Assessment of InvitroPropagation and Conservation of *SwertiaChirayata*

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ABSTRACT

A protocol for *in vitro* propagation and conservation was developed for *Swertiachirayita,* an endangered medicinal plant. The sterilized explants (leaves) cultured on MS medium supplemented with 0.1 mg/l NAA and 3.0 mg/l BA gave best results for *in vitro* callus induction. Shoot regeneration was obtained from the callus on the same medium. The *in vitro* shoots cultured on MS medium supplemented with 2.5 mg/l BA and 0.1 mg/l Kinetin gave best results for *in vitro* shoot multiplication. The MS medium supplemented with 0.1 mg/l NAA and 3.0 mg/l BA medium was found to be the best for direct shoot regeneration from *in vitro* leaves. 80.30% root induction from *in vitro* grown shoots was obtained on half strength MS medium supplemented with 400 mg/l activated charcoal and 0.1 mg/l NAA. *In vitro* conservation was carried out by using two different approaches namely slow growth by changing media composition (sucrose and abscisic acid), at low temperature and cryopreservation following vitrification.With increase in concentration of sucrose and ABA decrease in growth of *in vitro* shoots was observed. No shoot multiplication with average leaf size of 0.35 cm and shoot length 0.67 cm was observed on half MS containing 90 g/l sucrose. Similarily, in case of media containing half strength MS salts and 3.0 mg/l ABA showed no shoot multiplication,0.83 cm average leaf size and 0.83 cm shoot length. At low temperature the *in vitro* shoots incubated at 4o C, showed 100% retrieval, with 1.00 cm average number of shoots, 0.86 cm shoot length and 0.34 cm leaf size. *In vitro* shoots incubated at 10o C, showed 100% retrieval, with 1.00 cm average number of shoots, 0.76 cm shoot length and 0.23 cm leaf size. During studies the vitrified shoot gave retrieval of 42.33% when precooled at 4o C while only 22.37% vitrified shoots were retrieved from those precooled at 10o C.

Keywords: Invitro Propagation, Conservation and *SwertiaChirayata*

INTRODUCTION

*Swertiachirayita*Buch.- Hams. ex Wall. belongs to family Gentianaceae. It is commonly known as "Chirata" and in sanskrit it is called as Anaryatikta, Ardhatikta, Bhunimba, Chiratika, and Viktaka. It is an indigenous species of temperate Himalaya found at an altitude of 1200 to 3000 m from Kashmir to Nepal and also distributed in Bhutan, Khasi hills and Sikkim. (The Wealth of India, 1976; Garg, 1987; Kirtikar and Basu, 1998). It is an annual/ biennial medicinal herb (Joshi and Dhawan, 2007) but still it is not clear whether the plant behaves differently due to climatic conditions or varying genotype.

The plant has an erect, about 2-3 ft long stem and the whole plant is bitter in taste. It has lanceolate acute leaves with orange brown or purplish coloured stem, and contains large continuous yellowish pith. The roots are simple, tapering , stout, short and almost 7 cm long.

The flowering & fruiting occurs between July to September. Flowers of *Swertiachirayita*are in the form of numerous small, axillary, opposite, lax cymes arranged as short branches small, stalked, green-yellow, tinged with purple colour, rotate and tetramerous. The corolla is twice as long as the calyx and divided near the base into four ovate–lanceolate segments. The upper surface of the petal has a pair of nectaries covered with oblong scales and ending as fringes. Fruit is a small, one-celled capsule with a transparent yellowish pericarp. It dehisces from septicidally into two valves. Seeds are numerous, minute many-sided and angular. Floral characteristics such as colourful corolla and presence of nectaries support cross-pollination in the species. *Swertiachirayita*contains a yellow bitter ophelic acid and two bitter glucosideschiratin (Pant *et al.*, 2010).

It can be traced through the medicinal history as a safe ethnomedicinal herb. It is used in the treatment of various ailments like chronic fever, malaria, liver and stomach disorders, cold and cough, asthma and joint pains. It has blood-purifying, antifungal and antihelmintic qualities (Pant *et al*., 2011).

*Swertiachirayita*is difficult to propagate on mass scale via seed owing to non-availability of seeds due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. Also, the low viability and germination percentage of seeds and the necessary delicate field handling of the seedlings are some of the factors that discourage agro technological development and commercial cultivation of the species. There is an increasing pharmaceutical demand of the species both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. Destructive harvesting and loss of habitat has diminished the existing populations of *Swertiachirayita*. The species is among the highly prioritized medicinal plants of India as identified by National Medicinal Plant Board, Govt. of India and according to the new International Union for Conservation of Nature and Natural resources (IUCN) criteria, this priority plant has been designated as critically endangered (Pant *et al.*, 2010). Conventional approaches of propagation alone cannot guarantee the re-establishment and recovery of this important plant species, consequently, the application of alternative reproducible micropropagation strategies has become inevitable for mass propagation and sustainable utilization of this age-old medicinal plant.

Plant tissue culture provides a viable alternative for managing these valuable resources in a sustainable manner. It serves as an alternative means of secondary metabolite production through cell and organ cultures. Most importantly, micropropagation provides an efficient method for *ex situ* conservation of plant biodiversity and multiplication of the endangered species from a minimum of available plant material. In fact, the technique of micropropagation has been effectively employed for the multiplication and conservation of endangered and commercially exploited medicinal plants such as *Picrorhizakurroa*(Lal*et al*., 1988), *Aconitum heterophyllum*(Giri*et al*., 1993) , and *Saussureaobvallata*(Joshi and Dhar, 2003). However, very few micropropagation studies dealing with large-scale propagation of *S. chirayita*or its allied species have been reported. This situation has remained practically unchanged since 1970s when *in vitro* culture of *Swertia japonica*, a species of genus *Swertia*(Miura *et al.*, 1978), was reported for the first time.

The literature reveals that there is no standard method of cultivation of this valuable medicinal plant. Moreover, indiscriminate exploitation in the past has resulted in depletion of

the resources and collection has become uneconomical, so the species require immediate attention for its protection, conservation and large scale cultivation. Plant tissue culture offers an attractive and quick method for rapid multiplication and further conservation also. Due to scarcity of work on this important medicinal plant the present studies were focused to develop an efficient protocol for mass multiplication of species. Further studies were done to conserve the species using slow growth and cryopreservation techniques so the plant material could be stored for short and long term so that it can be made available on sustainable basis.

There are only some reports on *in vitro* propagation of *Swertiachirayita.* Ahuja*et al*. (2003) and Koul*et al*. (2009) have reported micropropagation of *Swertiachirayita*via field-grown nodal explants while seedling derived shoot tip explants were also used in the study. Similarly direct shoot organogenesis from *in vitro* leaves (Wang *et al*., 2009) was also reported. So developing an *in vitro* regeneration protocol for *Swertiachirayita*is urgent to promote large-scale production for *ex situ* conservation and for satisfying the pharmaceutical needs.

