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Modification of Plant Architecture of *Hemidesmus indicus* (L.) R. Br. (*Iramusu*) by *In vitro* Colchicine Treatment

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ABSTRACT. *The effects of different concentrations of colchicine on shoot and root development of nodal explants and germinating somatic embryos of Hemidesmus indicus (L.)R. Br. (Iramusu) was investigated. MS medium solidified with 8 g/l agar was superior to the liquid medium in all the concentrations of colchicine. In nodal derived H. indicus plants, the highest number of shoots per explant (4.23±1.49) and the longest shoot length (6.36±4.29 cm) were achieved at 5 mg/l colchicines forming a compact bushy type plant architecture. In almost all the treatments an increment of shoot length and number of shoots per explant could be observed compared to the control plants. Furthermore, 1 mg/l colchicine was more effective in germinating somatic embryos to produce more compact plant type under in vitro condition. There was no significant difference observed in different concentrations of colchicine on root length and root number of both nodal derived plants and also in plants derived from germinating somatic embryos.*

INTRODUCTION

Hemidesmus indicus (L.) R. Br. (*Iramusu*) is one of the wild plant species in Sri Lanka possessing high medicinal value. It belongs to family Asclepiadacea and widely distributed in India, Bangladesh and Sri Lanka. In Sri Lanka, it thrives well only up to an elevation of 2500 m. It is common in deciduous scrubland and deciduous forest of the dry regions as well as rubber, coconut and *Pinus* plantations (Gunatilleke *et al*., 2002). The plant is a perennial, semi-shrubby tawnier with a woody rootstock. The stem is very long, prostrate or ascending and slightly twining. Internodes are 1.5 - 7.2 cm in length. Leaves are simple, opposite and variable from oblong-oval to linear. Flowers are regular, bisexual and contain numerous bracts (Jayaweera, 1982).

This plant species can be used as an ornamental foliage plant as well as a medicinal plant, due to its several morphological characteristics such as shape and white color margin along the mid rib of the leaves. Eventhough the medicinal properties of this plant species have been identified and used for many centuries in the ayurvedic, unani and homeopathic medicines (Siddique *et al*., 2003) at present, not much attention is paid to its conservation in their natural habitats. One of the reasons for less popularity as an ornamental plant is due to its vine like growth habit. Therefore, if it can be modified into a bushy type it will be easy to popularize it as an ornamental potted plant in Sri Lanka.

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Chromosomal doubling is a common technique which is used in plant hybridization programmes. It allows induction of polyploid plants possessing superior agronomic traits over their diploid counterparts. Ploypoid plants possess larger leaves and flowers, thicker stems and roots, darker green leaves, an increased width-to-length ratio of the leaves, a more compact growth habit and a higher tolerance to environmental stress (Kehr 1996; Kermani *et al.* 2003; Shao *et al*. 2003). Furthermore, autotetraploids and triploids are sterile or frequently have low fertility. It is a more important character in clonally propagated ornamental plants since it avoids contamination of the flowers by the pollen and fruit set (Guofeng *et al*., 2007).

Colchicine is one of the pharmaceutically important alkaloids and a useful agent in the treatment of acute attacks of gout. Traditionally, colchicine was used for its antimitotic properties (Sivakumar *et al*., 2004) and treatment of familial Mediterranean fever. Colchicine has a well-known lipophilic drug action on tumors (Sivakumar *et al*., 2004). Derivatives of colchicine have shown promise as anticancer agents (Sivakumar *et al*., 2004). Colchicine was used for gene expression and gene amplification (Sivakumar *et al*., 2004). Colchicine disrupts mitosis by binding to tubulin, thus inhibiting the formation of microtubules and the polar migration of chromosomes, resulting in a cell with a doubled chromosome number. Now it is one of the most commonly used spindle inhibitors and has been used for the induction of tetraploids from diploid plants of a number of woody species including rose (Roberts *et al*. 1990; Kermani *et al*., 2003), mulberry (Chakraborti *et al*., 1998), rhododendron (Vainola, 2000), pear (Kadota and Niimi, 2002), and pomegranate (Shao *et al*., 2003).

