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IN VITRO PROPAGATION OF *RUSCUS*: A REVIEW

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ABSTRACT. *Ruscus is an important foliage plant cultivated commercially in several countries, especially at Florida, Israel, Columbia and Middle East. The Ruscus is a genus belonging to Asparagaceae family with well-known species frequently researched i.e. Ruscus aculeatus, R. hypoglossum and R. hypophyllum. Conventional propagation vegetatively and generatively of the plant is not suitable for commercial purposes, so in vitro propagation was promisingly addressed. In vitro plant culture as important methods can be applied for preserving germplasm followed by reintroducing endangered germplasm back into the wild and preparing high qualified planting materials for commercial purposes. Several in vitro culture of the Ruscus has been reported with different important results, however comprehensive in vitro propagation of the Ruscus was a few. This brief review provides a short synopsis of the advances made and problems exist thus far in the in vitro culture of Ruscus. This review expected can be used as main consideration to develop reliable an in vitro mass propagation protocol of Ruscus to answer germplasm and commercial future challenges.*

KEY WORDS: explant, germplasm, *Asparagaceae,* planting materials, *Ruscus.*

INTRODUCTION

Ruscus is a genus belonging and classified in the *Asparagaceae* family, subfamily *Nolinoidae*. However the genus has fluctuated between different families and by turns located in *Liliaceae*, *Convallariaceae* and *Ruscaceae* (Veronese 2015). This genus has hasapproximately 10 species with the most known being seven as follows: *R. aculeatus* (Butcher's Broom) originating from Mediterranean region is spread in western and southern Europe and Africa (Palazón et al. 2006, Banciu et al. 2009, Manole &

Banciu 2015); *R. colchicus*, common in Caucasus; *R. Hypoglossum* native to the Euro-Mediterranean, North Italy to Austria and Slovakia and east to Turkey and Crimea (Ivanova et al. 2013); *R. hypophyllum* widespread in the Iberian Peninsula and north-west Africa; *R. Streptophyllum* found in Madeira; and *R. hyrcanus*, endemic in the Talish Mountaine, Azarbaijan (Banciu & Aiftimie-Păunescu 2012). The plants derived from the genus are generally used as medicinal and ornamental plants (Brezeanu & Banciu 2010, Ivanova et al. 2015, Manole & Banciu 2015) as floral design, lanscape and indoor plants (Stamps 2001). The plant leaveslike cladodes with lack's spine and used as cut foliage in floral arrangements due to unique, esthetic with long vase-life for one month or more (Purwito et al. 2005, Winarto & Setyawati 2014). Internationally, *Ruscus* is produced commercially in Florida, Israel, Colombia and the Middle East and sold at Dutch auctions (Stamps 2001). In Rijnsburg auction, the *Ruscus* large leaf is sold £0.41 per stem and £7.63 per bunch for soft *Ruscus* 40 cm; £5.19 per bunch for soft *Ruscus* 60 cm; £7.28 per bunch for soft *Ruscus* short (Trianglenursery 2017). In Japan market, the *Ruscus* cut foliage was auctioned up to 21,015 stems with US $ 3,524.8 values. In Indonesia, the foliage plants were commercially cultivated under screen house or in opened-field at Pasir Sarongge and Cipanas-Cianjur; Cisarua-Bogor; Cihidueng-Bandung in West Java province. Bandungan- Semarang, Central Java; Batu-Malang, East Java. At Cihideung areas, farmer can produce more than 500 bunches of stems per week. The leaves of the plant were sold between 8,000-25,000 rupiahs per bunch dependence on the quality (Winarto & Setyawati 2014). Conventionally, the *Ruscus* are propagated generatively by seeds and vegetatively by cuttings and division of underground rhizomes (Stamps 2001). The generative propagation using seeds, though it results in high number of regenerants, the method produces varied-regenerants with ununiformity, not true-to-type, different characters and performances of them (Hartmann et al. 1997, Redaksi Agromedia 2007). The vegetative method *via* cuttings and division of underground rhizomes, even though produce true-to-type regenerants from mother plants. This technique produces limited number of plants with a low quality; need rooting; laborious and time consuming (Högberg 2003). These methods is not suitable for commercial purposes, therefore preparing high quality and quantity planting materials in shorter time with high sustainability can be carried out by applying tissue culture as a method. Ruscus tissue culture works in accordance with establishment of *in vitro* propagation protocol were initiated on *R. hypophyllum* (Ziv 1983, Jha & Sen 1985, Jhaet al. 1989). After the studies, different researches to explore potency of tissue culture application in Ruscus were reported on *R. hypoglossum* (Ivanova et al. 2013, Abou-Dahab et al. 2005); *R. aculeatus* (Moyano et al. 2006, Palazón et al. 2006, Banciu et al. 2009, Brazeanu & Banciu 2010, Banciu & Aiftimie-Păunescu 2012, Ivanova et al. 2015, Monale & Banciu 2015) and *R. hypophyllum* (Purwito et al. 2005, Winarto & Setyawati 2014). Though there were still few *in vitro* culture studies reported on Ruscus, several findings from those researches were important points applying to strengthen developing new *in vitro* mass propagation protocol having high effect in preparing high qualified and sustainable planting materials for commercial scale. From these studies it was revealed that establishment of *in vitro* mass propagation of *Ruscus* was clearly constrained by several factors *viz*. explant sources, disinfection agents and procedures, culture incubation, initiation medium to plantlet preparation and acclimatization of plantlets. Due to each step of tissue culture activities is interesting and give significant effect on establishing the *in vitro Ruscu*s mass propagation, all success and problems in the each step in the propagation are revealed comprehensively in this review. The recent findings and problems expected can be used as main considerations to develop *in vitro* mass propagation to answer future challenges on germplasm management and commercial purposes.