MATERIALS AND METHODS

IN VITRO PROPAGATION

Plant Material

Collection of plant material

Young and healthy leaves of *Swertiachirayita*plants were collected in the month of August, September and October 2012 from nursery of Forest Product Department of Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.).

Preparation of culture media

Cleaning of glassware

The new glass-wares were cleaned by washing them in a solution of 10.0% teepol in hot water. Remove the adhering dust with the help of bottle brush. The glass-wares were than soaked in the solution of chromic acid for overnight and rinsed thoroughly with hot water. Finally rinsed with double distilled water and dried at 150ºC in hot air oven for an hour.

The used glass wares containing the contaminated cultures were first autoclaved to kill the contaminating microorganisms. The molten warm medium was disposed off to empty the culture vessels. The culture vessels were washed with hot water containing 10.0% teepol and rinsed with distilled water followed by sterilization in hot air oven at 150ºC for one hour.

Media preparation

Explants were cultured on Murashige and Skoog (1962) medium (APPENDIX-I) supplemented with different concentrations of growth hormones.

Separate stock solutions of inorganic nutrients and organic nutrients were prepared (APPENDIX-II). Organic nutrients were dispensed in 10 ml aliquots and stored at 4ºC for maximum of one month. Plant growth regulators were prepared fresh each time; auxins were titrated into solution with alcohol, where ascytokinins were dissolved in dilute NaOH or aqueousethanol.

In preparing medium each stock is added one by one in specified quantity in double distilled water, after bringing them to room temperature. Sucrose and meso-inositol were added in the concentration of 30.0 g/l and 100.0 mg/l respectively. Then add the standardized amount of different growth hormones depending upon type of medium to be prepared such as establishment, multiplication and rooting. Then pH of the medium was adjusted in between 5.6 to 5.8 with 1 N HCl or 1 N NaOH. Final volume was adjusted with double distilled water. Thereafter agar-agar (8.0 g/l) was added and homogenized by heating the media. The media was finally dispensed into sterilized culture vessels and plugged the mouth of vessels with nonabsorbent cottonplugs.

Sterilization of culture medium

Culture vessels containing the medium were sterilized in autoclave at a pressure of 15 lbs per $inch^2$ at 121.6ºC for 15 -20 minutes. After cooling the media were stored in dark condition at 25ºC $\pm$2ºC for about a week to check the contamination beforeuse.

Aseptic manipulations and cultural condition

Sterilization of equipments

All the equipments, metal instruments which were used in culturing were wrapped in aluminum foil and sterilized by autoclaving. All the aseptic manipulations were carried out in laminar air flow chamber fitted with Ultra

Violet light, HEPA filters (High efficiency Particulate Air Filters) and Fluorescent tube (KlenzaidsBioclean Devices (P) Ltd., Model 1504). Before starting the aseptic manipulations the working surface was thoroughly wiped with 70% alcohol. Thereafter, culture vessels containing medium, autoclaved equipments, cotton, 70% alcohol and spirit lamp were kept inside the laminar air flow cabinet and UV light was switched on before 15 minutes ofculturing.

During culturing UV light was switched off, and fluorescent tube and air flow were switched on. Wiped the hands with 70% alcohol, thereafter flame sterilized the working surface and metal instruments prior to culturing. After culturing, flame sterilized the rim of the culture vessel and quickly plugged the mouth of the vessel. In all the experiments cultures were incubated in culture room at temperature of 25 $\pm$ 2ºC, with 60 $\pm$ 5 per cent relative humidity under 35 µmol/ms photosynthetic photon flux white fluorescent light emitted by 40 W Phillips tubes, programmed for 16 hour photoperiod and 8 hour dark conditions.

Plant regeneration

Sizing of explants

Explants were procured from the 1.0 year old plant of *Swertiachirayita*. Outer older leaves were carefully removed with the help of sharp sterilized scalpel blade to keep the juvenile leaves with or without leafbase.

Surface sterilization

Explants were washed thoroughly under running tap water with teepol for about half an hour to remove the soil and dust particles from the plants. After that explants were washed with sterilized distilled water to make the plant material free from superficial contamination. Rest of the sterilization procedure was carried out in laminar air flow. Explants were transferred to laminar air flow and rinsed the explants with 70% alcohol for about 1-2 minutes. Thereafter washed the explants with autoclaved distilled water 2 to 3 times to remove the alcohol. After that explants were treated with 0.3% bavistin for different time durations, followed by washing with autoclaved distilled water to remove the tracesof

bavistin. Therefore, treatment of 0.5% sodium hypochlorite was given for different time durations, followed by washing with autoclaved distilled water to remove the particles of sodium hypochlorite. This experiment was carried out in three replications with 24 explants in each. Observations were recorded for:

i) Effect of different treatment durations of sterilants on per centsurvival of explant.
ii) Effect of sterilants on per cent contamination of theexplant.

In vitro establishment of callus

The establishment of culture was done by inoculating the surface sterilized leaves with leaf base on solid MS medium supplemented with different concentrations of BA and NAA in combinations. This experiment was carried out in three replications containing 24 explants in each replication. The cultures were incubated at temperature of 25 $\pm$2ºC under 16 hour photoperiod for four weeks. The observations were recordedfor:

i) Effect of growth hormones on establishment of cultures in terms of per cent establishment wasrecorded.

In vitro shoot bud induction

The calli were subcultured on solid MS medium supplemented with different concentrations of BA and NAA in combinations. This experiment was carried out in three replications containing 24 explants in each replication. The cultures were incubated at temperature of 25 $\pm$ 2ºC under 16 hour photoperiod for four weeks. The observations were recorded for:

i) Effect of growth hormones on establishment of shoot regenerationin terms of per cent establishment wasrecorded.
ii) Average shoot length (cm) of multipliedmicroshoots.
iii) Average number of shoots perexplant.

In vitro multiplication of shoots

Multiplication of established shoots was carried out on MS medium supplemented with different combinations and concentrations of BA and Kinetin.

This experiment was carried out with 24 explants replicated 3 times. After four weeks of incubation observations were recorded for:

i) The effect of growth hormones on multiplication of *invitro* raisedmicroshoots.

ii) Average shoot length (cm) of multipliedmicroshoots.

iii) Average number of shoots perexplant.

Direct shoot regeneration

The leaves were separated from *in vitro* regenerated shoots and cultured on solid MS medium supplemented with different concentrations of BA and NAA in combinations. This experiment was carried out in three replications containing 24 explants in each replication. The cultures were incubated at temperature of 25 $\pm$2ºC under 16 hour photoperiod for four weeks. The observations were recordedfor:

i) Effect of growth hormones on establishment of cultures in terms ofper cent shoot regeneration wasrecorded.

ii) Average number of shoots perexplant.

iii) Average shoot length (cm) of multipliedmicroshoots.

