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In vitro plant regeneration from hypocotyl of Arben (*Rubus fraxinifolius* Poir.)

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Abstract

Rubus fraxinifolius, a group of wild raspberry, was improved as a fresh fruit in Indonesia. The purpose of this study was to develop a protocol for micropropagation of *R. fraxinifolius*. Cultures were subcultured from hypocotyls (1 cm) which is initiated from seeds. The experiments were focused on the effect of plant growth regulators concentrations and combinations. The highest germination by *in vitro* was obtained using MS medium supplemented with 10 mg/L of GA_3. Furthermore, based on five parameters, i.e. callus height, callus diameter, number of leaves, plantlet height and number of roots, medium 3 (0.5 mg/L GA_3, 2.5 mg/L 2-iP and 0.1 mg/L NAA) shown the best results during the development explants of *R. fraxinifolius*. Effectivity of *R. fraxinifolius* regeneration through *in vitro* was affected by 2-iP and NAA concentration. If both of plant growth regulators were high (medium 8) or low (medium 1), it made less growth.

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Keywords: *Rubus fraxinifolius*, *in vitro*, hypocotyl, 2-iP, NAA, GA_3.
Abbreviations: 2-Ip_N^6-(2-Isopentenyl) adenine; GA3_Gibberelic acid; MS_Murashige & Skoog Medium; NAA_Naphtaleine acetic acid.

Introduction

Rubus is one of the most diverse plant genera of Rosaceae, consist of 12 subgenera and large number of species (Jennings et al. 1990). *Rubus fraxinifolius*, distributed in Malesia region from Borneo, Java, Philippines, Celebes, Lesser Sunda Island, Moluccas, New Guinea, Solomon Islands and Bismarck Archipelago (Kalkman1993), is group of wild raspberry. Among local people in Cibodas (West Java), this wild raspberry is known as arben and they already sell it as fresh fruit to the visitor in Cibodas Botanical Gardens since many years ago (Fig. 1).

An efficient cost and reliable method for producing a large number of plantlets is needed for commercialization purposes. Currently, the propagation of *R. fraxinifolius* through seedling that growing surround the garden. Moreover, the propagation method by seed takes a long time, as reported by Surya (2012) that within 12 weeks after sowing, the seedling's height of *R. fraxinifolius* range between 0.96 – 3.56 cm. In the other hand, the germination of *R. fraxinifolius* using *in vitro* method was faster than the conventional method (Surya et al. 2015).

Regeneration of adventitious shoots of Rubus spp. from different explants has been reported for leaves (Fiola et al. 1990, Swartz et al. 1990, Owens y de Novoa and Conner 1992), petioles (Cousineau and Donnelly 1991), leaf discs and internodal stem segments (McNicol and Graham 1990), cotyledons (Fiola et al. 1990, Gingas and Stokes 1993) and mature embryos (Fiola and Swartz 1986). Due to the lack of tissue culture information on *in vitro* regeneration of *R. fraxinifolius*, explants with different medium growth were used to determine the propagation methods for *in vitro* multiplication. The objective of this study was to develop a simple and efficient protocol for micropropagation of *R. fraxinifolius* using seeds and seedling parts as explants.

Results and Discussion

As it can be observed from Table 1, the highest germination was achieved when seeds were culture on MS medium supplemented with 10 mg/L GA_3 (95%) followed by MS medium supplemented with 20 mg/L GA_3 (92.50%) and MS 0 (91.25%). Moreover, the average number of germination seed was also observed four times i.e. 14, 18, 22, and 26 days after planting. The results show that the highest number of germination was MS medium supplemented with 10 mg/L GA_3. The addition of GA_3 was found more effective to increase seeds germination compare to MS with lack of GA_3 (MS 0). This result is similar to other studies on several species that the application of GA_3 is an effective method to promote germination and to increase the germination capability (Perez-Garcia and Duran 1990, Upreti and Dhar 1996, Khan and Ungar 1998, Hernandez-Verdugo et al., 2001, Miransari and Smith 2009, Prakash et al., 2011). Furthermore, Akhtar et al., (2008) reported that the addition of GA_3 were able to increase the potential embryo growth and endosperm weakening, cell elongation, cell division, and to promote growth and development of many plant species.