EXPLANT SOURCES

Explant soucre utilized in tissue culture propagation depends on the age, size and behaviour has an important role in establishing *in vitro* propagation protocol of each plant. Each plant has specific explant type of it having high response *in vitro* culture. Based on physiological aspects of explant, younger explant condition have higher regeneration capacity. Apical meristem was high responsive explant by *in vitro* culture of Gerbera than vegetative buds (Naz et al. 2012); stem node in Lavender (Kara & Baydar 2012); nodal segments for *Ananas comosus* (Ibrahim et al. 2013); nodal explants for *Poulawnia tomentosa* (Ozaslan et al. 2005). In *in vitro* culture of ruscus, Ziv (1983) determined that shoot tip induced higher shoot regeneration up to 6-10 fold than infloresecence on *Ruscus hypophyllum*. The explant was also succesffully applied for the Ruscus in the similar purpose (Purwito et al. 2005); stem segments taken from young shoots was proved by Jha et al. (1989); and young stem nodes by Winarto & Setyawati (2014) for the same Ruscus. High shoot induction was recorded form rhizome segments of *R. aculeatus* (Moyano et al. 2006, Banciu & Aiftimie-Păunescu 2012) and R. hypoglossum (Ivanova et al. 2013) that were utilized as explant source. From those studies, it was clearly revealed that most of the regenerated-shoots derived from the rhizome, nodes and stems were produced indirectly from induction callus (Jha & Sen 1985, Jha et al. 1989; Purwito et al. 2005, Moyano et al. 20061, Palazón et al. 2006, Banciu & Aiftimie-Păunescu 2012, Ivanova et al. 2013, Winarto & Setyawati 2014). While the rest of shoots were regenerated directly via axillary shoot proliferation (Ziv 1983, Manole & Banciu 2015, Ivanova et al. 2015).

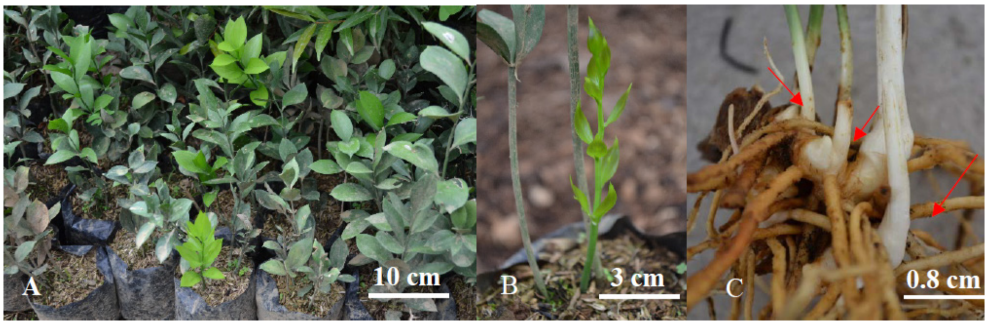

Figure 1. Mother plants and explant types of *R. hypophylum*. A. Mother plants of *R. hypophyllum*. B. Young shoot. C. Different sizes of rhizome used in *in vitro* culture. New photos prepared by Winarto.