With this information, the main objective of the present study was to obtain genetic variability of *H. indicus* by colchicine treatment. In this research a reliable protocol is presented for obtaining *in vitro* cultured *H. indicus* plants with modified growth habit of bushy appearance.

METHODOLOGY

Donor plants

The nodal explants were taken from plants grown in the plant house, two days after spraying of 0.1% Bavastin™ (Carbendazim) solution. The nodal explants were surface sterilized by washing 3 times with Teepol™, dipped in 0.1% Bavastin™ solution for 30 minutes, vacuum sterilized with 10% Clorox™ and two drops of Tween™ 20 for 5 minutes and thoroughly shake with 10% Clorox™ for 5 minutes. This was followed by three to four times washing with sterilized distilled water.

After the sterilization process 1-2 cm nodal segments were inoculated on Murashige and Skoog (1962) (MS) basal medium supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l α-naphthaleneacetic acid (NAA), 3% (w/v) Sucrose, 100 mg/l Myoinositol, 15 mg/l adenine sulphate, 0.1% streptomycin, 0.1 g/l ascorbic acid solidified with 8 g/l agar. Those cultures were incubated at 25±2 ^{0}C under warm fluorescent light with intensity varying from 900 to 1500 lux and 16 h:8 h (day: night) photoperiod. Shoots were multiplied on MS basal medium supplemented with 2 mg/l BAP (Nagahatenna and Peiris, 2007).

Effects of colchicine on regeneration of nodal explants on liquid and solid medium

Prior to the colchicine treatment, shoots were transferred to hormone-free MS medium for 4 weeks to eliminate the effect of the BAP in the previous medium. Nodal segments (1-2 cm) excised from those shoots were cultured on MS medium with different concentrations of colchicine (0, 0.5, 1, 2, 5 mg/l). The effectiveness of the liquid and solid medium on the effect of colchicine was also investigated. Since colchicine is a heat-labile substance, it was added to the autoclaved medium following filter sterilization.

Duration of the treatment was 4 days. All the cultures were kept under the same conditions mentioned above. After 4 days time period, they were transferred to fresh medium free from colchicine for 7 days to eliminate the toxic effects of the drug by prolonged exposure. Recovered shoots were multiplied on MS medium containing 2 mg/l BAP, 3% (w/v) Sucrose and 100 mg/l Myoinositol and solidified with 8 g/l agar.

Two months after transferring to the multiplication medium, the plants were cultured on ½ strength MS medium containing 1.5 mg/l IBA for two months for root formation (Nagahatenna and Peiris, 2007). When the root system was fully developed, plantlets were individually labeled and they were used for the chromosome counting and acclimatization to identify the performances at the outside environment. Treatments were replicated three times. Performances of the plants were observed at weekly intervals and shoot length, root length, number of shoots/explant and number of roots per explant were recorded 12 weeks after the treatment.

Effect of colchicine on germinating somatic embryos

Somatic embryos of *H. indicus* were produced from *in vitro* excised leaf explants (data not shown) and they were used as the source tissues for this experiment. Rooted somatic embryos which were on hormone-free MS medium were transferred on to MS medium with different concentrations of colchicine (0, 0.5, 1, 2, 5 mg/l) solidified with agar (8 g/l). Duration of the treatment was 4 days. Cultures were kept under the same conditions mentioned above.

Following the treatment, colchicine treated somatic embryos were transferred to solidified MS medium free from colchicine and kept for 7 days to eliminate the toxic effects of the drug created by prolonged exposure. Then they were transferred to hormone-free solid MS medium for the induction of the shoot system. Germinated shoots were transferred to the MS medium containing 2 mg/l BAP for the *in vitro* multiplication for one month. Individually labeled plants were then used for the chromosome counting and acclimatization to identify the performances under normal conditions. Treatments were replicated three times and performances of the plants such as germination percentage of somatic embryos shoot length, root length, number of shoots/explant and number of roots per explant were recorded 8 weeks after the treatment.

