South Western Journal of Horticulture, Biology and Environment
P-Issn: 2067- 9874, E-Issn: 2068-7958

Vol.5 , No.2, 2014
pp. 63-82

YOUNG SHOOT NODES DERIVED ORGANOGENESIS *IN VITRO* IN MASS PROPAGATION OF *Ruscus hypophyllum* L.

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ABSTRACT. *Young shoot nodes derived organogenesis in vitro in mass propagation of Ruscus hypophyllum L. was successfully established. High callus formation derived from the young shoot nodes was mostly induced in surounded-areas at the basal leaf-like cladodes on quater-strength Murashige and Skoog (MS) medium supplemented with 200 ml/l Coconut Water (CW), 0.5 mg/l N6-benzyladenine (BA), 0.5 mg/l indole-3-acetic acid (IAA), 20 g/l sucrose and 1.8 g/l Gelrite and successfully stimulated 100% explant regeneration with high callus score, short callus initiation time (14 days after culture) and 14.8 initial shoots per explant. The initial shoots were easily regenerated to produce high number of shoots per explant (8.6 shoots) on three-fourth strength Winarto-Teixeira (WT) medium containing 0.25 mg/l BA, 0.5 mg/l Kinetin (Kin), 0.05 mg/l IAA, 20 g/l sucrose, 1 g/l activated-charcoal (AC) and 1.8 g/l Gelrite. The proliferation of shoots up to 9.2 shoots per explant with 22.4 leaf-like cladodes and 2.7 cm height of shoots was slightly improved by culturing the shoots on half-strength MS medium hormone free with full vitamin. Producing the high quality of shoots could be maintained up to the fifth subculture then reduced thereafter. Well rooted-shoots were easily stimulated and successfully transferred to ex vitro condition with 82% survivability on burned-rice husk medium.*

KEYWORDS: *shoot node, medium, subculture, micro-propagation, Ruscus hypophyllum L.*

INTRODUCTION

Ruscus hypophyllum L. (Family: Liliaceae) is an evergreen, semiwoody groundcover native principally to northwest Africa that can be used as floral design, landscape and indoor plant (Stamps 2001). The plant has leaf-like cladodes with lack a spine and are popular used as cut foliage in floral arrangements due to unique, esthetic and long vase-life for one month or more (Stamps & Boone 1992, Purwito et al. 2005). In Indonesia, the *R. hypophyllum* was cultivated commercially under screen house at Pasir Sarongge and Cipanas areas of Cianjur District; Cisarua-Bogor District; and Cihideung-Bandung District in West Java Province. In Bandung District, especially at Cihideung areas, farmer can produce more than 500 bunches of stems per week and be sold between 8,000-14,400 rupiahs per bunch depended on the quality.

Traditionally, the *R. hypophyllum* are propagated by seeds, cuttings and division of the underground rhizomes (Stamps 2001). The propagation using seeds results in varied-regenerants, while cuttings and the division of rhizomes that can produce off-springs identically to characteristics of original plant only produce limited-new plants. The conventional methods have been usually utilized by the Indonesian Ruscus farmers that also rose solved the problem in producing high quality products of the plants commercially. Therefore the use of tissue culture technique in vegetative propagation of the plants can be considered as an alternative and suitable method to obtain high quantities and qualities of *R. hypophyllum* commercially (Abou-Dahab et al. 2005).

There were few reports of *in vitro* propagation of *R. hypophyllum* published previously. Ziv (1983) used shoot tips and inflorescence explants that pre-cultured in agitated Linsmaier and Skoog liquid medium containing 1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and N6-benzylaminopurine (BAP) and subcultured every 15 days in solid medium with a higher (5:1) cytokinin:auxin ratio for shoot development. Regenerated *in vitro* shoots derived from mature embryos were successfully established on MS medium fortified with 2 mg/lα-naphthalene acetic acid (NAA) (Ivanova et al. 2008). Purwito et al. (2005) successfully produced 9.2-9.4 shoots derived

from shoot tips as explant sources on Murashige and Skoog (1962, MS) medium containing 1-2 mg/l N6-benzylaminopurine (BAP) and 0.2 mg/l NAA after 8 weeks of culture. The shoots were then rooted on half strength MS containing 1.5 mg/l indole-3-butiryc acid (IBA) and acclimatized in a mixture of soil and burned-rice husk (1:1, v/v). MS medium supplemented with 0.05 mg/l 2,4-D and 0.5 mg/l BAP was also suitable medium for initiation and proliferation of shoots of *R. hypophyllum* (Jha & Sen 1985). Therefore developing a new *in vitro* propagation protocol for the *R. hypophyllum* is essentially addressed especially in accelerating the availability and supplying the sustainability of high quality planting materials commercially for Indonesian farmers.

Other *in vitro* propagation studies of *Ruscus* reported that high number of shoots per explant of *R. hypoglossum* was recorded on half-strength MS after the third subculture (Tarek & Abou-Dahab 2004). MS medium containing 1.5 mg/l BA in the same *Ruscus* produced 6.8 shoots per explant (Abou-Dahab et al. 2005). The MS medium augmented with 0.5 mg/l thidiazuron (TDZ) and 60 g/l sucrose successfully resulted in high number of shoots per explant up to 8 shoots in the *R. hypoglossum* (Ivanova 2012, Ivanova et al. 2013). MS medium supplemented with 0.5 mg/dm^{-3} 2,4-2,4-D and 1 mg/dm^{-3} Kin was suitable shoot formation for *R. aculeatus* (Moyano et al. 2006).