STERILIZATION OF EXPLANT FROM THE *EX VITRO MILIEU*

Obtaining sterile explants derived from *ex vitro* material, such as greenhouse or field-grown plants with high potential regeneration capacity for varied-purposes are the most important point in establishing of an effective tissue culture system (George & Debergh 2008). Though it is fact that field-grown plants contain more soil- and air-borne contaminants than the greenhouse-grown plants (Niedz & Bausher 2002), soil-grown organ plants such as rhizomes, corms, bulbs and tubers than hidroponic plants, disinfection process need to all plant materials used for *in vitro* culture (Srivastavaet al. 2010,Teixeira Da Silva et al. 2016). The disinfection process is usually initiated by initial washes and removal of rough contaminants (Hall 1999), followed by (a) pre-sterilization with a disinfection solution [e.g. 70% ethanol/alcohol; 1-2% soap solution and 1% pesticide solution (for tropical condition)] then either washing in sterile distilled water (SDW) or not; (b) sterilization usually carried out in laminar air flow cabinet with another disinfectant [e.g. sodium hypochlorite (NaOCl); mercury chloride ($HgCl_2$); hydrogen peroxide (H_2O_2)] and finally (c) rinses in SDW at least three times. Furthermore the type, order and concentration of disinfectants utilized, their combinations and their exposure period were applied in different variations (Hall 1999, Onwubiko et al. 2013). The age,

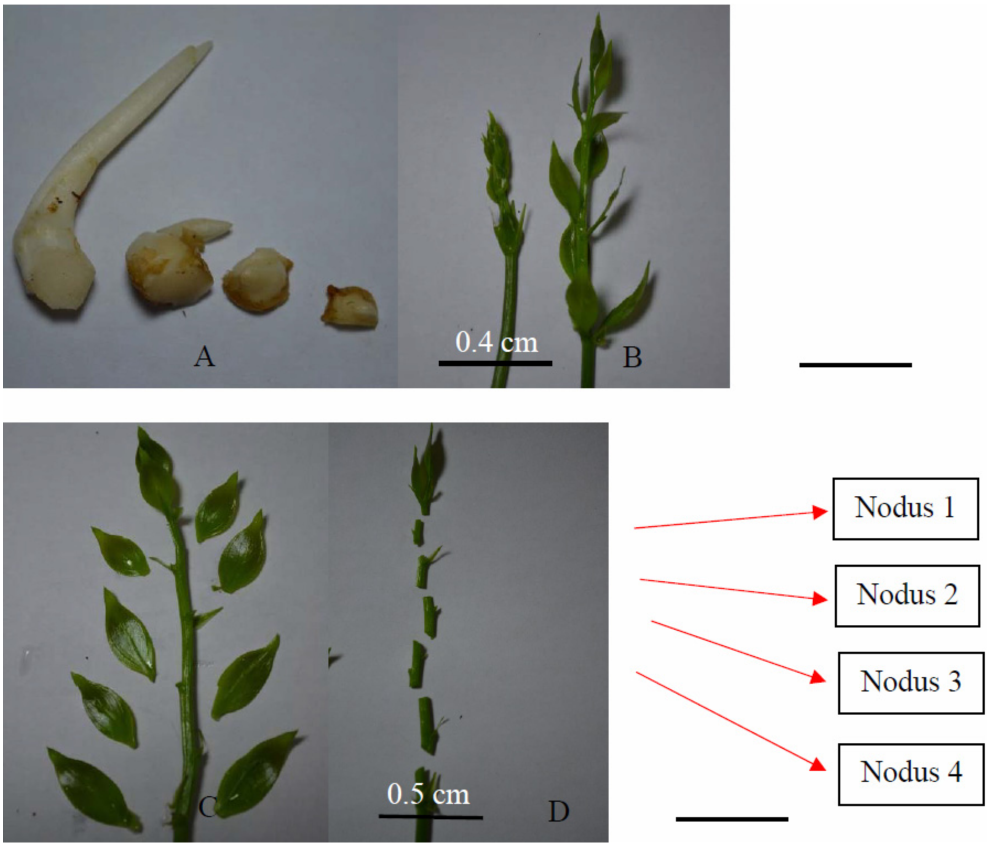

Figure 2. Type of explants on developing *in vitro* propagation of*Ruscus* and preparation of them. A. Different size of rhizome used by*in vitro* culture of *Ruscus*. B. Young shoot utilized in as explant source. C. Removing leaflikes cladodes before or after sterilization of explant. D. Young nodes (1 to 4) usually cultured on media for callus regeneration. New photos prepared by Winarto.