Rooting of microshoots

3.1.4.7.1 *In vitro* rooting

Shoots of variable length ranging in length 3.0 to 3.5 cm were excised at different stages of subculturing and transferred to full strength MS medium and half strength MS medium containing 400 mg/l activated charcoal and NAA (0.1 mg/l - 0.4 mg/l) for root induction. Experiment consisted of 24 microshoots in three replications followed by completely randomized design. The cultures were incubated at 25 $\pm$ 2ºC under 16 hours photoperiod. After four weeks of incubation observations were recorded for:

i) Per cent rooting which was based upon number of microshootsforming roots among total culturedshoots.

ii) Numbers of roots permicroshoot.

iii) Average root length(cm).

Hardening

Preparation of potting mixture

The survival and establishment of shoots after *in vitro* rooting was studied after transplanting the plantlets into potting media consisting soil and cocopeat.

Potting media was brought to homogenous consistency and the media was sterilized in an autoclave at a pressure of 15 lbs per $inch^2$ at 121º C for half an hour. After sterilization mixture was transferred to small plastic pots. Than potting mixture was drenched with ¼th MS medium to keep the mixture moist and nutritive so that it can support the plantgrowth.

Washing of roots

In vitro raised microshoots of length 2.5 to 3.5 cm along with 3.0 to 4.0 leaves were taken out from the tubes in such a way that roots were not damaged. Roots were washed under running tap water to remove the adhering medium. After that, plants were kept in water for about 30 minutes, so they do not wilt after transfer to soil. Thereafter, roots of the *in vitro* raised shoots were dipped in 0.2% bavistin solution to avoid any fungalattack.

Transplantation into pots

Well developed plantlets were transferred to plastic cups of diameter 5.0 cm. The plants were covered with jam bottles to maintain the relative humidity. The plants were watered at every alternate day and observed.

***IN VITRO* CONSERVATION**

3.2.1 By Slow growthmethod

3.1.2.1 By changing the media composition

In vitro raised shoots of *Swertiachirayita*were cultured on full and half strength MS media with different concentration of sucrose (30 g/l,60g/l and

90g/l) and abscisic acid (1.0 – 3.0 mg/l) to slow down the growth of *in vitro* raised shoots.

Observations were recorded after 30 days of incubation for percentage survival of microshoots.

3.1.2.1 By storing at low temperature

In vitro raised shoots of *Swertiachirayita*were tried for their survival at lowertemperatureat4^{o}Cand10^{o}C.*Invitro*multiplyingshootsonM$_4$medium (full strength MS medium supplemented with 0.1 mg/l Kn and 2.5 mg/l BA solidifiedwithagar)wereincubatedat4^{o}Caswellason10^{o}C.Theflaskswere closed with cotton plugs which were wrapped with parafilm that was swabbed with rectified spirit to prevent the desiccation of medium.

Observations were recorded after 30 days of incubation for percentage survival of microshoots.

3.2.2 Cryopreservation following vitrification of *in vitro*shoots

*Invitro*raisedshootsof*Swertiachirayita*weredividedintotwosetsand precooled separately at 4^{o}C and 10^{o} C in an incubator for one month. Henceforth, themanipulationswerecarriedoutaseptICallyinlaminarflowchamber.Pre-cooled *invitro*raisedshootsweretakenandapproximately3.0mmlongexplantsi.e.shoot buds were prepared. Autoclaved petriplates with filter paper were taken and 1.5-2.0 ml of precultured medium, named V$_1$ (MS salt+ 0.5 M sucrose) was added. The pre-cooled shoot segments were placed on it, sealed with parafilm and incubated overnight indark at 4^{o} C temperature inside a refrigerator. Henceforth, the manipulations were carried out aseptically in laminar flow chamber. The shoot segments were put into pre-cooled cryovials containing V$_2$ solution (MS salts + 0.4 Msucrose+3Mglycerol)andcryovialswereincubatedat4^{o}Cfor20-30minutes.

After this V$_2$ solution was pipette out from cryovials leaving the shoot segments behind and 1.5-2 ml of vitrification solution V$_3$ (MS salts + 30% glycerol + 13% DMSO + 15% ethylene glycol) was added. After this the cryovials were placed on laptop cooler (at ice temperature) for 60 minutes. Finally the cryovials were placed in small muslin cloth bags andthe

bags containing cryovials were quickly plunged into a cryocan containing liquid nitrogen for 60 minutes. For retrieval the cryovials were taken out and thawing was done quickly with constant agitation at 40^{o} C.

Henceforth the manipulations were carried out aseptically in laminar flow chamber. V_3 solution was discarded using a micropipette and 4 -5 washings were given using V_4 solution (MS salts + 1.2 M sucrose) to completely eradicate the effects of cryoprotectants. After 20 minutes V_4 solution was discarded and the shoots were placed on an autoclaved filter paper inside a petridish containing 1 mlofliquidshootmultiplicationmediumconsistingofMSbasal+0.1mg/lKn +2.5 mg/l BA. The petridish was sealed with parafilm and kept overnight in dark at 25 ± 2 ^{o} C.

These were then transferred to fresh, solid shoot multiplication medium in flasks and were incubated in diffused light for seven to ten days. Finally the shoot tips were transferred to full light at 25 ± 2^{o}C.

Observations were recorded with respect percent retrieval of microshoots after cryopreservation vitrification.

3.3 STATISTICALANALYSIS

The experiments were conducted in a completely randomized design (CRD). The data recorded for different parameters was subjected to analysis of variance (ANOVA) using completely randomized design [Gomez and Gomez, 1984 (Appendix-III)]. Data transformation was carried out as needed to satisfy ANOVA requirements. Arcsine transformation was performed on percentage data (derived from count data) lying in the range of both zero to thirty per cent and seventy to hundred per cent while square-root transformation was performed on data consisting of small whole numbers i.e. data counting in rare events and for percentage data (derived from count data) lying within the range of 0 to 30 per cent and 70 to 100 per cent, but not both. The data that have been transformed were expressed in original units for presentation in theTables.

RESULTS AND DISCUSSION

IN VITRO PROPAGATION

Source plant material

The plant material of *Swertiachirayita*was collected from the nursery of Forest Product Depatrment of Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). Plant of *Swertiachirayita*is shown in Plate 1.

Sterlization of explants

Leaves were used as explant for *in vitro* propagation of *Swertiachirayita*. Surface sterilization of explants was done using bavistin and sodium hypochlorite. The leaves were cut from the plant and washed thoroughly with tap water to remove adhering soil. The explants were prepared and washed with 2-3 drops of teepol as detergent under running tap water for 15-20 minutes. There after, under laminar air flow cabinet the explants were treated with 0.3% bavistin for different duration (10-25 minutes) followed by treatment with 0.5% sodium hypochlorite for 5-10 minutes as shown in Table 1.