Reliable induction and regeneration of callus derived from node explants, followed by shoot multiplication, root formation and acclimatization, are the main objectives to develop a new *in vitro* propagation protocol for *R. hypophyllum.*

MATERIALS AND METHODS

Materials

Ruscus hypophyllum L. mother stock plants (1.5 years old) were obtained from Indonesian Ornamental Crops Research Institute (IOCRI) germplasm cultivated in a mixture of soil, burned rice husk and bamboo peat (1:1:1, v/v/v) under screen house with 35% reduced light intensity. The plants were watered twice a week and fertilized 15 g/plant once a month using granule fertilizer (2 g/l of N:P:K, 20:15:15;

Nusa Tani, Ltd., Jakarta, Indonesia). Young shoots with 5-6 leaf-like cladodes were harvested from donor plants and used as explant sources.

Explant sterilization and culture conditions

The young shoots were cut in each node containing one leaf. The explants were then put under tap water for 30 min, then immersed in a 1 % pesticide solution of 50 % benomyl (Benlox_50WP, Dharma Guna Wibawa Ltd., Jakarta, Indonesia) and 20 % streptomycin sulphate (Agrept_ 20WP, Mastalin Mandiri Ltd., Jakarta, Indonesia) for 30 min and rinsed with distilled water 5 times (5 min each rinse). After pretreatment, the explants were sterilized by immersing them in 1 % sodium hypochloride (NaOCl, Bayclin-Johnson Home Hygiene Products Ltd., Jakarta, Indonesia) for 10 min, 2 % NaOCl for 5 min, 80 % alcohol for 30 s, followed by 5–6 rinses in sterile distilled water (SDW, 5 min each rinse). After sterilization, the explants were prepared by cutting and removing leaves and damage tissues of explant due to disinfectant effect using tissue culture blade into ± 1.0 cm in length, then cultured in initiation medium (Fig. 1A).

Cultures of explants were incubated in the dark for ± 2.0 months for callus initiation; thereafter, callus cultures were then placed under fluorescent lamps (TL-Philips, The Netherlands) under ~13.5 µmol/m/s (light intensity measured with a Digital Lux Meter, Lutron LX 101, Taiwan) for shoot regeneration, multiplication and root formation with a 12-h photoperiod, 23.5 ± 1.1°C, and 60.6 ± 3.8 % relative humidity (temperature and relative humidity measured by a HaarSynth-Hygro thermo-hygrometer, Germany). Lux was converted to µmol/m/s using the conversion factor for cool white fluorescent lamps i.e. 9 0.0135 (Thimijan & Heins 1982). Callus cultures were also maintained in these conditions for regenerating shoots and roots.

Experiment 1, effect of different initiation media for callus formation

Initiation medium (IM) tested in the experiment were half-strength MS containing (1) 10 mg/l 2,4-D, 1 mg/l BA and 0.05 mg/l NAA (IM-1), (2) 10 mg/l TDZ, 1 mg/l BA and 2 mg/l IAA (IM-2), (3) 10 mg/l BA, 2 mg/l 2,4-D and 0.05 mg/l NAA (IM-3), (4) 10 mg/l picloram, 1 mg/l BA, and 0.05 mg/l IAA (IM-4), (5) 150 ml/l CW, 0.5 mg/l BA, and 0.05 mg/l NAA (IM-5), (6) 1 mg/l BA, 0.5 mg/l TDZ and 0.5 mg/l IAA (IM-6), (7) 100 ml/l CW, 0.5 mg/l BA and 0.1 mg/l IAA (IM-7), three fourth strength MS supplemented with 100 ml/l CW, 0.5 mg/l TDZ, and 0.1 mg/l IAA and (9) quarter strength MS augmented with 200 ml/l CW, 0.5 mg/l BA and 0.5 mg/l IAA (IM-9). To each medium 20 g/l sucrose and 1.8 g/l Gelrite were added and the pH was adjusted to 5.8. Thereafter, 10 ml were poured into a culture vessel (5 cm in

diameter; 7 cm in height; 80 ml in volume) and sterilized at 121°C, 15 kPa for 20 min using a Pressure Steam Sterilizer Vertical Cylindrical LS. 001 (SMIC, Shanghai, China).

The experiment was arranged in a complete randomized design with four replications. Each treatment consisted of five bottles and each bottle contained four young node explants. While parameters observed in the study were (1) percentage of explant regeneration (%), callus score, (3) callus initiation time (day), (4) range of initial number of shoots per explant and (5) number of initial shoots per explant. Callus scores were – to ++++, where - there is no callus formation, + 1-25% of total of explant produced callus, ++ 26-50% of total explant produced callus, +++ 51-75% of total explant produced callus, and ++++ more than 75% of total explant produced callus. Periodical observation was conducted to check for each alteration of explants cultured during incubation. All data were collected two months after dark incubation.

Experiment 2, effect of regeneration media on shoot formation

For regeneration of callus derived from the first experiment, especially callus initiated from IM-9 into shoot formation, there were several regeneration media (RM) investigated of (1) Winarto-Teixeira (WT) medium (Winarto et al. 2011, RM-1), (2) three fourth strength WT medium (RM-2), (3) New Winarto-Teixeira (NWT) medium (Winarto et al. 2011, RM-3), (4) three fourth strength NWT medium (RM-4), (5) half strength Murashige and Skoog (MS) medium (RM-5), (6) Growmore (GM) medium (100 mg/l 32N:10P:10K; New Century Drive, Gardena, CA-USA; RM-6); (7) GM medium (200 ml/l 32N:10P:10P + 12.5 mg/l 18N:18P:18K + 25 mg/l 6N:30P:30K, RM-7); and (8) Bregadium water PA-27 medium (10 ml/l; PT. Sekar Wangi Nusantara, Jakarta-Indonesia; RM-8). RM-6 and RM-7 were added by 1 mg/l glycine, 50 mg/lmyo-inositol and 25 µ/l Enervon C (PT. Eagle Indo Pharma, Tangerang-Indonesia) solution. All media were supplemented with 0.25 mg/l BA, 0.5 mg/l Kin, 0.05 mg/l IAA, 20 g/l sucrose, 1 g/l activated-charcoal and 1.8 g/l Gelrite.