Estimation of ploidy level

The ploidy level of the *H. indicus* plantlets was estimated by chromosomal counting in root tips. The plantlets were randomly sampled from each treatment and the tips of young roots were collected at 9:00 to 10.00 a.m. The root tips were pretreated for 24 hours in iced water

(0 ^{0}C) fixed in acetic acid, chloroform, 95% ethanol (1:3:6). Hydrolysis was performed in 1 N HCl for 10 minutes at 60 ^{0}C before staining with Shiff solution and squashing in 0.6% propionic carmim. Microscope slides were examined at 400 X magnification under inverted phase contrast microscope.

Data analysis

The experiments were set up in a completely randomized design. Fifteen replicates were used for each treatment. The effect of colchicine on shoot length and root length were analyzed by using one way ANOVA with SAS statistical software. The effect of colchicine on germination percentage, number of shoots per explants and number of roots per explant were analyzed by chi-square contingency test with MINITAB. Mean comparisons were done with Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Effect of colchicine on regeneration of nodal explants on solid medium

In the present study, solid medium was more superior to the liquid medium for the nodal explants of *H. indicus* in all the colchicine concentrations. Toxic effects such as severe browning were observed in nodal explants in liquid medium at all the colchicine concentrations during the duration of the treatment. Only few nodal explants were recovered after the treatment, but they also died back within two weeks. Repeated trials also justified the same results. On solid medium, 100% of the nodal explants were recovered and they grew vigorously. In contrast to these results, it has been demonstrated that the liquid medium containing colchicine was more effective in the regeneration of tetraploid plants in protocorms of *Cymbidium* (Wimber and Van Cott, 1966), *Dendrobium* (Sanguthai *et al.*, 1973), *Phalaenopsis* (Griesbach, 1981; 1985) and *Paphiopedilum* (Watrous and Wimber, 1988).

The concentrations of the drug as well as the duration of the treatment are more important because higher concentrations of the drug or prolonged treatments may be lethal to sensitive plant tissue (Derman, 1940). In the present study significant differences were observed with respect to the shoot length and number of shoots per explant compared to the control. When different concentrations of colchicine were applied to the nodal explants, a higher shoot growth was achieved. Colchicine treated nodal explants gave rise to increased number of shoots per explant than the control. The highest shoot length and the highest number of shoots per explant were achieved at 5 mg/l colchicine (Plate 1, Table 1).

According to Alejandro *et al*., (2007), shortness and compactness have been observed in tetraploid *Mecardonia tenella* plants regenerated from the colchicines-treated nodal explants under *in vitro* conditions compared to the wild diploid plants. However, no other differences have been reported with respect to the diameter and covering capacity between these plant species. *Platanus acerifolia* seedlings treated with colchicine showed reduced stem elongation, stem growth and slower node development, compared to the control seedlings and, in many cases, the first 1–2 true leaves were morphologically abnormal, e.g. wrinkled; while subsequent leaves appeared normal. However, in *P. acerifolia*, when colchicine is

Table 1. Effect of different concentrations of colchicine on shoot growth of *H. indicus*, at 12 weeks after the treatment.

Colchicine concentration (mg/l)	Shoot length (cm)	Number of shoots/explant	Root length (cm)	Number of roots/ explant
0	3.66 ±2.50 [a]	2.23 ±1.06 [a]	3.89 ± 1.24 [a]	3.54 ± 2.23 [a]
0.5	5.87±4 [b]	2.65±1.66 [b]	3.51 ± 1.29 [a]	3.64 ± 2.01 [a]
1	5.45± 3.54 [b]	2.09±0.91 [c]	3.64 ± 1.11 [a]	3.79 ± 1.43 [a]
2	5.54±4.11[b]	3.75±1.37 [d]	3.76 ± 1.19 [a]	3.46 ± 0.56 [a]
5	6.36±4.29 [c]	4.23±1.49 [e]	3.53 ± 1.20 [a]	3.53 ± 1.65 [a]

Note: Data expressed as Mean ± SE from 15 replicates. Within columns, values followed by the same letter are not significantly different at the 5% level.

