittacorum* cv. RHIZOMATOSA.

Thorough and effective surface sterilization of shoot tip explants is in evitable since the shoot tips accumulate lot of soil and dirt which harbour microorganisms. Bacterial contamination mainly due to *Ralstonia* sp. has long been a major hindrance in the *in vitro* culture establishment of *Heliconia* (Rodrigues, 2005). In the present study also, bacterial contamination was encountered in severe proportions. Hence mercuric chloride was used at different concentrations (0.08 to 0.10 per cent) for varying periods of time (5 to 12 minutes) for achieving surface sterilization of the shoot tip explants. The variety St. Vincent Red was selected for standardization of surface sterilization. Double sterilization with mercuric chloride was found to be more effective with respect to survival percentage (Table 5).

Table 5 : Effect of surface sterilants on the control of *in vitro* microbial contamination in shoot tip explants of *Heliconia* (Var. St. Vincent Red)

Treatments	Blackening (%)	Contamination (%)		Survival (%) after four weeks
		Fungal	Bacterial	
HS_1	0.00	33.33	66.67	0.00
HS_2	16.67	33.33	33.33	16.67
HS_3	0.00	50.00	50.00	0.00
HS_4	16.67	16.67	50.00	16.67
HS_5	33.33	0.00	33.33	33.33
HS_6	50.00	0.00	16.67	33.33
HS_7	33.33	0.00	33.33	33.33
HS_8	33.33	0.00	16.67	50.00
HS_9	50.00	0.00	16.67	33.33
HS_{10}	16.67	0.00	16.67	66.67

The data represents the mean value of 6 replications
Treatment details are given in Table 1

Treatment with 0.10 per cent mercuric chloride for 10 minutes (before trimming of the explants) followed by dipping in 0.05 per cent mercuric chloride for 5 minutes (after trimming) recorded the highest survival percentage (66.67). Babu (2005) suggested dipping in absolute alcohol for one minute before mercuric chloride treatment for better survival of explants. Contrary to this finding, in the present study, when absolute alcohol dip was given to the explants before mercuric chloride treatment, only 33.33 per cent survival was recorded.

Physical injury treatments were found to be favourable for the release of axillary buds in this monocot plant. In order to study the effect of physical injury, shoot tips of the variety Sexy Pink from *ex vitro* and *in vitro* were tried. Physical injury to *ex vitro* shoot tips resulted in drying of the explants and they did not survive beyond two weeks. However, shoot tips from *in vitro* cultures responded positively to the injury treatments. Among the different injury treatments tried, longitudinal cutting of the shoot tip with apical dome into two halves and separately culturing them in MS medium supplemented with BA 5.00 mg l^{-1} yielded the highest number of axillary buds (3.25 buds from each half) in a period of four weeks. This treatment also recorded a survival of 75.00 per cent after four weeks (Table 6). The present finding supports the earlier reports made by Diniz *et al*. (2004) in *H. stricta* and Bhaskar (1991) in banana.

Table 6 : Effect of physical injury on *in vitro* culture establishment of shoot tip explants in *Heliconia* var. Sexy Pink

Treatments	Number of shoot buds developed in four weeks	Survival (%) after four weeks
HD_1	1.50	75.00
HD_2	2.00	75.00
HD_3	1.00	50.00
HD_4	3.25	75.00
F	25.571**	
S.E.±	0.191	
C.D. (P=0.05)	0.588	

The data represents the mean value of 4 replications.
Treatment details are given in Table 2.
** indicate of significance of value at P = 0.01

Media browning and explant blackening were severe during the culture establishment phase of *Heliconia*. Hence in the present study, activated charcoal was added to the establishment medium at two different concentrations *viz*., 0.05 (EAC_1) and 0.10 per cent (EAC_2) to study its effect. It was observed that both concentrations were equally effective not only in reducing the media browning and explant blackening but also in

Table 7 : Effect of activated charcoal on *in vitro* culture establishment in *Heliconia*

Treatments	St. Vincent Red			Golden Torch Adrian			Sexy Pink		
	Explant blackening and media browning (%)	Minimum days for shoot initiation	Survival (%) after four weeks	Explant blackening and media browning (%)	Minimum days for shoot initiation	Survival (%) after four weeks	Explant blackening and media browning (%)	Minimum days for shoot initiation	Survival (%) after four weeks
Control	83.33	6.83	16.67	66.67	6.67	33.33	66.67	5.00	33.33
EAC_1	66.67	4.17	33.33	50.00	4.00	50.00	50.00	3.67	50.00
EAC_2	66.67	4.17	33.33	50.00	3.83	50.00	50.00	3.67	50.00
F		20.320**			54.600**			11.429**	
SE		0.342			0.215			0.228	
C.D. (P=0.05)		1.029			0.648			0.686	

(Medium – MS + inositol 100 mg l^{-1} + sucrose 30.00 g l^{-1} + agar 6.30 g l^{-1} + BA 5.00 mg l^{-1})
The data represents the mean value of 6 replications.
Treatment combinations are given in Table 3.
** indicates significance of value at P = 0.01

enhancing the survival percentage of explants (Table 7).

In all the three varieties, explants in the charcoal media exhibited earlier shoot initiation. From the results, it can be concluded that addition of activated charcoal at a concentration of 0.05 per cent is sufficient to prevent the detrimental effect of brown exudates. The present findings agree with the report of Hosni (2001) and Costa *et al*. (2006) (application of 1.00 per cent activated charcoal to the culture medium for controlling oxidative browning in *Strelitzia reginae* and 0.30 per cent in banana (cv. GRAND NAIN) shoots, respectively).

Acknowledgement :

The first author wishes to thank Heliconia Society International for providing Research Grant and Jawaharlal Nehru Memorial Fund, Teen Murti House, New Delhi for providing fellowship for doctoral studies.

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