The experiment was arranged in a complete randomized design with four replications. Each treatment consisted of five bottles and each bottle contained four explants. The explants were callus with initial shoots derived from IM-9 medium. While parameters observed in the study were number of shoots per explant, height of shoots and score of initial shoots. Score of initial shoots was – to ++++, where – there is no initial shoots regenerated; + 1-5 initial shoots per explant; ++ 6-10 initial shoots per explant; +++ 11-25 initial shoots per explant and ++++ more than 25 initial shoots per explant. Periodical observation was conducted to check for each

alteration of explants cultured during incubation. All data were collected two months after culture.

Experiment 3, effect of multiplication media on shoot proliferation

Multiplication media (MM-1) tested in the experiment were (1) half-strength MS medium hormone free with full vitamin (MM-1), (2) half-strength MS medium with full vitamin supplemented with 1 g/l AC (MM-2), (3) Vacin and Went (VW, 1949) medium containing 150 ml/l CW (MM-3), (4) New *Phalaenopsis* (NP) medium containing 1.5 mg/l GA_3 and 0.5 mg/l IAA (MM-4), (5) NP medium supplemented with 1.5 mg/l BA and 0.01 mg/l NAA (MM-5), (6) half-strength MS medium containing 65 g/l potato extract, 2 g/l peptone and 1 g/l AC (MM-6), and (7) three fourth NWT medium 0.25 mg/l BA, 0.5 mg/l Kin, 0.05 mg/l IAA as control. All media were supplemented with 20 g/l sucrose and 1.8 g/l Gelrite.

The experiment was arranged in a complete randomized design with four replications. Each treatment consisted of five bottles and each bottle contained four explants. The explants were the shoots derived from RM-2 medium. While parameters observed in the study were number of shoots per explant, total number of leaf-like cladodes and height of shoots. Periodical observation was carried out to check for each alteration of explants cultured during incubation. All data were collected 2.5 months after culture.

Experiment 4, proliferation of shoots under periodical subcultures

The experiment was applied to know the effect of periodical subcultures (every ± 2.0 months) on growth and proliferation of shoots. In the study, shoots as explant sources was cultured on half-strength MS medium hormone free with full vitamin as optimal and selected-medium for proliferation. The medium was supplemented with 20 g/l sucrose and 1.8 g/l Gelrite. The pH was adjusted to 5.8. Afterward, 30 ml were poured into a culture vessel (7 cm in diameter; 13 cm in height; 350 ml in volume). In the study in each subculture, there were ten bottles and in each bottle 3 shoots were cultured with 4 replications. The shoots were derived from regenerated-shoots on the half-strength MS medium hormone free with full vitamin. Parameters observed in the experiment were number of shoots per explant, number of leaf-like cladodes per shoot, height of shoots and number of roots per cluster of shoots. All data were collected 2.5 months after culture.

Rooting of shoots

Rooting of shoots was prepared by culturing them in half-strength MS medium hormone free with full vitamin. The 20 g/l sucrose and 1.8 g/l Gelrite was added in

the selected medium, while the pH was adjusted to 5.8. Afterward, 30 ml were poured into a culture vessel (7 cm in diameter; 13 cm in height; 350 ml in volume). In the study, there were fifteen bottles and in each bottle three clusters of shoots were cultured with 4 replications. Parameters observed in the experiment were number of roots per shoot cluster and length of roots. All data were collected ± 2 months after culture.

Acclimatization of plantlets

Transferring plantlets from *in vitro* to *ex vitro* condition was started from taking them from the culture vessels, washing plantlets under tap water to release remains of agar, treating them with 1 % pesticide solution of 50 % benomyl and 20 % streptomycin sulphate for 3 min, then culturing them in plastic pot (15 cm in diameter) containing burned-rice husk and *Cycas rumphii* bulks (2:1, v/v). The pots were then covered with transparent plastic for ± 7 days and placing under reduced-light intensity area (100–120 µmol/m/s using 65% shading net) in a glass house. In one of plastic pot there were 15 shoot clusters cultured and replicated 10 times. In the experiment percentage of survivability and number of survival shoot clusters were recorded 2 months after acclimatization.

Data analysis

All data collected from the experiments were analyzed by analysis of variance (ANOVA) with SAS program Release Windows 9.12. Significant differences between means were assessed by Tukey's Studentized Range (HSD) at $P = 0.05$ (Mattjik & Sumertajaya 2006).

RESULTS AND DISCUSSION

Effect of initiation media on callus formation

Through periodical observation it was known that initial callus formation was occurred 15-20 days after culture, especially in surrounded-areas of basal part of leaf-like cladodes. After ± 35 days of culture, small size of callus (± 0.3 × 0.3 × 0.6 cm; w × h × l) was easily measured (Fig. 1B). All callus derived from the study were organogenic callus with different percentage of explant regeneration, callus scores, callus initiation time, range of initial number of shoots and number of initial shoots per explant.

High percentage of explant regeneration up to 100% with the shortest callus initiation time (14 days), the highest callus score, 10-17 range of initial number of shoots per explant and 14.8 initial shoots per explant was recorded in IM-9 medium (quarter strength MS medium containing 200 ml/l CW, 0.5 mg/l BA and 0.5 mg/l IAA) (Table 1; Fig. 1C). The medium gave significantly effect statistically compared to others. The second best medium was noted in explants that were cultured in the IM-1 medium (half-strength MS medium supplemented with 10 mg/l 2,4-D, 1 mg/l BA and 0.05 mg/l NAA), while the lowest response of callus initiation was determined in explants cultured on IM-6 medium (half-strength MS medium containing 1 mg/l BA, 0.5 mg/l TDZ and 0.5 mg/l IAA. The experiment also reveals that high concentration of hormone combination did not always have high correlation to high explant response in callus initiation. The similar condition was also recorded in low concentration of them.