In this experiment, we used MS modification by 2-iP, NAA, and GA_3. Based on statistical analysis, the treatment of initiation medium and growth medium on *in vitro* propagation of *R. fraxinifolius* shows that a few parameters observed were significant on 5% and 1% level (Table 2). The

Table 1. Rate of seed germinationof *Rubus fraxinifolius.*

Germination times/days	Mean number of germinated seed			Germination rate(%)		
	MS 0	MS +GA_3 (10 mg/L)	MS + GA_3 (20 mg/L)	MS 0	MS + GA_3 (10 mg/L)	MS + GA_3 (20 mg/L)
14	2.25	3.25	3	11.25	16.25	15
18	13	13	11.5	65	65	57.5
22	15.75	18	16.5	83.75	90	82.5
26	16.75	19	18.5	91.25	95	92.50

Fig 1. *Rubus fraxinifolius* (a) plant, (b) flower and (c) fruits.

effect of initiation medium was significant to callus height on 8 and 12 weeks after subculture, callus diameter on 8 weeks after subculture, and number of roots on 4 weeks after subculture. Moreover, the effect of growth medium was significant to callus height on 12 weeks after subculture, number of leaves on 12 weeks after subculture, plant height on 12 weeks after subculture, and number of roots on 4, 8 and 12 weeks after subculture. Furthermore, a significant interaction between initiation and growth medium showed on three parameters i.e. callus height on 12 weeks after subculture, callus diameter on 8 weeks after subculture, and number of roots on 4 weeks after subculture. In the other hands, our experiment have not given the best formulation for medium with combination of plant growth regulators and the concentration to produce the number of buds.

Callus is the massive growth of cells and accumulation of callose associated with wounding (Ikeuchi et al., 2013). The composition of the medium employed for initiation of callus tissue is one of the key factors that induce callus formation in *in vitro* culture. A majority of explants type require addition of plant growth regulators into culture medium to initiate callus formation (Dziadczyk et al., 2013). Moreover, Brown (1990) reported that auxins; IAA, 2,4-D or NAA and cytokinins; kinetin, BAP or zeatin in various combination have most often been applied. In our experiment, we used eight combinations of plant growth regulator added to basal Murashige and Skoog medium with vitamins in order to obtain *Rubus* callus. The results show that MS combined with 0.5 mg/L GA_3, 1.5 mg/L 2-iP and 0.1 or 0.5 mg/L NAA was the best medium to produce callus. Those modified MS medium referred to medium 1 and medium 2. Morante-Alarcònetet et al., (2014) reported that hypocotyls explants were the most responsive in term of friable callus induction by auxins or cytokinin.

On the *in vitro* propagation of *R. fraxinifolius*, the highest number of leaves were obtained in medium 3 and medium 7 which are MS combined with 0.5 or 1.0 mg/L GA_3, 2.5 mg/L 2-iP and 0.1 mg/L NAA (Table 3). Our results show that the production of leaves does not affected by GA_3 concentration, but 2-iP (Cytokinins) and NAA (Auxin). The higher concentration of cytokinin (2.5 mg/L) and lower concentration of auxin (0.1 mg/L) affected to leaves number. Najaf-Abadi and Hamidoghli (2009) reported that in tissue cultures (as well as in intact plants and plant organs), cytokinin appear to be necessary for plant cell division. Cytokinins are very effective in promoting direct or indirect shoot initiation. To encourage the growth of axillary bud and reduce apical dominance in shoot cultures, one or more cytokinis are usually incorporated into the medium at proliferation stage (Machakova et al., 2008).