size, type, topophysis of donor plant, genotype, temperature, relative humidity (RH), photoperiod, light intensity, irrigation, fertilization, season have to be considered in the disinfection process (Traore et al. 2005, George & Debergh 2008, Dobránski & Teixira Da Silva 2010, Mihaljevic et al. 2013).

In *in vitro* culture of *Ruscus*, several disinfection processes, agents and period of treating were applied to obtain sterile explants (Winarto & Setyawati 2014) and those are also influenced by type of explants. Using rhizomes as explant source generally have higher problem due to contaminants and latent contaminents compared to others (Abou-Dahab et al. 2005, Purwito et al. 2005, Moyano et al. 2006, Banciu & Aiftimie-Păunescu 2012). The higher contaminants were due to position of rhizomes as underground organs covered by media (Moyano et al. 2006). Therefore application of disinfectant agents with high effect to eliminate contaminants and less influence to survivability of explants as $HgCl_2$ generally applied (Abou-Dahab et al. 2005, Moyano et al. 2006, Banciu & Aiftimie-Păunescu 2012). While utilization of stems and nodes as explant source resulted in low and no contamination (Jha & Sen 1985, Winarto & Setyawati 2014).

CULTURE INCUBATION

Culture incubation is an environmental condition needed to stimulate explants in different behaviour in *in vitro* culture (Khanet al. 2006, Winartoet al. 2011). Generally incubation of explants in the dark condition lead to regeneration of callus leading to adventitious shoot formation (Yeet al. 2012) and/or somatic embryogenesis (Khanet al. 2006). While light incubation of explants stimulate direct and/or indirect organogenesis either axillary shoots, adventitious shoots or embryos (Konstaset al. 2003, Ozaslan et al. 2005, Kara & Baydar 2012, Ibrahim et al. 2013).In in vitro culture of Ruscus, dark incubation at 25°C under periodical subcultures every 4 weeks regenerated callus derived from rhizome of *R. aculeatus* (Palazón et al. 2006); the dark incubation of node explants of R. hypophyllum for ± 2.0 months for callus initiation and the initiated-callus were then placed in a 12-h photoperiod under fluorescent lamps with ~13.5 µmol/m/s, 23.5 ± 1.1°C, and 60.6 ± 3.8 % relative humidity (Winarto & Setyawati 2014). While light incubation was applied in the most of

Table 1. Varied-sterilization processes by*in vitro* culture of *Ruscus* and percentage of contaminated explants recorded.

No.	Explant source	Pre- sterilization	Sterilization	Percentage of contaminated-explants (%)	References
1.	Rhizomes of *R. hypophyllum*	Rinsing for 24 h under running water and dipping in 70% ethanol	Dipping in 9% $CaHClO_4$ for 15 rain was followed by rinsing 3 times in sterile water	Not stated	Ziv 1983
2.	Stems of *R. hypophyllum*	Washing in 5% Teepol for 15 min	Soaking in 0.1% $HgCl_2$ for 8 min followed by 5-6 washing in sterile distilled wate	Not stated	Jha & Sen 1985
3.	Rhizomes of *R. hypophyllum*	Brushing them gently using smooth brush, followed by immersing them in 2% pesticide solution (2 g/l of Dithane-M45 and Agrimycin) for 24 h then rinsing by sterile water 3 times (@ 3 min).	Immersing in 70% alcohol for 2 min, dipping in 10% clorox for 10 min then rinsing by sterile water 3 times; soaking in 5% clorox for 10 min then rinsing with sterile water	Not stated	Purwito et al. 2005
4.	Rhizomes of *R. hypoglossum*	Washing in soap water using septol soap for 30 minuets and rinsed with running tap water for two hours	Dipping in 0,4% $HgCl_2$ or 50-60% chlorox for 20 min and rinsing 3 times with distilled water	20%	Abou-Dahab et al. 2005

Table 1. (continued)