The interesting point in the callus initiation stage was due to noted that application of CW and strength reduction of MS medium. Increasing CW concentration from 100 ml/l to 200 ml/l and reducing MS medium from half to quarter strength gave high significant effect on the callus formation. The alteration successfully accelerated the callus initiation time, growth and

Figure 1. *In vitro* propagation protocol of R. hypophyllum using young node stem as explant source. A. Node explant in initial culture, B. Small size of regenerated callus (± 0.3 × 0.3 × 0.6 cm; w × h × l) on quarter strength MS medium containing 200 ml/l, 0.5 mg/l BA and 0.5 mg/l IAA (IM-9) ± 35 days after culture, C. Regenerated-callus on the IM-9 ± 2 months after culture, D. High number of shoots per explant on three fourth strength of WT medium ± 1.5 months after culture, E. Improved number of shoots per explant on half-strength MS medium hormone free with full vitamin ± 2.5 months after culture, F. High number of shoots per explant on the half-strength MS medium hormone free with full vitamin in the fifth subculture period, ± 2.5 months after culture, G. Roots produced on the half-strength MS medium hormone free with full vitamin ± 2 months after culture, H. Rooted-shoot condition for acclimatization puspose, I. Immersing plantlets in 1% fungicide and bactericide solution for 3 min before planting them, J-K. Survival plantlet performance ± 1 month after culture, L. Growth of plantlets ± 3 months after culture.

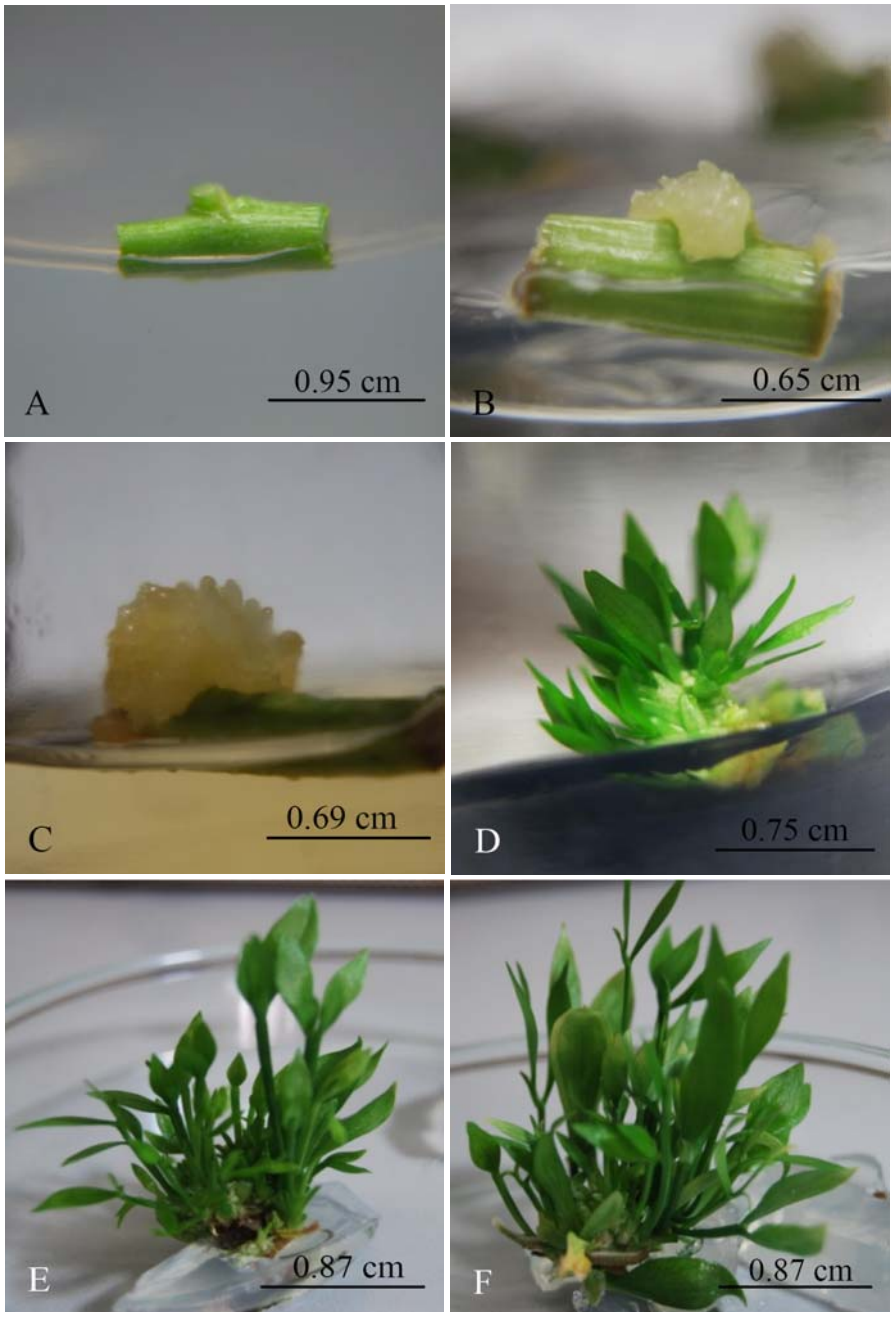

Figure 1. (A-F)