Plantlet height is one of the effects of growth medium during the development explants of *R. fraxinifolius* (Table 3). Our experiment show that the highest plantlet was found in medium 3, which is MS combined with 0.5 mg/L GA_3, 2.5 mg/L 2-iP and 0.1 mg/L NAA. Sigarroa-Rieche and Garcia-Delgado (2011) reported that the greatest elongation of *Rubus glaucus* was due to the presence GA_3 in the medium and Jadan et al., (2015) also reported that the combination concentration of cytokinin and auxin gave higher number of internodes and shoot elongation of *R. glacus*. Physiologically, gibberellins affect cell elongation, mainly in internodes and increase the production of endogenous auxin. Gaspar et al., (1996) reported that some GA effects are caused by increase or decrease in the biosynthesis and activity of specific enzymes. When GAs is added to plant tissue culture media, they often diminish or prevent the formation of roots, shoots, or somatic embryos, although the opposite has also been seen. In the other hands, GA_3 promotes the petioles elongation of *Rubus chamaemorus* but an addition of GA_3 reduced the number of shoots per explant (Martinussen et al., 2004).

The highest roots numbers were obtained in medium 2 (4.333b) and medium 6 (4.500b). Both of medium had similar combination in having a highest concentration of auxin (0.5 mg/L) and lower concentration of cytokinin (1.5 mg/L), but different concentration of GA_3. Auxin exert a strong influence over processes such as cell growth expansion, cell wall acidification, initiation of cell division, and organization of meristems giving rise to defined organs. In organized tissue, auxins appear to be key players in maintaining apical dominance, affecting abscission, and

Table 2. Significances of initiation medium, growth medium and interaction on *in vitro* propagation of *Rubus fraxinifolius.*

No	Parameters	Initiation Medium (IM)	Growth Medium (GM)	IM x GM
1.	Callus Height (mm)			
	a. 4 week after planting	ns	ns	ns
	b. 8 week after planting	**	ns	ns
	c. 12 week after planting	**	*	*
2.	Callus Diameter (mm)			
	a. 4 week after planting	ns	ns	ns
	b. 8 week after planting	**	ns	*
	c. 12 week after planting	ns	ns	ns
3.	Number of Buds			
	a. 4 week after planting	ns	ns	ns
	b. 8 week after planting	ns	ns	ns
	c. 12 week after planting	ns	ns	ns
4.	Number of Leaves			
	a. 4 week after planting	ns	ns	ns
	b. 8 week after planting	ns	ns	ns
	c. 12 week after planting	ns	**	ns
5.	Plantlet Height (mm)			
	a. 4 week after planting	ns	ns	ns
	b. 8 week after planting	ns	ns	ns
	c. 12 week after planting	ns	**	ns
6.	Number of Roots			
	a. 4 week after planting	**	**	**
	b. 8 week after planting	ns	*	ns
	c. 12 week after planting	ns	**	ns

Remarks: ns = not significant; * = significant at p<0.05; ** significant at p<0.01.

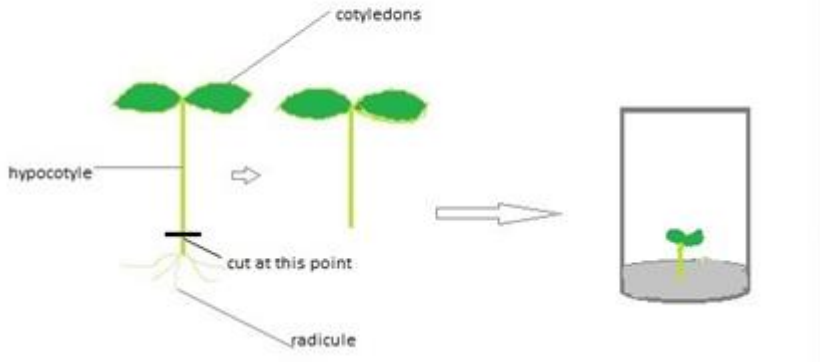

Fig 2. An overview of *in vitro* regeneration.

promoting root formation (Gaspar et al., 1996, Martinussen et al., 2004).