No.	Explant source	Pre- sterilization	Sterilization	Percentage of contaminated-explants (%)	References
5.	Rhizomes of *R. aculeatus,*	-	Immersing in 70 % ethanol for 30 s, then in HgCl2 (1%) for 15 min and NaClO (3 %) for 30 min, and rinsing 5 times with sterile water	< 30%	Moyano et al. 2006
6.	Rhizomes of *R. aculeatus*	-	Soaking in ethyl alcohol (70%) for 30 seconds, immersing in 5% NaOCl for 3 minutes, 1% $HgCl_2$ for 2 minutes, and rinsing with 4-6 vigurous washes with sterile distilled water for 2 minutes	Exceeded 40%	Banciu & Aiftimie-Păunescu 2012
7.	Nodes of *R. hypophyllum*	Putting under tap water for 30 min, then immersing in a 1 % pesticide solution of 50 % benomyl and 20 % streptomycin sulphate for 30 min and rinsing with distilled water 5 times (5 min each rinse)	Dipping them in 1 % NaOCl 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)	Not stated	Winarto & Setyawati 2014

micropropagation of *Ruscus*. An environment-controlled chamber, under a 14-h photoperiod of 1.5 × 10^{-3} W.cm^{-2} at a constant temperature of 20 ± 1°C for adventitious shoot proliferation of *R. hyophyllum* (Ziv 1983); 16-h photoperiod of fluorescent lamps with 32 × 108 μmol/m^2/s regenerating callus and adventitious shoots derived from stem explants (Jha & Sen 1985); 16-h photoperiod under illumination intensity of 3000 lux with 24±2°C day/night producing callus derived from rhizome to produce adventitious shoots of *R. hypoglossum* (Abou-Dahab et al. 2005); 24-h photoperiod under fluorescent lamps with 1500 lux, 20-22ºC, 55-60% RH to produce callus and adventitious shoots of *R. hypophyllum* (Purwito et al. 2005); 16-h photoperiod irradiance of 28-36 μmol m^{-2} s^{-1} and at a temperature of 25°C for callus formation from rhizome *of R. aculeatus* (Moyano et al. 2006); 16-h illumination periods at 2000 lux light intensity inducing organogenic callus and axillary proliferation of *R. aculeatus* (Banciu & Aiftimie-Păunescu 2012);16-h photoperiod at 3000 lux light intensity at 22±2°C stimulating axillary shoots of *R. aculeatus* (Manole & Banciu 2015).

MICROPROPAGATION OF *RUSCUS* USING RHIZOME AS EXPLANT SOURCES

Development of mass propagation of Ruscus using rhizome as explant sources was initiated by culturing shoot tip of the explant of *R. hypophyllum* by Linsmaier & Skoog (1965) augmented with 1.0 mg/l 2,4-dichloroacetic acid (2,4-D) and 5.0 mg/l kinetin (Kin) succesfully producing 6.8 shoots per explant 58 days after culture (Ziv 1983). Application of MS medium by Murashege & Skoog (1962), supplemented with 4.0 mg/l 6-benzylaminopurine (BAP) and 0.4 mg/l indole-3-acetic acid (IAA) induced 2.4 initial shoots for two weeks of culture (Purwito et al. 2005); 10 shoots per explant with 2.75 cm shoot length and 9.5 leaves per shoot of *R. Hypoglossum,* regeneratedon MS medium containing 1.5 mg/l 6-benzyl aminopurine (BAP) for 18 weeks of incubation (Abou-Dahab et al. 2005); ±28 shoots of *R. aculeatus,* stimulated on MS medium augmented with 0.5 mg/l kinetin (Kin) for 16 months of incubation without subculture (Ivanova et al. 2011); 5.0 shoots per explant of *R. hypoglossum* on MS medium fortified by 0.5 mg/l thidiazuron (TDZ) and 1.0 mg/l α-naphthalene acetic acid (NAA) for 8 weeks of culture (Ivanova et al. 2013); 24.2 shoots per explant of *R.*