Table1: Effect of different chemical sterilants on sterilization of leaf explant of *Swertiachirayita*after four weeks ofincubation

Sr. No	Treatment	Sterilant	Concentration	Duration	Percentage of uncontaminated leaf after 4 weeks of culturing	Percent survival of explants
1	T1	Bavistin (50% carbendazim)	0.3%	10 minutes	6.25 (14.47)	10.00 (18.41)
		$NaoCl_2$	0.5%	5 minutes		
2	T2	Bavistin (50% carbendazim)	0.3%	15 minutes	18.75 (25.64)	22.20 (28.08)
		$NaOCl_2$	0.5%	5 minutes		
3	T3	Bavistin (50% carbendazim)	0.3%	20 minutes	25.00 (29.98)	34.45 (35.92)
		$NaOCl_2$	0.5%	5 minutes		
4	T4	Bavistin (50% carbendazim)	0.3%	25 minutes	37.35 (37.65)	45.45 (42.39)
		$NaOCl_2$	0.5%	5 minutes		
5	T5	Bavistin (50% carbendazim)	0.3%	10 minutes	45.55 (42.42)	58.45 (49.84)
		$NaOCl_2$	0.5%	10 minutes		
6	T6	Bavistin (50% carbendazim)	0.3%	15 minutes	64.25 (53.26)	64.54 (53.43)
		$NaOCl_2$	0.5%	10 minutes		
7	**T7**	**Bavistin (50% carbendazim)**	**0.3%**	**20 minutes**	**87.55 (69.32)**	**95.00 (84.35)**
		$NaOCl_2$	**0.5%**	**10 minutes**		
8	T8	Bavistin (50% carbendazim)	0.3%	25 minutes	98.45 (83.102)	75.42 (60.04)
		$NaOCl_2$	0.5%	10 minutes		
$CD_{0.05}$					**2.05**	**1.73**
SE±					**0.67**	**0.57**

Values expressed in parentheses are the arc sine transformation of percentage

Fig 1.Swertiachirayita

The explants were than cultured on MS medium incubated at 25 ±2ºC at 16 hours photoperiod. Data presented in Table 1 shows that treatment T8 of 0.3% bavistin for 25 minutes and 0.5% sodium hypochlorite for 10 minute resulted maximum percent uncontaminated explants (98.45%) but with less survival (74.45%) of explants after 4 week of culturing. While 87.55% uncontaminated explants were observed on treatment T7 of 0.3% bavistin for 20 minutes and 0.5% sodium hypochlorite for 10 minutes with 95% survival of explants. It may be noted that increase in time duration of sterilization lead to death of explants, however decrease in time of treatments (T1, T2, T3, T4, T5, T6) resulted in less percent of uncontaminatedexplants.

Therefore, out of all the treatments, T7 was found to be the best and statistically significant for surface sterilization of explants. Hence for further experiment sterilization of explants was done using 0.3% bavistin for 20 minutes and 0.5% sodium hypochlorite for 10 minutes.

In vitro callus induction

For callus induction the surface sterilized leaves were cut into small pieces of 0.5-1.0 cm size and cultured on MS medium supplemented with differentconcentrationsofBA(2.0-3.5mg/l)andNAA(1.0-0.5mg/l).Atotalof 17 different medium combinations were tested (Table 2). The cultures were incubated at 25 ± 2ºC for 16 hours photoperiod. There were 24 explants in each treatment replicated thrice following completely randomized design.

In all the 17 treatments the explants showed swelling of explant to a considerable extent after 4 weeks of incubation. Callus initiation took place from cut ends of explants with in a week. Results presented in Table 2 show that on control medium MC_1 without any photohormones 77.78% callus formation was observed. While 100% callus induction was observed on treatment MC_4 containing 3.0 mg/l BA and 0.1 mg/l NAA with in 2 weeks of incubation which was statistically significant. The callus formed on both the media was compact initially green in colour which later on turned brown.

Table 2: Effect of different concentrations of BA and NAA on callus induction from *ex vitro* leaf explants after 4 weeks of incubation

Sr. No	Medium code (MS basal)	BA mg/l	NAA mg/l	Percent of Callus Induction
1	MC_1	-	-	77.78 (61.94)
2	MC_2	2.0	0.1	33.33 (35.25)
3	MC_3	2.5	0.1	22.22 (29.7)
4	**MC_4**	**3.0**	**0.1**	**100 (90.00)**
5	MC_5	3.5	0.1	33.33 (35.14)
6	MC_6	2.0	0.2	22.22 (28.02)
7	MC_7	2.5	0.2	11.11 (19.21)
8	MC_8	3.0	0.2	33.33 (33.49)
9	MC_9	3.5	0.2	22.22 (28.02)
10	MC_{10}	2.0	0.3	44.44 (43.38)
11	MC_{11}	2.5	0.3	31.25 (35.14)
12	MC_{12}	3.0	0.3	44.44 (41.78)
13	MC_{13}	3.5	0.3	11.11(19.21)
14	MC_{14}	2.0	0.4	0.00 (0.00)
15	MC_{15}	2.5	0.4	11.11 (19.21)
16	MC_{16}	3.0	0.4	44.44 (43.38)
17	MC_{17}	3.5	0.4	11.11(23.61)
$CD_{0.05}$				**6.36**
SE±				**2.20**

Values expressed in parentheses are the arc sine transformation of percentage

It may be noted that treatment MC_4 consisting of 3.0 mg/l BA and 0.1 mg/l NAA was statistically significant and showed 100% callus formation along with maximum shoot induction from leaf explants, hence was considered best for callus induction. Callus induction from leaf explant is presented in Plate 2(a-b).

In vitro shoot bud induction

The calli were subcultured on the same medium under same cultured condition for 4 weeks. For *in vitro* shoot regeneration callus was subcultured on same medium and incubated at 25± 2ºC of 16 hours photoperiod. There were 24 explants in each treatment replicated thrice following completely randomized design.

Results presented in Table 3 show that on control treatment (MC_1) 66.67% shoot bud induction was observed while 100% shoot bud induction was observed in MC_4 treatment containing 3.0 mg/l BA and 0.1 mg/l NAA with 4.50 cm average number of shoots, 1.72 cm shoot length. No shoot bud induction was observed in any othertreatment.