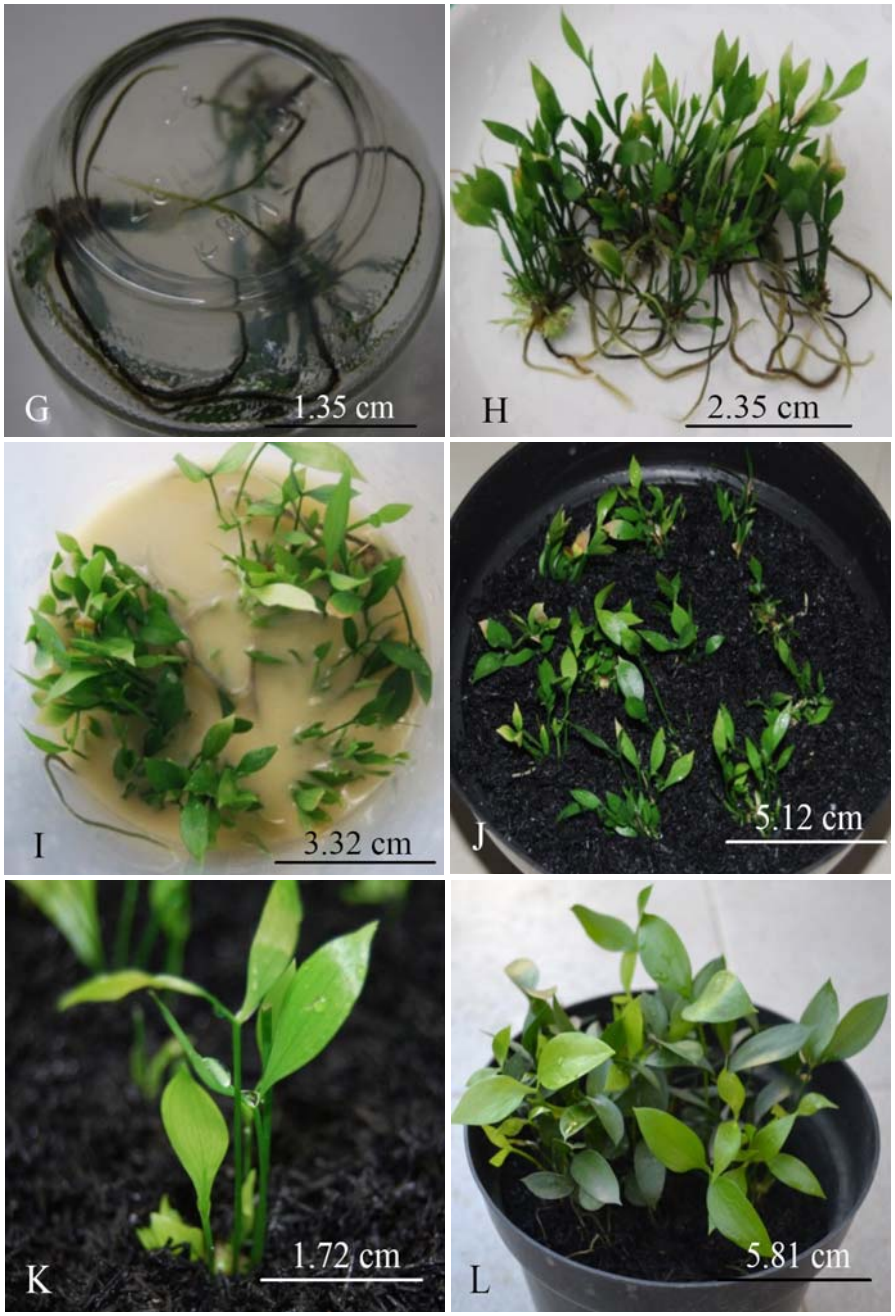

Figure 1. (G-L)

increased number of initial shoots per explant (Table 1), however subculturing the callus in the medium caused continuing callus growth in the callus stage with increasing size and volume with low capacity in regeneration of shoots. Therefore finding suitable regeneration media was addressed in the next experiment.

Table 1. Effect of initiation media on callus formation.

Initiation medium (IM)	Percentage of explant regeneration (%)	Callus score	Callus initiation time (day)	Range of initial number of shoots per explant	Number of initial shoots per explant
IM-1	88.8 a	+/++	17.3 ef	6-11	8.8 b
IM-2	100 a	+	23.3 cb	0-5	3.8 cde
IM-3	100 a	+	27.0 a	0-3	1.6 de
IM-4	56.3 b	+	18.9 ed	2-5	4.1 cd
IM-5	58.8 b	++	16.2 f	4-11	7.3 b
IM-6	82.5 a	+	24.5 b	0-3	1.3 e
IM-7	100 a	+	22.6 c	3-5	4.0 cde
IM-8	100 a	+/++	20.0 d	3-8	6.0 bc
IM-9	100 a	++/+++	14.0 g	10-17	14.8 a

Note: Cultures were incubated under dark condition for ± 2 months. Initiation media tested were half-strength MS containing (1) 10 mg/l 2,4-D, 1 mg/l BA and 0.05 mg/l NAA = IM-1, (2) 10 mg/l TDZ, 1 mg/l BA and 2 mg/l IAA = IM-2, (3) 10 mg/l BA, 2 mg/l 2,4-D and 0.05 mg/l NAA = IM-3, (4) 10 mg/l picloram, 1.0 mg/l BA, and 0.05 mg/l IAA = IM-4, (5) 150 ml/l CW, 0.5 mg/l BA, and 0.05 mg/l NAA = IM-5, (6) 1 mg/l BA, 0.5 mg/l TDZ and 0.5 mg/l IAA = IM-6, (7) 100 ml/l CW, 0.5 mg/l BA and 0.1 mg/l IAA = IM-7, (8) three fourth strength MS supplemented with 100 ml/l CW, 0.5 mg/l TDZ, and 0.1 mg/l IAA = IM-8 and (9) quarter strength MS augmented with 200 ml/l CW, 0.5 mg/l BA and 0.5 mg/l IAA = IM-9. Means followed by the same letter in the same column are not significantly different based on Tukey's test (P=0.05).