aculeatus on MS medium added by 5.0 mg/l BAP and 1.0 mg/l NAA within 8 weeks after culture (Manole & Banciu 2015). In the initiation stage, shoots derived from the rhizome explant were generally regenerated directly and/or indirectly and always faced by contamination (Abou-Dahab et al. 2005, Banciuet al. 2009, Banciu & Aiftimie-Păunescu 2012) and necrosis (Ivanova et al. 2013) as man in important problem. Percentage of explant contamination was varied from 20-100% (Abou-Dahab et al. 2005, Banciu & Aiftimie-Păunescu 2012, Banciu et al. 2013) and 6.7-50.0% for explant necrosis (Ivanova et al. 2013).The problems were reduced by increasing concentration of chlorox from 30% to 60-70% or $HgCl_2$ from 0.1% to 0.4% (Abou-Dahab et al. 2005) and applying 5% NaOCl (Banciu & Aiftimie-Păunescu 2012). While to lower necrosis was carried bysubstituting BAP and Kin with TDZ (Ivanova et al. 2013). Proliferation and multiplication of shoots in establishing of *in vitro* mass propagation protocol are an important step to produce high qualified planting materials as much as posible dealing with the purpose under periodical subcultures (Hamad & Taha 2008; Winarto 2012; Vujovic et al. 2012). Generally, subculture gave effect on multiplication rate of *in vitro* cultures varied from one species to another. Short or long number of the periodical subcultures is affected by type of explant, physiological alteration of explant, culture medium and incubation (Hamad & Taha 2008; Vujovic et al. 2012). Growth and gradual increase in number of explant and rate of multiplication are generally recorded fromfirst subculture to seventh subculture and declined therafter (Kofi & Adachi 1993; Singh & Manual 2000). The such studies were reported on *Dendrobium* (Winarto 2012), *Dendrobium* 'Gradita 31' (Winarto & Teixiera Da Silva 2015), *Phalaenopsis* 'AMP 17' (Pramanik et al. 2016), *Phalaenopsis* (Winarto et al. 2016), *Gerbera jamesonii* (Winarto & Yufdy 2017). From those studies, declining proliferation and multiplication stage of explants was indicated by lowering number of shoots produced, increasing root formation capacity of shoots, appearing and enhancing number of old leaf senescences. In *in vitro* propagation of *Ruscus* using rhizome as explant source, proliferation of shoots was carried out under different culture media. Abou-Dahab et al. (2005) noted that success proliferation of shoots of *R. hypoglossum* was recorded on explant cultured on MS medium supplemented with 1.5 mg/l BA. In the first subculture, number of shoots per explant was 4.5 shoots, increased to 6.0 shoots per explant in the second subculture and 10.0 shoots per explant in the third subculture with 2.54 cm shoot length and 7.25 leaves per shoot, however best results based on shoot length and number of leave as per shoot were proved on MS medium containing 4.5 and 6.0 mg/l Kin. Use ofMS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA resulted in 9.4 shoots per explant during 8 weeks of incubation (Purwito et al. 2005). The study also did not report when declining number of shoots was occurred. MS medium fortified by 0.5 mg/l TDZ, 1.0 mg/l NAA and 60 g/l sucrose was the most suitable medium for proliferation of shoots of *R. hypoglossum* (Ivanova et al. 2013). The medium successfully regenerated 8.4 shoots per explant with 6,7% callogenesis, 2.7 growth index, no mrophological variation and 6.7% necrotic explants; however the study did not explore multiplication rate of shoots under periodical subculture. Other studies only showed partial activities on callus initiation, regeneration followed by root formation on *R. hypophyllum* (Purwito et al. 2005), callus initiation and regeneration on *R. aculeatus* (Palazón et al. 20061), callus initiation, shoot regeneration and root formation of *R. aculeatus* (Moyano et al. 20061), axillary initiation and shoot proliferation under long period of incubation (16 months without subculture) on *R. aculeatus* (Ivanova et al. 2011). From these studies it was clearly known that there were no proliferation and multiplication studies to reveal rate of it, no paying attention on howmany times the initial shoots established can be subcultured and in which subculture period the number of shoots in the peak point and reducing growth performance of shoots occurred. Root formation and plantlet acclimatization are final stages established in tissue culture works. Successful of the stage is significantly influenced by culture media, physilogical stage of explant and culture incubation (Winarto 2012, Winarto & Teixeira Da Silva 2015, Winarto et al. 2016, Winarto & Yufdy 2017). Medium with less or no hormone and easy-to-root shoots with higher light intensity are applied to prepare well-plantlets ready for acclimatization, while hardenning (optional) and selecting plantlets, treating roots with pesticide solution, applying porous media with enough water holding capacity and nutrition gave high affect on acclimatization of plantlets. In establishing micropropagation of *Ruscus* using rhizome as explant by Purwito et al. (2005) cultured of shoots on half-strength MS medium containing 2.0 mg/l indole-3-butyric acid (IBA) successfully induced 6.6 roots per shoot with 3.4 cm root length, 4.1 cm plant height of *R. hypophyllum*. MS medium with 2 mg/l NAA was applied to shoot rooting of *R. aculeatus* (Moyano et al. 2006). There was no more rooting studies reported on *in vitro*of *Ruscus* using rhizome explant. While successful acclimatization of plantlets using mixture of soil and burned-rice husk (1:1, v/v) was clearly reported on *R. hypophyllum* with 60-100% survivability under different treatments (Purwito et al. 2005). The results indicated that developing complete and comprehensive study on establishing *in vitro* propagation protocol using rhizome as explant source were still few.