Table 3: Effect of different concentrations of BA and NAA on *in vitro* shoot bud induction from callus explant after 4 weeks of incubation

Sr. No	Medium code (MS basal)	BA mg/l	NAA mg/l	Percent of shoot bud induction	Number of shoots	Shoot length
1	MC_1	-	-	66.67 (54.68)	2.34	1.32
2	MC_2	2	0.1	0.00	0.00	0.00
3	MC_3	2.5	0.1	0.00	0.00	0.00
4	**MC_4**	**3**	**0.1**	**100 (89.39)**	**4.50**	**1.72**
5	MC_5	3.5	0.1	0.00	0.00	0.00
6	MC_6	2	0.2	0.00	0.00	0.00
7	MC_7	2.5	0.2	0.00	0.00	0.00
8	MC_8	3	0.2	0.00	0.00	0.00
9	MC_9	3.5	0.2	0.00	0.00	0.00
10	MC_{10}	2	0.3	0.00	0.00	0.00
11	MC_{11}	2.5	0.3	0.00	0.00	0.00
12	MC_{12}	3	0.3	0.00	0.00	0.00
13	MC_{13}	3.5	0.3	0.00	0.00	0.00
14	MC_{14}	2	0.4	0.00	0.00	0.00
15	MC_{15}	2.5	0.4	0.00	0.00	0.00
16	MC_{16}	3	0.4	0.00	0.00	0.00
17	MC_{17}	3.5	0.4	0.00	0.00	0.00
$CD_{0.05}$				**0.46**	**0.20**	**0.90**
SE±				**0.16**	**0.07**	**0.03**

Values expressed in parentheses are the arc sine transformation of percentage

Thus treatment MC_4 consisting 3.0 mg/l BA and 0.1 mg/l NAA showed maximum shoot regeneration of 100% which is statistically significant and found to be best among all. *In vitro* shoot bud induction from callus is presented in Plate 2 (c-d).

In vitro shoot multiplication

After four weeks the *in vitro* shoots regenerated were separated and cultured measuring 0.5-1.0 cm on MS medium supplemented with different concentration and combination of BA and Kinetin. There were 17 treatments with 24 explants in each treatment replicated thrice.

Results presented in Table 4 show that 3 shoots per explant was observed in control medium without any phytohormone. However treatment MS_5 containing 2.5 mg/l BA and 0.1 mg/l Kinetin showed maximum average number of shoots per explants (5.66) and shoot length (3.5) as compared to all other treatments after 30 days of incubation. No shoot multiplication was observed on treatment M_{12} andM_{17}.

Table 4: Effect of different concentrations of BA in combination with Kinetin on *in vitro* shoot multiplication after 4 weeks of incubation

Sr. No	Medium	BA mg/l	Kn mg/l	Number of shoots	Shoot length (cm)
1	M_1	-	-	3.00	3.00
2	M_2	1.0	0.1	3.66	1.00
3	M_3	1.5	0.1	5.00	1.50
4	M_4	2.0	0.1	4.33	2.00
5	**M_5**	**2.5**	**0.1**	**5.66**	**3.50**
6	M_6	1.0	0.2	2.66	2.50
7	M_7	1.5	0.2	2.33	2.00
8	M_8	2.0	0.2	2.66	1.50
9	M_9	2.5	0.2	2.66	1.50
10	M_{10}	1.0	0.3	2.00	0.95
11	M_{11}	1.5	0.3	1.33	0.86
12	M_{12}	2.0	0.3	1.00	0.95
13	M_{13}	2.5	0.3	0.33	0.84
14	M_{14}	1.0	0.4	0.66	1.0
15	M_{15}	1.5	0.4	1.33	1.5
16	M_{16}	2.0	0.4	1.66	1.0
17	M_{17}	2.5	0.4	1.00	0.86
$CD_{0.05}$				**0.49**	**0.34**
SE±				**0.17**	**0.11**

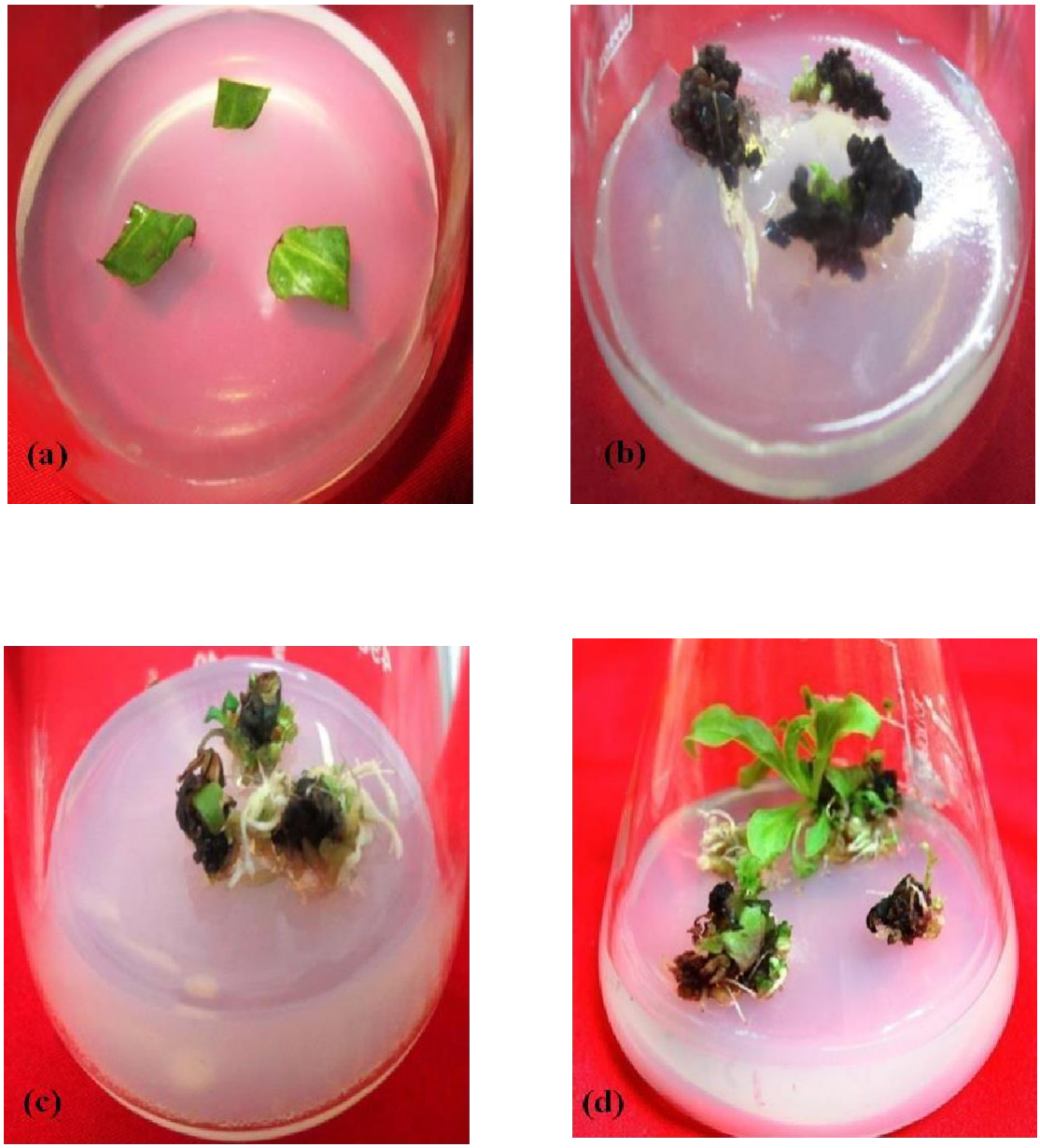

Fig.2 **Swertiachirayita**Explants were cultured in MS medium

Thus it may be noted that maximum number of shoots (5.66) with maximum average shoot length (3.50) observed on M_5 medium was statistically significant in comparison to other treatments. Hence MS medium supplemented with 2.5 mg/l BA and 0.1 mg/l NAA was found out to be best for *in vitro* multiplication of shoots. Multiplications of shoots are presented in Plate 3.