Regeneration of shoots

Based on periodical observation it was revealed that regeneration of shoots indicated by growing leaf-like cladodes derived from the initial shoots and height of shoot gradually 16-20 days after culture. Number of the leaf-like cladodes grew from 0 in initial culture to 6 leaf-like cladodes ± 2 months after culture with height of shoots from 0.1 to 2.7 cm (Fig. 1D). Percentage of shoot regeneration recorded in the experiment was 14.3% to 76.9% with

1-14 number of shoots per explant and 6-28 total number of leaf like-cladodes.

In the experiment, application of regeneration media gave significant effect statistically in all parameters observed (P=0.05). Each medium stimulated different effect in each parameter observed. The highest percentage of shoot regeneration up to 71.8% was recorded in RM-4 (three fourth strength of NWT medium), the highest number of shoots per explant of 8.6 shoots in RM-2 (three fourth strength of WT medium), 23.5 total number of leaf-like cladodes in RM-1 and 2.00 cm height of shoots in RM-7 (GM medium with 200 mg/l 32N:10P:10P, 12.5 mg/l 18N:18P:18K and 25 mg/l 6N:30P:30K) (Table 2).The high quality of regenerated-shoots with greener cladodes, relative homogeny in size and performance was significantly observed in callus cultured on RM-4, while heterogenic shoots growth with varied-size and performance were measured in most of media utilized and low regeneration capacity of initial shoots was noted in RM-7.

Table 2. Effect of regeneration media on shoot formation.

Regeneration medium (RM)	Percentage of shoot regeneration (%)	Number of shoots per explant	Total number of leaf-like cladodes	Height of shoots (cm)
RM-1	62.0 b	7.9 abc	23.5	1.25 bc
RM-2	54.7 c	8.6 a	20.3	1.36 abc
RM-3	43.3 e	6.5 abcd	16.3	1.62 abc
RM-4	71.0 a	6.8 abcd	22.3	1.22 c
RM-5	44.0 de	7.5 abcd	18.8	1.50 abc
RM-6	50.9 cd	6.1 cde	14.5	1.98 a
RM-7	18.1 f	4.0 e	7.5	2.00 a
RM-8	50.1 cde	5.6 de	17.8	1.51 abc

Note: Cultures were incubated under light condition for ± 2 months. Regeneration media (RM) investigated were (1) WT medium (RM-1), (2) three fourth strength WT medium (RM-2), (3) NWT medium (RM-3), (4) three fourth strength NWT medium (RM-4), (5) half strength MS medium (RM-5), (6) GM medium (100 mg/l 32N:10P:10K; RM-6); (7) GM medium (200 mg/l 32N:10P:10P + 12.5 mg/l 18N:18P:18K + 25 mg/l 6N:30P:30K, RM-7); and (8) Bregadium water PA-27 medium (10 ml/l; RM-8). RM-6 and RM-7 were added by 1 mg/l glycine, 50 mg/l myo-inositol and 25 µ/l Enervon C solution. All media were supplemented with 0.25 mg/l BA, 0.5 mg/l Kin, 0.05 mg/l IAA, 20 g/l sucrose, 1 g/l AC and 1.8 g/l Gelrite. Means followed by the same letter in the same column are not significantly different based on Tukey's test (P=0.05).

Proliferation of shoots

Application of multiplication media to increase proliferation of shoots slightly improved regeneration capacity of shoots. From new six media tested in the experiment it was revealed that light improvement in multiplication of shoots was indicated by subculturing shoots on half-strength MS medium hormone free with full vitamin (MM1). The medium could produce 9.2 shoots per explant subcultured with 22.4 total number of leaf-like cladodes and 2.69 cm height of shoots (Table 3; Fig. 1E). The effect was higher than MM-7 as control medium and other media. Most of new multiplication media tested in the study tended to reduce the proliferation capacity of shoots with low response growth of shoots determined on MM-4 and MM-6. In the both media, there was no initial shoots regenerated.

Table 3. Effect of media on multiplication of shoots.

Multiplication medium (MM)	Number of shoots per explant	Total number of leaf-like cladodes	Height of shoots (cm)
MM-1	9.2 a	22.4 a	2.69 ab
MM-2	6.5 b	17.3 c	2.85 a
MM-3	5.2 c	16.0 cd	2.23 ab
MM-4	4.2 c	15.5 d	2.69 ab
MM-5	4.5 c	16.6 cd	1.74 b
MM-6	4.1 c	14.9 d	1.89 ab
MM-7	7.5 b	20.3 b	2.46 ab

Note: Cultures were incubated under light condition for ± 2.5 months.MM-1, half-strength MS medium hormone free with full vitamin; MM-2, half-strength MS medium with full vitamin and 1 g/l AC; MM-3, VW medium containing 150 ml/l CW; MM-4, NP medium containing 1.5 mg/l GA3 and 0.5 mg/l IAA; MM-5,NP medium supplemented with 1.5 mg/l BA and 0.01 mg/l NAA; MM-6, half-strength MS medium fortified 65 g/l potato extract, 2 g/l peptone and 1 g/l AC and MM-7, three fourth strength NWT medium 0.25 mg/l BA, 0.5 mg/l Kin, 0.05 mg/l IAA as control. All media were supplemented with 20 mg/l sucrose and 1.8 g/l Gelrite. Means followed by the same letter in the same column are not significantly different based on Tukey's test (P=0.05).