IN VITRO PROPAGATION OF *RUSCUS* USING YOUNG STEM OR NODES AS EXPLANT SOURCES

Establishment of micropropagation protocol of *Ruscus* utilizing young stem or node as explant source was a few. So far there were three published research works internationally by Jha & Sen (1985), Jha et al. (1989) and Winarto & Setyawati (2014) for *R. hypophyllum*. From these studies it can be summarized as follow:

Initiation stage was carried by culturing stem explants on MS medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l BAP (Jha & Sen 1985, Jhaet al. 1989). The treatment induced callogenesis up to 60% compared to others. The callus were then regenerated on MS medium containing 0.05 mg/l 2,4-D and 0.5 mg/l BAP with 80% callus successfully produced shoots up to 10-12 shoots per culture and 6.6 cm shoot length after 8-12 weeks of incubation. Initial callus formation derived from node explants was occurred 15-20 days after culture and produced obvious callus 35 days after culture on quarter strength of MS medium containing 200 mg/l coconut water (CW), 0.5 mg/l BA and 0.5 mg/l IAA. The medium induced percentage of explant regeneration up to 100% with ++/+++ callus score, 14 days callus initiation time, 10-17 initial number of shoots and 14.8 initial shoots per explant (Winarto & Setyawati 2014). The initial shoots were further and optimal regenerated on three fourth strength Winarto & Teixeira Da Silva (Winarto et al. 2011) medium fortified by 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 resulting 71% shoot regeneration, 6.8 number of shoots, 22.3 leaf-like cladodes and 1.22 cm shoot height. From these studies, there was no contamination explant recorded and callus initiation was significantly affected by medium strength, combination and concentration of plant growth regulator.

Proliferation of shoots under periodical subcultures was carried out by subculturing regenerated shoots on MS medium hormone free with full vitamin (Winarto & Setyawati 2014). Using the medium, average of shoot proliferation rate was 9.2 shoots per explant subcultured with 2.9 leaves per

Table 2. Summary on *in vitro* propagation protocol of *R. hypophyllum.*

Explant type	Callus initiation and regeneration	Proliferation of shoots	Rooting of shoots	Plantlet acclimatization	Reference
Rhizomes of *R. hypophyllum*	MS medium supplemented with 4.0 mg/l BAP and 0.4 mg/l IAA; 2.4 initial shoots for 2 weeks of culture	MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l IAA; 9.4 shoots per explant after 8 weeks of incubation	½ MS medium containing 2.0 mg/l IBA; 6.6 roots per shoot with 3.4 cm root length, 4.1 cm plant height	mixture of soil and burned-rice husk (1:1, v/v); 60-100% survivability after 4 weeks of acclimatization	Purwito et al. 2005
Young stem of *R. hypophyllum*	MS medium containing 1 mg/l 2,4-D and 0.1 mg/l BAP; 60% of explant produced callus	MS medium supplemented with 0.5 mg/l 2,4-D and 0.5 mg/l BAP; 10-12 shoots per cluster for 8-12 weeks of culture	MS medium hormone free; 100% shoots rooting	Soil; 80% survival plantlet after 3-4 months	Jha & Sen 1985
Young nodes of *R. hypophyllum*	¼ MS medium containing 200 ml/l CW, 0.5 mg/l BA, 0.5 mg/l IAA; 100% callus formation of explant with 14.8 initial shoots per explant during 14 days after culture; ¾ WT medium 0.25 mg/l BA, 0.5 mg/l Kin and 0.05 mg/l IAA; 8.6 shoots per explant.	½ MS medium hormone free with full vitamin; 9.2 shoots per subculture period with 22.4 leaf-like cladodes and 2.7 cm shoot height	½ MS medium hormone free with full vitamin; 2.7 roots per shoot with 1.53 cm root length	Burned-rice husk; 73-93% survival plantlet	Winarto & Setyawati 2014