Direct shoot regeneration from *in vitro* leaves

The experiment was conducted to study the effect of different concentrations of BA along with 0.1 mg/l NAA on direct shoot regeneration from

in vitro leaf.

The leaves were separated from *in vitro* regenerated microshoots and cultured on MS medium supplemented with different concentrations of BA (1.0-3.5 mg/l) along with NAA (0.1 mg/l).The cultures were incubated at 25 $\pm$ 2º C under 16 hours photoperiod. There were 7 treatments and 24 explants in each treatment replicated thrice following completely randomized design.

Direct shoot regeneration from leaves initiated after 2 weeks of incubation. The results presented in Table 5 show that 77.78% shoot regeneration was observed on control medium (MS) devoid of any phytohormone. However,

Table 5: Effect of different concentrations of BA and NAA on direct shoot regeneration from *in vitro* leaf explant after 4 weeks of incubation

Sr. No	Medium code (MS basal)	BA mg/l	NAA Mg/l	Percent shoot regeneration	Number of shoots	Shoot length (cm)
1	MS_1	-	-	77.78 (61.85)	2.33	1.50
2	MS_2	1.0	0.1	66.67 (54.71)	3.66	0.67
3	MS_3	1.5	0.1	44.44 (41.78)	5.00	0.83
4	MS_4	2.0	0.1	33.33 (35.24)	7.66	1.00
5	MS_5	2.5	0.1	44.44 (41.79)	4.33	1.30
6	**MS_6**	**3.0**	**0.1**	**88.89 (70.54)**	**9.33**	**2.50**
7	MS_7	3.5	0.1	33.33 (35.24)	1.33	0.67
$CD_{0.05}$				**1.52**	**0.70**	**0.37**
SE±				**0.49**	**0.23**	**0.12**

Values expressed in parentheses are the arc sine transformation of percentage

maximum shoot regeneration (88.89%) was observed on treatment MS_6 containing 3.0 mg/l BA and 0.1 mg/l NAA which is statistically significant as compared to other treatments. Maximum number of shoots (9.33) with maximum shoot length of 2.5 cm was observed on same medium. However, treatment MS_7 containing 3.5 mg/l BA and 0.1 mg/l NAA showed minimum percent of shoot regeneration from *in vitro* leaves. Direct shoot regeneration from *in vitro* leaves is presented in Plate4.

Therefore, treatment MS_6 containing 3.0 mg/l BA and 0.1 mg/l NAA was statistically significant and showed maximum direct shoot regeneration and average number of shoot per explant as compared to all other treatments hence found to be best treatment among all.

4.1.7 *In vitro*rooting

In this experiment *in vitro* raised shoots were cultured on half strength medium supplemented with 400 mg/l activated charcoal and different concentration of NAA (0.1-0.4 mg/l). The experiment was carried out to study the effect of different treatments on percent of *in vitro* rooting of microshoots, average root length and average number of root per shoot. There were 5 treatments with 10 explants. Each experiment was replicated thrice following completely randomizeddesign.

Table 6: Effect of different media on *in vitro* root induction after 4 weeks of incubation

Sr. No	Medium	Composition	Rooting percentage	Number of roots	Root Length (cm)
1	MR_1	Half strength+ 400 mg/l AC	42.33(40.55)	3.5	0.9
2	**MR_2**	**Half strength + 400 mg/l AC +0.1 NAA mg/l**	**80.30(63.64)**	**6.5**	**1.5**
3	MR_3	Half strength + 400 mg/l AC+ 0.2 NAA mg/l	62.20(52.04)	4.5	1.2
4	MR_4	Half strength + 400 mg/l AC + 0.3 NAA mg/l	0.00(0.00)	0.0	0.0
5	MR5	Half strength ++ 400 mg/l AC + 0.4 NAA mg/l	0.00(0.00)	0.0	0.0
$CD_{0.05}$			**1.60**	**0.20**	**0.27**
SE±			**0.50**	**0.60**	**0.08**

Values expressed in parentheses are the arc sine transformation of percentage

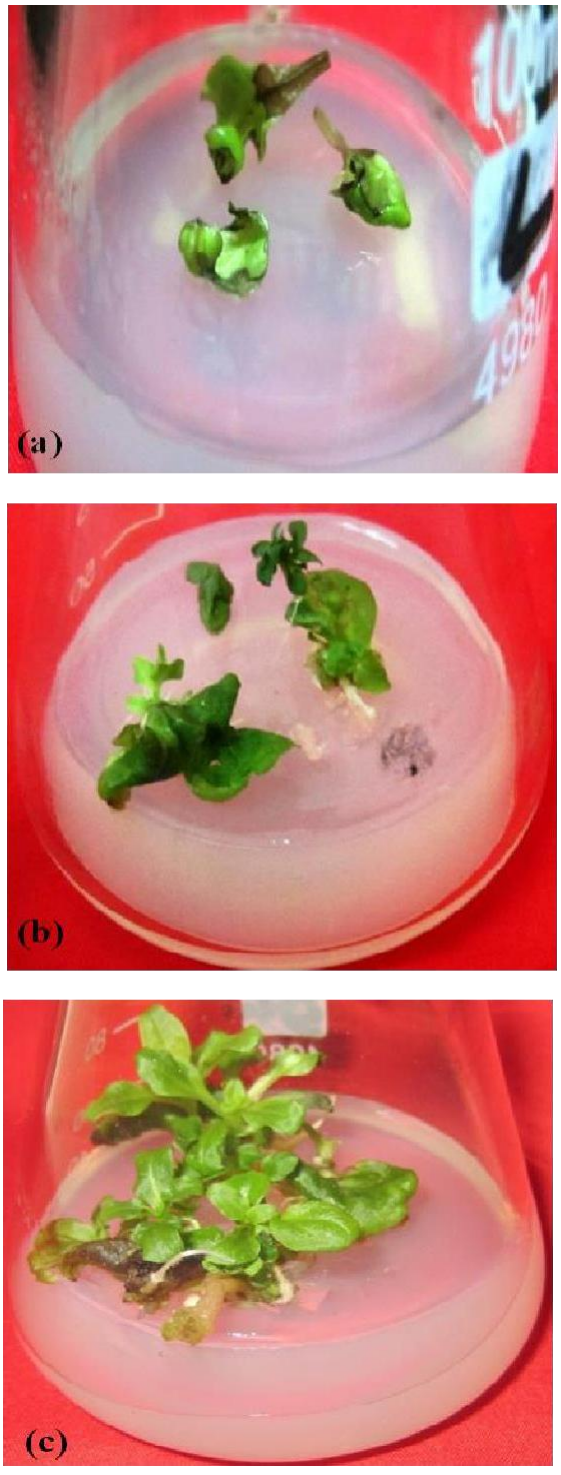

Fig.4. **Swertiachirayita grown in after inoculation of Explants in the MS medium**