Proliferation of shoots under periodical subcultures in the half-strength MS medium hormone free with full vitamin almost gave similar results in all parameter observed. Number of shoots per subculture period was ± 9.2

shoots per explant subcultured with 2.9 leaves per shoot, 2.92 cm height of shoots and 1.5 roots per explant subcultured (Fig 1F; Fig. 2). The high data recorded in all parameters were recorded in the third and fourth subculture period and decreased thereafter. Root formation was induced gradually due to subculture periods. The roots were observed firstly in the third subculture and increased gradually in the next subcultures. Although number of shoots produced in each subculture period indicated almost similar results, reducing quality of shoots was initially recorded in the sixth subculture indicated by altering color leaves from green to yellowish light green. In the seventh subculture, changing color leaves from the green to yellowish light green or yellowish green increased gradually. Percentage of the problem was 2.8-3.5% in the sixth subculture and increased up to 8.6% in the seventh subculture. The reducing leaf-like cladodes quality could be utilized to control quality production of shoots and applied for proliferation purpose of shoots in *R. hypophyllum*.

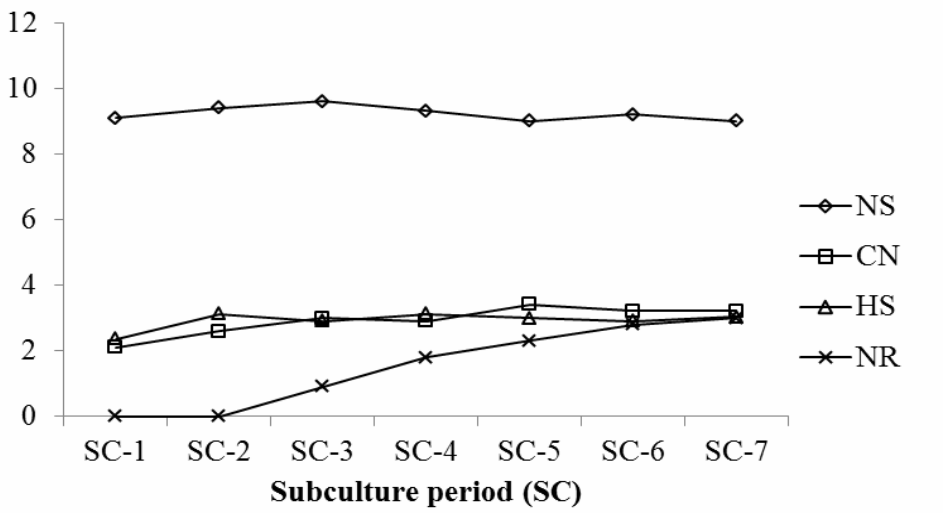

Figure 2. Shoot growth on half-strength MS medium with full vitamin during periodical subcultures. NS- number of shoots per explant subcultured, CN- number of leaf-like cladodes per shoot, HS-height of shoots and NR- number of roots per cluster of shoots

Rooting of shoot clusters

Root formation was easily prepared by culturing shoots in half-strength MS medium hormone free with full vitamin. Initial roots were clearly observed 15-20 days after culture in the basal part of shoots. The initial roots continued to grow in number, size and length. After ± 3 months of culture, number of roots was about 1-5 roots per shoot cluster and 2.7 in average number of roots with 0.5 – 3.5 cm root length and 1.53 cm in average (Fig. 1G).

Acclimatization of plantlets

Transferring plantlets from *in vitro* to *ex vitro* condition started from washing plantlets under tap water to release remains of agar (Fig. 1H), treating them with 1% fungicide and bactericide solution (Fig. 1I), culturing on burned-rice husk in plastic pot, covering the plastic pot with transparent plastic and placing under reduced-light intensity area of glass house resulted in high survivability of them. In the experiment all plantlets acclimatized had varied-survivability from 73-93% with 82% in average (Fig. 1J and 1K). The survived-plantlets continually grew, increased in leaf and root number and length and height of shoots. Three months after acclimatization the acclimatized-plantlets were ready to be planted individually in bigger plastic pots (Fig. 1L).

Morphogenesis of varied-explants in *in vitro* propagation of plants is influenced by several important factors and one of them is culture medium (George et al. 2007). High suitability of explant and culture medium will lead to obtaining high regeneration of it involving callus formation as reported previously. Jha & Sen (1985) reported that high callus formation was successfully established on MS medium supplemented with 1 mg/l 2,4-D and 0.1 mg/l BAP, while Purwito et al. (2005) determined that callus formation followed by adventitious shoot formation on the *R. hypophyllum* was initiated by culturing shoot tips on MS medium supplemented with 2 mg/l BAP and 0.2 mg/l IAA. In *R. hypoglossum*, high callus formation was established on MS medium containing 0.5 mg/l TDZ (Ivanova et al. 2011). In other Liliaceae's plants, callus formation was easily regenerated on B5 medium supplemented with 2 mg/l NAA and 0.5 mg/l Kin for *Gloriosa*

superba (Rishi 2011), MS medium fortified with 2 mg/l 2,4-D for the same plant (Venkatachalam et al. 2012), MS medium augmented with 0.5 mg/l Kin and 0.5 mg/l 2,4-D for *Aloe vera* (Chaudary et al. 2011), 5 µM Picloram and 5 µM BA for *Lilium martagon* (Kedra & Bach 2005). In the study, high callus formation was stimulated on a quarter strength MS medium containing 200 ml/l CW, 0.5 mg/l BA and 0.5 mg/l IAA; however subculturing regenerated-callus in the medium could not induce callus to produce shoots, but continue to stimulate callus proliferation.