Figure 3. *In vitro* propagation of *R. hypophyllum* using young nodes as explant source. A. Node explant in initial culture, B.Regenerated callus on quarter strength MS medium containing 200 ml/l, 0.5 mg/l BA and 0.5 mg/l IAA ± 35 days after culture, C. Regenerated-initial shoots on the similar medium ± 20 days under light incubation, D. Regenerated-initial shoots on the similar medium ± 35 days

under light incubation, E. Improved number of shoots per explant on half-strength MS medium hormone free with full vitamin ± 2.5 months after culture, F. Cluster shootsprepared for proliferation, G. Regenerated-initial shoots ± 20 after culture, H. 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, I. Rooted-shoot performances for acclimatization puspose, J. Immersing plantlets in 1% fungicide and bactericide solution for 3 min before planting them, K. Survival plantlet performance ± 1 month after acclimatization, L. Growth of plantlets ± 3 months after acclimatization. All photos were different results of Winarto *Ruscus* research; Winarto & Setyawati (2014).

shoot, 2.92 cm shoot height and 1.5 roots per subcultured in seven subculture periods. Initial root formation was noted in the third subculture, while reduction of shoot quality was recorded in the sixth subculture indicated by immersing leaf senescence with yellowing leaves. Whereas Jha & Sen (1985) subcultured slicing callus periodically every 12 weeks for two year without reduction of regenerative ability of them. From both studies it was revealed that there were different problems exist in multiplication of explants. From Winarto & Setyawati (2014) reduction of shoot quality occurred due to periodical subcultures, whereas no reduction of regenerative capacity of callus was not followed by regeneration shoot information of them (Jha & Sen 1985). Rooting of shoots was not be induced on individual shoots detached from the calli. To efficient root formation, shoot rooting was carried out by culturing 2-4 shoots on MS medium hormone free (Jha & Sen 1985). Increasing root formation was established on half-strength MS medium containing 2 mg/l IBA resulting 100% shoots rooted successfully. While half-strength MS medium hormone free with full vitamin stimulated 2.7 roots per shoot cluster with 1.53 cm root length (Winarto & Setyawati 2014).

Transferring plantlets to *ex vitro* condition was successfully done starting from washing plantlets under tap water, treating 1% pesticide solution, culturing on burned-rice husk in plastic pot watered sufficiently, covering transparent plastic and placing under reduced-light intensity area in glasshouse (Winarto & Setyawati 2014). Under the process survivability of plantlets were 73-93%. While successful acclimatization of plantlets up to 80% was recorded after a month of hardening of plantlets under day light and day temperature (28-32°C) (Jha & Sen 1985).

CONCLUSIONS AND FUTURE PERSPECTIVES

From these review it is clear revealled that complete and comprehensive *in vitro* mass propagation studies of *Ruscus* were still limited based on rhizomes and young stems or nodes as explant sources. Based on rhizomes, *Ruscus* of micropropagation was established on *R. hypophyllumvia* callus initiation, shoot multiplication, elongation and plantlet preparation and plantlet acclimatization (Purwito et al. 2005); *R. aculeatus* from callus initiation, shoot regeneration, rooted shoots and acclimatization of plantlets (Moyano et al. 20061, Banciu & Aiftimie-Păunescu 2012).Under young stem or node explants there were *in vitro* propagation of *R. hypophyllum* via indirect organogenesis with callus initiation and regeneration, shoot proliferation, rooting shoots and plantlet acclimatization (Jha & Sen 1985; Winarto & Setyawati 2014).

For the future, development of reliable *in vitro* mass propagation of *Ruscus* to prepare and fulfill good quality planting materials for commercial purposes shall more pay attention to type of explant used, minimize contamination problem, prolong high quality proliferated-shoots under periodical subculture. Selecting young stem/node as explant sources prefer than rhizome. Though MS medium usually used in *Ruscus in vitro* culture, different medium salts can be tested to explore and obtain new information. New combination and concentration of BA/BAP and IAA can be diversified with other hormones and additives to develop new route of *in vitro* propagation protocol. A reliable of *in vitro* propagation *R. hypophyllum* established can be applied to develop new micropropagation protocol for other *Ruscus*.

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