Data presented in Table 6 shows that treatment MR_2 containing half strength MS media, 400 mg/l activated charcoal (AC) and 0.1 mg/l NAA was statistically significant and resulted in maximum percent (80.30) rooting in *in vitro* raised shoots. Maximum average number of roots (6.5) per shoot and average root length (1.5 cm) was obtained on same medium. However, on half strength MS basal containing 400 mg/l activated charcoal without NAA 42.33 percent of rooting was observed. No rooting was observed on treatment MR_4 and MR_5 with half strength MS medium containing 400 mg/l Activatedcharcoal with 0.3 and 0.4 NAA mg/l. *In vitro* rooting in microshoots is presented in Plate 5a-b.

4.1.8 Hardening of *in vitro* grownplantlets

Rooted plants of *Swertiachirayita*were taken out of rooting media and washed properly to remove the agar. Before transferring to pots they were treated with 0.1% bavistin for 30 minutes with constant shaking. Finally the plants were transferred to plastic pots containing autoclaved potting mixture of soil: cocopeat in the ratio of 1:1 (Plate 5c).

4.2 *IN VITRO*CONSERVATION

4.2.1 By slow growthmethod

4.2.1.1 By changing the mediacomposition

4.2.1.1.1 High concentration of sucrose

The experiment was conducted to study the effect of different concentrations of sucrose (3 to 9 %) on growth of *in vitro* raised shoots of *Swertiachirayita*. The cultures were incubated at 25±2ºC under 1 6 hours photoperiod.

In vitro raised shoots measuring 0.5 cm were cultured on full and half strength MS media with different concentration of sucrose to slow down the growth of *in vitro* raised shoots.

Results presented in Table 7 shows 100% survival of shoots on all the treatments. With increase in concentration of sucrose decrease in growth rate, number of shoots per explant, shoot length and leaf size was observed. On control

medium FS_1 (MS medium supplemented with 3% sucrose) 5.66 shoots per explant with average shoot length of 2.5 cm and leaf size of 3.67 cm was observed after 4 weeks of incubation. However, minimum growth was observed on treatment HS_3 containing half strength MS medium supplemented with 9% sucrose as no shoot multiplication occurred and each shoot remained as single shoot with average shoot length of 0.67 cm and leaf size of 0.35 cm.(Plate6)

Table 7: Effect of different concentrations of sucrose on growth and development of *in vitro* shoots after 4 weeks of incubation

Sr. No	Medium	Composition	Number of shoots	Leaf size (cm)	Shoot length (cm)	Morphological variation	Survival rate
1	FS_1	MS full strength	5.66	3.67	2.50	Normal growth	100%
2	FS_2	MS full strength + 60g/l sucrose	2.00	2.30	1.50	Light green colour , root formation	100%
3	FS_3	MS full strength + 90g/l sucrose	1.33	1.17	1.40	Root formation,leaves darkened	100%
4	HS_1	MS half strength+ 30g/l sucrose	1.67	1.83	1.50	Normal growth	100%
5	HS_2	MS half strength+ 60g/l sucrose	1.33	1.67	0.97	Light green, root formation	100%
6	**HS_3**	**MS half strength+ 90g/l sucrose**	**1.00**	**0.35**	**0.67**	**Light green, root formation**	**100%**
$CD_{0.05}$			**0.16**	**0.12**	**0.23**		
SE±			**0.05**	**0.04**	**0.07**		

4.2.1.1.1 Effect of Different concentrations of ABA

The experiment was conducted to study the effect of different treatments of ABA on growth of *in vitro* raised shoots of *Swertiachirayita*.

In vitro raised shoots were isolated and cut to the size of 0.5 cm and cultured on full and half strength MS media and supplemented with different concentrations of ABA (1.0 -3.0mg/l) to slow down the growth of *in vitro* raised shoots. The cultures were incubated at 25±2ºC under 16 hours photoperiod.

Fig.5. **Grown of Swertiachirayita from MS medium to pot culture**

Fig. 6. F53 and 650 grown stages

Results presented in Table 8 show 100% survival of shoots in all the treatments. With increase in concentration of ABA decrease in growth rate, number of shoots per explant, shoot length and leaf size was observed. On control medium (MS medium supplemented with 3% sucrose) 5.66 shoots per explant with average shoot length of 2.5 cm and leaf size of 3.67 cm was observed after 4 weeks of incubation. However with increase in concentration of ABA from 1.0 mg/l to 3.0 mg/l decrease in number of shoots per explant from 2.63 to 1.00 was observed. No shoot proliferation was observed on treatment FA_3 and HA_3 containing full strength and half strength MS medium supplemented with 3.0 mg/l ABA respectively. Minimum average shoot length (0.83 cm) and leaf size of (0.24 cm) was observed on HA_3 treatment containing full strength MS medium supplemented with 3.0 mg/l ABA.(Plate7)

Table8: Effect of different concentrations of Abscisic acid on growthand development of *in vitro* shoots of after 4 weeks ofincubation

Sr. No	Medium	Composition	Number of shoots	Leaf Size (cm)	Shoot length (cm)	Morphological variation	Survival rate
1	Control	MS full strength	5.66	3.67	2.5	Normal growth	100%
2	FA_1	MS full strength +1.0 mg/l ABA	2.63	2.50	2.00	Root formation	100%
3	FA_2	MS full strength +2.0 mg/l ABA	1.67	1.30	1.00	Small root formation	100%
4	FA_3	MS full strength +3.0 mg/l ABA	1.00	1.00	1.00	Small root Formation	100%
5	HA_1	MS half strength+ 1.0 mg/l ABA	2.67	1.47	1.30	Normal growth	100%
6	HA_2	MS half strength+ 2.0 mg/l ABA	1.00	1.17	0.83	Light green, adventitious roots	100%
7	HA_3	MS half strength+3.0 mg/l ABA	1.00	0.83	0.83	Normal growth	100%
$CD_{0.05}$			**0.36**	**0.52**	**0.41**		
SE±			**0.11**	**0.17**	**0.13**		

4.2.1.2 By storing at low temperature

The experiment was conducted to study the effect low temperature to slow down the growth of *in vitro* growing shoots.

In vitro multiplied shoots were isolated cut to size of 0.5 cm and cultured on shoot multiplication medium (MS + 2.5 mg/l BA +0.1 mg/l Kn) and kept for incubation at two different temperatures i.e. 4^{o}C and 10^{o}C to slow down the growth of *in vitro* growing shoots.