Coconut water, containing unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones, is traditionally used as a growth supplement in plant tissue culture (Yong et al. 2009, Gbadamosi & Sulaiman 2012) and induce cell division, promote morphogenesis and accelerate the multiplication of callus as reported in *Cleisostoma racemiferum* (Temjensangba & Deb 2006), *Cymbidium pendulum* (Kaur & Bhutani 2011), *Phalaenopsis amabilis* (L.) Bl. cv. 'Golden horizon' (Sinha & Jahan 2011) and *Dendrobium* 'Alya Pink' (Nambiar et al. 2012). The high effect of the CW application for callus proliferation reduced regeneration capacity of the callus to produce shoots and the condition also determined in the first experiment.

In the study, substituting medium quarter strength MS medium containing 200 ml/l CW, 0.5 mg/l BA and 0.5 mg/l IAA to three fourth strength WT medium fortified with 0.25 mg/l BA, 0.5 mg/l Kin, 0.05 mg/l IAA, 20 g/l sucrose, 1 mg/l AC and 1.8 g/l Gelrite successfully induced high regeneration of callus into shoots (Table 2). The shoot formation was then slightly improved by subculturing the shoots on half-strength MS medium hormone free with full vitamin. Changing medium in obtaining high response of explant both for regeneration and proliferation purposes was also applied in other *in vitro* culture of Ruscus. Abao-Dahab et al (2004) utilized MS medium augmented with 1.5 mg/l BA for initial shoot formation then MS medium supplemented with 1.5 mg/l BA and 2 mg/l Kin to improve the shoot proliferation of *R. hypoglossum*. MS medium containing 0.5 mg/l TDZ and 1 mg/l NAA was suitable for shoot regeneration and MS medium hormone free with 60 g/l sucrose was appropriate for high proliferation of shoots up to 8 shoots per explant for the same Ruscus (Ivanova 2012;

Ivanova et al. 2013), while Tarek & Abao-Dahab (2004) applied full strength MS medium for initiation of shoots and half-strength MS medium for multiplication of shoots of The *R. hypoglossum*.

Producing high number and quality of shoots in in vitro culture of Ruscus could be obtained by subculturing of shoots in the optimal medium. In the research, high number and quality of shoots was produced by periodical subculturing shoots on half-strength MS medium hormone free with full vitamin up to fifth subculture then reduced the quality of shoots produced thereafter. Tarek & Abao-Dahab (2004) recorded that subculturing shoots on half-strength MS medium, the number of shoots per explant of *R. hypoglossum* was significantly increased from 2.86 to 8.14 shoots until the third subculture then declined afterwards. In other Liliaceae's studies, high quality of *Aloe vera* shoots could be maintained up to the fifth subculture and then reduced in the next subculture (Gantait et al. 2010), high number of shoots (35 shoots) of *Chlorophytum borivilianum* was established on MS medium containing 2 mg/l BAP in four weeks of periodical subculture (Sharma & Mohan 2006).

Well developed-plantlets with healthy roots are pre-requisite condition in obtaining high survivability during transferring *in vitro* plants to *ex vitro* condition. In the study, periodical subculturing shoots in the half-strength MS medium hormone free with full vitamin was successfully induced healthy roots and high number of roots up to 5 roots per shoot cluster recorded at the seventh subculture. Purwito et al. (2005) recorded that high number of roots was established on the half-strength MS medium hormone free of *R. hypophyllum*. MS medium supplemented with 1.8 mg/l IBA and 0.02 mg/l Kin was suitable for root formation of *R. aculeatus* (Banciu & Aiftimie-Păunescu 2012), MS medium augmented with 2 mg/l NAA was also used for the *R. aculeatus* (Mayano et al. 2006, Ivanova et al. 2008).

Acclimatization of plantlets derived from *in vitro* culture of *R. hypophyllum* was not a critical point in the study. High percentage of survivability of plantlets cultured in burned-rice husk with 82% in average was recorded in the experiment. In other studies, 90% survivability of the *R. hypophyllum* was established in a mixture medium of soil and burned rice husk (1:1, v/v) (Purwito et al. 2005), 92% survivability of acclimatized-

plantlets in a medium containing 60% forest soil and 40% perlite was noted for *R. aculeatus* ((Banciu & Aiftimie-Păunescu 2012), while Mayano et al. (2006) with the same plants only got 58% survival plantlets. The 90% survivability of plantlets was successfully established for *R. hypoglossum*, but it only reached 50% for *R. aculeatus* (Ivanova 2012). The reduction of the survivability was significantly affected by condition of plantlets and healthy plantlets of the *R. hypoglossum* gave high result in acclimatization stage (Ivanova 2012).

CONCLUSSION

Finally from the study it was concluded that *in vitro* in mass propagation of *Ruscus hypophyllum* L. was successfully established. High callus formation derived from the young shoot nodes was mostly induced on quater-strength Murashige and Skoog (MS) medium supplemented with 200 ml/l CW, 0.5 mg/l BA, 0.5 mg/l IAA, 20 g/l sucrose and 1.8 g/l Gelrite. The initial shoots were easily regenerated on three-fourth strength Winarto-Teixeira (WT) medium containing 0.25 mg/l BA, 0.5 mg/l Kin, 0.05 mg/l IAA, 20 g/l sucrose, 1 g/l AC and 1.8 g/l Gelrite. The proliferation of shoots was slightly improved by culturing the shoots on half-strength MS medium hormone free with full vitamin. Producing the high quality of shoots could be maintained up to the fifth subculture then reduced thereafter. Well rooted-shoots were easily stimulated nd successfully transferred to *ex vitro* condition with 82% survivability on burned-rice husk medium.

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