In general, medium 3 (0.5 mg/L GA_3, 2.5 mg/L 2-iP and 0.1 mg/L NAA) show the best results during the explants development of *R. fraxinifolius* (Fig 3). Effectivity of *R. fraxinifolius* regeneration through *in vitro* was affected by concentration of 2-iP and NAA. If both plant growth regulators were high (medium 8) or low (medium 1) in concentration, it will result poor growth on *R. fraxinifolius* (Table 4). The effect of plant growth regulators are rarely specific in their ultimate influence on growth and development, and the responses of cells, tissue, and organs *in vitro* may vary with culture condition, type of explant and genotype (Gaspar et al., 1996). During the induction of callus growth, auxin promotes cell dispersion in suspension culture, while cytokinins tend to cause cell aggregation. The relatively high levels of auxin added to liquid media to obtain dispersion will prevent morphogenesis, but might induce embryogenesis if the cells are still competent. Whereas cytokinins tend to promote the formation of chlorophyll in callus and suspension cultures, auxins can be an inhibitor. An auxin is almost invariably required to promote the initial growth of meristem and shoot tip explants. A low concentration of auxin is often beneficial in conjunction with high level of cytokinin when organ culture such as shoot multiplication is required, although in some case cytokinin alone is sufficient (Machakova et al., 2008).

Materials and Methods

Plant material

The experiment was conducted in the Laboratory of Cibodas Botanic Gardens. The seeds of *Rubus fraxinifolius* which

Table 3. Effect of growth medium on the *in vitro* propagation of *R. fraxinifolius* on 12 weeks after planting.

Medium	Callus Height (cm)	Callus Diameter (cm)	Number of Leaves	Plantlet Height (cm)	Number of Roots
Medium 1	0.88±0.08b	1.30±0.12	3.60±1.69ab	0.46±0.26ab	1.20±0.58a
Medium 2	0.79±0.12b	1.52±0.08	6.22±1.54bc	1.47±0.43bc	4.33±1.26b
Medium 3	0.59±0.07a	1.30±0.09	7.85±1.11c	2.28±0.58c	2.61±0.47ab
Medium 4	0.66±0.09ab	1.48±0.09	2.46±0.87a	0.60±0.27ab	2.31±0.67ab
Medium 5	0.60±0.12a	1.25±0.19	3.17±0.98ab	0.48±0.21ab	1.17±0.83a
Medium 6	0.64±0.06ab	1.39±0.13	4.60±1.15bc	1.36±0.33bc	4.50±1.51b
Medium 7	0.81±0.05b	1.22±0.10	7.90±2.68c	1.54±0.47bc	0.70±0.30a
Medium 8	0.64±0.05ab	1.24±0.08	0.27±0.27a	0.03±0.03a	0.82±0.35a

*Values represent means±SE. Means followed by the same letter within columns are not significantly different by LSD test at the 5% probability level.

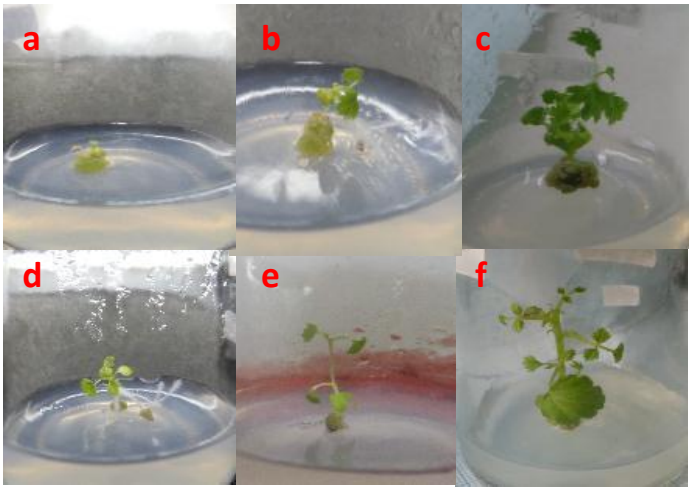

Fig 3. *R. fraxinifolius* growth on (a) medium 1, (b) medium 2, (c) medium 3, (d) medium 4, (e) medium 6 and (f) medium 7. Medium 1, 2, 4 and 6 were observed within 4 weeks after planting. Medium 3 was observed within 9 weeks after planting. Medium 7 was observed within7 weeks after planting.