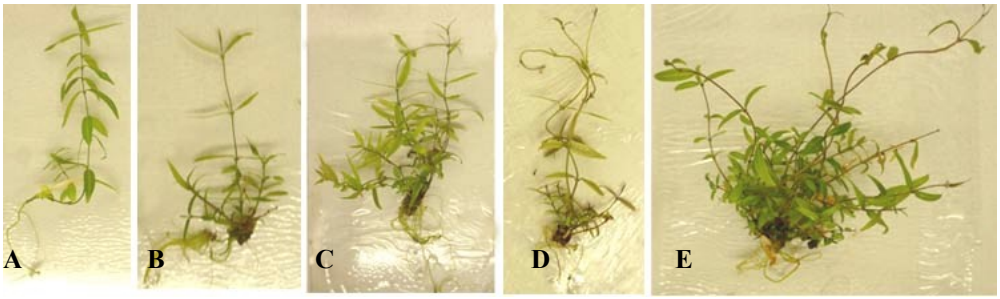

Plate 1. Variations of shoot initiation and shoot growth from nodal explants of *H. indicus* cultured on MS medium treated with different concentrations of colchicine 3 ½ months after culture establishment. (A) 0 mg/l (B) 0.5 mg/l (C) 1 mg/l (D) 2 mg/l (E) 5 mg/l

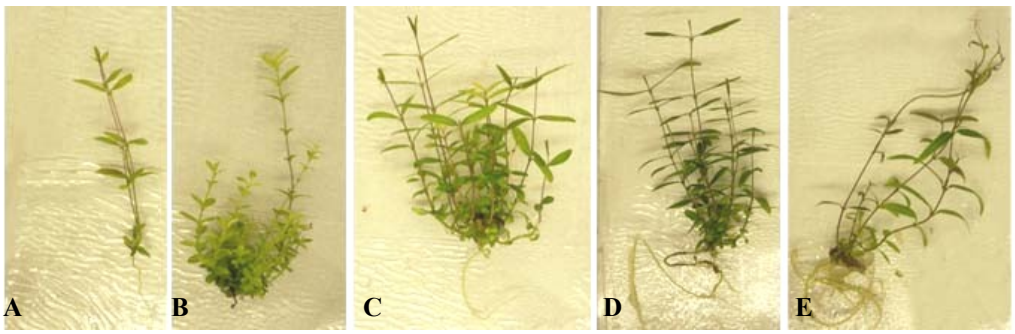

Plate 2. Shoot initiation and shoot growth of somatic embryos derived plants of *H. indicus* cultured on MS medium treated with different concentrations of colchicine 8 weeks after the treatment. (A) 0 mg/l (B) 0.5 mg/l (C) 1 mg/l (D) 2 mg/l (E) 5 mg/l

applied to nodal explants under *in vitro* conditions, they have failed to yield a single polyploid mature plant (Guofeng *et al*., 2007).

Based on the tested colchicine concentrations, compactness could be achieved in *H. indicus* plants at 5 mg/l colchicine concentration. Eventhough at this concentration reduced stem elongation was not observed, it has given rise to significantly higher number of shoots compared to the other treatments forming a bushy type appearance (p=0.05).

Although there is a significant impact of different concentrations of colchicine on shoot growth of *H. indicus*, no other changes were found with respect to the root length and root number.

Effect of colchicine on germinating somatic embryos of *H. indicus*

Due to total failure of colchicines-treated nodal explants of *H. indicus* on liquid medium, embryos were evaluated only on solid medium. It was clearly observed in the present study that different concentrations of colchicine have affected germination percentage, shoot length and number of shoots per explant significantly. Compared to the control, germination percentage was reduced at 0.5 and 2 mg/l colchicine whereas at 1 and 5 mg/l, it has been enhanced. The highest shoot length (5.33 ± 3.7) was achieved at 5 mg/l colchicine concentration while the lowest shoot length was at 0.5 mg/l (2.35 ± 3.14 cm). The highest shoot multiplication rate (5.67 ± 4.04) was recorded at 1 mg/l colchicine concentration (Plate 2 and Table 2).