After 4 weeks of incubation observation were recorded with respect to number of shoots per explant, shoot length and leaf size.

Data presented in Table 9 shows that no much growth of shoots was noted at both the temperatures after 4 weeks of incubation and statistically significant. On control medium, 5.66 shoots per explant with average shoot length of 2.5 cm and leaf size of 3.67 cm was observed after 4 weeks of incubation. Whereas, in case of shoots kept at 4^{o}C and 10^{o}C no shoot multiplication was observed and shoots remained as single shoot with average shoot length of 0.86 cm and 0.73 cm. Leaf size of 0.34 cm and 0.23 cm at 4^{o}C and 10^{o}C respectively, was noted. No morphological variation was observed in shoots incubated at both temperature (Plate 8).

Table 9: Effect of low temperature on growth and and survival percentage of shoots after 30 days of incubation

Sr. No.	Incubation temperature	Per cent survival after 30 days of Incubation	Average number of Shoots	Shoot length (cm)	Leaf Size (cm)	Morphological variation
1	Control	100%	5.66	2.5	3.67	No variation
2	4^{o}C	100%	1.00	0.86	0.34	No variation
3	10^{o}C	100%	1.00	0.76	0.23	No variation
CD $_{0.05}$			**0.61**	**0.11**	**0.18**	
SE +			**0.17**	**0.03**	**0.05**	

The shoots showed normal growth when brought back to normal culture conditions.

4.2.2 Cryopreservation following vitrification technique

In vitro raised shoots of *Swertiachirayita*were divided into two sets and precooled separately at 4^{o}C and 10^{o}C in a incubator for one month. Henceforth, the manipulations were carried out aseptically in laminar flow chamber. Pre-cooled *in vitro* raised shoots were taken and approximately 3.0 mm long explants

containing shoot buds were prepared. Autoclaved petriplates with filter paper were taken and 1.5-2.0 ml of precultured medium, named V_1 (MS salt+ 0.5 M sucrose) was added. The pre-cooled shoot segments were placed on it, sealed with parafilmandincubatedovernightindarkat4^{O}Ctemperatureinsideaincubator.

Henceforth, the manipulations were carried out aseptically in laminar flow chamber. The shoot segments were put into pre-cooled cryovials containing V_2 solution (MS salts + 0.4 M sucrose + 3 M glycerol) and cryovials were incubated at4^{O}Cfor20-30minutes.AfterthisV_2 solutionwaspipetteoutfromcryovials leaving the shoot segments behind and 1.5-2 ml of vitrification solution V_3 (MS salts + 30% glycerol + 13% DMSO + 15% ethylene glycol) was added. After this the cryovials were placed on laptop cooler (at ice temperature) for 60 minutes. Finally the cryovials were placed in small muslin cloth bags and the bags containing cryovials were quickly plunged into a cryocan containing liquid nitrogen for 60 minutes. For retrieval the cryovials were taken out and thawing was done quickly with constant agitation at 40^{O} C.

The cryopreserved vitrified shoots were kept for retrieval on M_5 medium (MS + 2.5 mg/l BA + 0.1 mg/l Kn). M_5 is the medium which had been found to be best for *in vitro* shoot multiplication.

Table 10: Percentage survival of cryopreserved vitrified shoot tips of *Swertiachirayita*pre-cooled at 4^{O}C and 10^{O}C

Sr. No	Treatment	Per cent survival
1	4^{O}C	42.33
2	10^{O}C	22.37
$CD_{0.05}$	**2.21**	
SE ±	**0.55**	

The shoot tips pre-cooled at 4^{O}C for one month showed multiplication within 3 weeks of culturing. The retrieval rate was 42.33 per cent. However, they attained their normal growth rate after subculturing. While the per centretrieval of shoot tips with prior incubation at 10^{O}C was very less (22.77) as comparedto that of shoots with prior incubation at 4^{O} C and they showed multiplication after 4 weeks of culturing (Table 10). The germination of vitrified buds is presented in Plate 9.

In view of the tremendously growing world population, increasing anthropogenic activities, rapidly eroding natural ecosystem, the natural habitat for a great number of herbs and trees are dwindling. Many of them are facing extinction. Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate. Thus mass multiplication of disease free planting material is a general problem. In this regard the micropropagation holds significant promise for true to type,

rapid and mass multiplication under disease free conditions. In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions.

*Swertiachirayita*commonly known as chirata is an important medicinal plant with an increasing pharmaceutical demand both, in the indigenous and the world market but lack of large-scale commercial plantations of the herb continues. *Chirayita*is difficult to propagate on mass scale via seed owing to non availability of seeds or due to harvesting of plants before seeds mature. The species is, therefore, deprived of natural regeneration. There are only some reports on *in vitro* propagation of *S. chirayita.*by Ahuja*et al*. (2003), Chaudhuri*et al*. (2007), Koul*et al.* (2009) and Pant *et al*. (2010).They have reported micropropagation of *S. chirayita*via field- grown nodal explants, while seedling derived shoot tip explants were used in the study by Joshi and Dhawan(2007a).

Efforts were made during present investigations entitled "*In vitro* propagation and conservation of *Swertiachirayita.***"** to standardize a protocol for *in vitro* propagation for mass multiplication and *in vitro* conservation for long term storage of *Swertiachirayita.* The results of the investigations have been discussed on the basis of available literature under the following sections:

***In vitro* propagation**

***In vitro* conservation**

IN VITRO PROPAGATION

In 1902, Haberlandt predicted that every single cell of plant body has got the potential to regenerate a whole new plant, and this property is known as totipotency. For different cells to express its totipotency it first undergoes dedifferentiation following redifferentiation. The various factors, which influence morphogenic differentiation under *in vitro* conditions, are explants, their physiological state, phytohormones, their concentration and their interaction with explants.

Establishment of callus culture and shoot regeneration

Manipulations of auxin and cytokinin levels are successful in defining a growth regulator balance necessary for the required behavior of culture. The ability of auxins to regulate the rate of cell elongation and cytokinin to control cytokinesis, accelerated the process of cell division in callus and thus emphasized the presence of both the growth regulators in the medium for callusinduction.

In the present investigation *ex vitro* leaf segments were used as explants for establishment of callus culture. It was observed that solid MS medium supplemented with 0.1 mg/l NAA and 3.0 mg/l BA induced 100 percent callus from mature leaf explant however callus induction (77.78 per cent) was also observed in MS basal media without any growth regulators which is not reported anywhere earlier.

Similarly, callus induction and shoot regeneration using stem as explant of *Swertiamussotii*Franch. on MS medium containing 3.0 mg/l BA and 0.5 mg/l NAA was reported by He *et al* (2012). Bisht and Bisht (2008) also reported callus induction and shoot regeneration in *Swertiaangustifolia*Buch-Ham. Using leaf and petiol as explant. They found that BA and NAA in the range of 0.5-3.0 mg/l