Table 4. Eight medium for in vitro propagation on *Rubus fraxinifolius.*

Name of medium	Basal medium	Concentrations of growth regulators		
		GA_3 (mg/L)	2-iP (mg/L)	NAA (mg/L)
Medium 1	MS + Vit.	0.5	1.5	0.1
Medium 2	MS + Vit.	0.5	1.5	0.5
Medium 3	MS + Vit.	0.5	2.5	0.1
Medium 4	MS + Vit.	0.5	2.5	0.5
Medium 5	MS + Vit.	1.0	1.5	0.1
Medium 6	MS + Vit.	1.0	1.5	0.5
Medium 7	MS + Vit.	1.0	2.5	0.1
Medium 8	MS + Vit.	1.0	2.5	0.5

were used for the experiments were collected from Situgunung Resort, Mount Gede Pangrango National Park.

Seed germination

Rubus seeds from mature fruits were first washed under running tap water, continued with detergent solution for 5 min to remove pulp, then soaked for 15 min Tween 80. In order to minimize fungal and bacteria contamination, seeds were treated with solution of fungicide (Benlox) and bactericide (Agrept) for 20 min by gentle shaking. Final steps of seeds surface sterilization were transferred to 70% ethanol for 1 min and followed by NaOCl solution (Sunklin® 20%) for 15 min. Each treatment was followed by repeated washings for a minimum of 3 times in sterile distilled water. The seeds were cultured in bottle containing 20 mL of MS medium (Murashige and Skoog 1962), consisted of different concentrations of GA_3 (10, 20 mg/L) which were added to the MS medium. The medium was also enriched with 30 g/L sucrose and solidified with 8 g/L agar. The pH was adjusted to 5.7 before autoclaving at 121ºC and 1 atm for 20 min. The experiments were repeated four times, each repetition comprising 20 seeds per bottles. The cultures were incubated under controlled conditions at 25 ±2ºC, 16:8 (light: dark). After 14, 18, 22, and 26 days of incubation, the number and percentage of seed germination was recorded.

Callus and shoot initiation

For callus and shoot initiation/organogenesis (multiplication), The seedling radicle was excised, explants consisting of 1 cm long hypocotyl with cotyledon (Fig. 2) were inoculated vertically on 8 different shoot/callus induction medium. MS medium containing 30 g/L sucrose and solidified with 8 g/L agar and supplemented with different concentrations of growth regulators 2-iP, GA_3 and NAA (Table 4). The pH of

the media was adjusted to 5.7. After 4, 8, and 12 weeks, the observations were made on number of shoot, leaves, roots, callus diameter, callus height, and plantlet height.

Statistical analysis

Experiments were conducted in completely randomized design (CRD). Each treatment consisted of 10 replications. The data were collected after 4, 8 and 12 weeks of culture incubation and analyzed statistically using one way analysis of variance (ANOVA) and values are expressed as mean±SE Significance of difference between means were tested by LSD ($P<0.05$).

Conclusion

The best medium for *R. fraxinifolius* regeneration was medium 3 and followed by medium 2, 6 and 7. Those auxin, cytokinin, and gibberellin concentration in cultures need to be carefully balanced and controlled. It due to auxin can inhibit cytokinin accumulation, whereas cytokinins can inhibit at least some of action of auxin, and gibberellin may also become an inhibitor or stimulator on the metabolism of auxin and cytokinin in tissue culture.

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