According to Wu and Mooney (2002), embryogenic callus growth, development and the rate of plantlet regeneration have been partly suppressed in the presence of colchicine at two concentrations tested (0.05 or 0.1%). Based on the present results on *H .indicus*, it was clear that this kind of suppression on germination percentage, shoot length and shoot multiplication have been achieved even at 0.5 mg/l colchicine. At 1 mg/l concentration, those parameters have increased significantly. Therefore, due to comparatively less height of the plants and highest multiplication rate, 1 mg/l colchicine concentration has produced bushy type *H. indicus* plants through somatic embryogenesis.

The success rate of producing non-chimeric autotetraploids from somatic embryogenic callus lines depends on the genotype, the growth status of the callus and the type, concentration and time of exposure to the anti-mitotic agent (Wu and Mooney, 2002). Wu and Mooney (2002) have demonstrated that the citrus genotypes have different levels of sensitivity to colchicine. The tetraploid 'Umatilla' plants exhibited a stunted growth habit and had no thorns on the new shoots. The leaves on these tetraploid plants were dark green, short, narrow and leathery compared to the broad-shaped leaves on control lines.

In the present experiment no significant differences were recorded with respect to the effect of different concentrations of colchicine on root growth and root number of *H. indicus*.

Estimation of ploidy level

Fixation is the most critical step in the cytological procedure. Prior to the use of cytologic stains, root tips were treated with a chemical fixative in order to kill cells, to make cell components water-insoluble, and to prevent leaching out of the tissue during subsequent

handling, and to facilitate the uptake of stains into the cytoplasm. In fixation ethanol-like highly penetrating molecules were used. Tissues were hydrolyzed by using HCl (hydrochloric acid). It removes purine bases of the strands of DNA, leaving a reactive aldehyde group at the number 1 carbon of deoxyribose sugar. When Schiff's reagent is applied, this aldehyde group of the DNA molecules reacts with the solution and gives a reddish-purple dye.

Table 2. Effect of different concentrations of colchicine on germination and growth of somatic embryos on MS medium containing 2 mg/l BAP 8 weeks after the treatment.

Colchicine concentration (mg/l)	Germination (%)	Shoot length (cm)	Number of shoots/explant	Root length (cm)	Number of roots/ explant
0	28	3.1±1.38 [a]	4.2 ± 4.44 [a]	3.53 ± 1.32 [a]	2.94 ± 0.98 [a]
0.5	18	2.35 ± 3.14 [b]	4.4 ± 1.67 [a]	3.29 ± 0.58 [a]	3.19 ± 1.26 [a]
1	37	3.26 ± 2.06 [a]	5.67 ± 4.04 [c]	3.34 ± 0.53 [a]	3.24 ± 1.34 [a]
2	12	3.81 ± 2.54 [c]	2.89 ± 1.36 [b]	3.48 ± 0.78 [a]	3.08 ± 0.86 [a]
5	50	5.33 ± 3.7 [d]	2.71 ± 1.6 [b]	3.59 ± 1.12 [a]	3.14 ± 0.94 [a]

Note: Data expressed as Mean ± SE from 20 replicates. Within columns, values followed by the same letter are not significantly different at the 5% level.

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Even though the colchicines-treated root tips of *H. indicus* could be successfully stained through the above procedure, it was very difficult to count the number of chromosomes in each cell more accurately due to poor magnification of the light microscope. Since the success of staining depends on several factors such as pH, temperature, osmotic balance and duration of fixation, variation of any factor gives inadequate staining.

CONCLUSIONS

Based on the results of this study, compact *in vitro H. indicus* plants can be regenerated from the nodal explants treated with 5 mg/l colchicine and incubated at 25 ^{0}C temperature under 16:8 hr photoperiod under 1000 lux light intensity. Further application of 1 mg/l colchicine on germinating somatic embryos of *H. indicus*, more compact bushy type plants can be successfully obtained under *in vitro* conditions.

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