Shoot Multiplication and Rooting Induction in *Carica pubescens* Lenne & K. Koch (Mountain Papaja)

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***Abstract*—An efficient micropropagation protocol in *Carica pubescens* Lenne & K. Koch known as an endemic species at Dieng highland and has a great commercial importance has been developed. In the present study the influence of growth regulator of culture medium on shoot multiplication and rooting induction was investigated. For shoot multiplication, lateral shoot explant was chosen. The research was carried out using a completely randomized factorial design with two factors. Different concentrations of 6-benzylamino purine (BAP) (1.0, 2.0, 3.0 and 4.0 µM) and gibberelic acid-3 (GA3) (0.1, 0.2, 0.3, and 0.4 µM) were added to Murashige and Skoog (MS) medium. The best shoot multiplication was showed on MS medium containing 2.0 µM of BAP and 0.3 µM of GA3. After 3 months of culture shoot initiation has been observed in all explants with 5.3 number of shoot per explant, and 3.9 cm shoot height. Further shoot multiplication in successive subcultures was also possible. For rooting induction, the research was conducted using a completely randomized design with one factor. Medium MS supplemented with 2 ppm (9.8 µM) of 3-indolebutyric acid (IBA) proved to be the best one for rooting of shoots, producing root initiation at 16 days after rooting, 4.50 number of roots per shoot and 7.57 cm root length. This protocol opens up the prospect of *in vitro* conservation of this endemic species.**

***Keywords*—*Carica pubescens*, Root Induction, Shoot multiplication,**

I. INTRODUCTION

Carica pubescens Lenne & K. Koch (syn. *Vasconcellea pubescens*) belongs to the Caricaceae. Currently, only about 33 thousands of *C. pubescens* plants were cultivated in Dieng (private communication with district government of Wonosobo, Central of Java, 2011), mainly concentrated at altitude of 2400 m above sea level like Sembungan and Sikunang village. These villages are few areas where *C. pubescens* could grow well and bears fruit optimally with a high antioxidant capacity [1]. *C. pubescens* possesses great economic potential due to their benefits as food and medicine materials. Its habitus is similar to *C. papaya* but the fruits are smaller than it (Fig. 1a) and have a strong characteristic flavor. The fruit mainly used to produce preserved fruit. An additional feature of this species is its ability to produce latex with a high level of papain, an important and valuable proteolytic enzyme in industrial and pharmaceutical [2].

Because of the economic potential and associated with its extensive usability, germplasm preservation is important strategy to guarantee not only the conservation, but also their sustainable use. *In vitro* conservation needs a large quantity of
shoots which can be induced to form roots.

This study aims to develop *in vitro* shoot multiplication and root induction protocol of *C. pubescens*. The multiplication of shoot and induction of root are greatly influenced by plant growth regulators content in the medium. Increase in volume associated with shoot multiplication and adventitious root growth must be the result of either cell division or cell enlargement, or both. Cytokinin was believed as division cell regulator and auxin known to be involved in cell enlargement. The requirement of type and concentration of each of plant growth regulator varies with different species [3]. Previous study showed that addition of auxin and cytokinin into Murashige and Skoog (MS) (1962) medium was not effective in shoot multiplication of *C. pubescens* (data no shown). Therefore, this study would examine the use of gibberellins (especially gibberelic acid-3 or GA3), and cytokinin (especially 6-benzylamino purine or BAP) in inducing shoot multiplication. The roles of GA3 related to photomorphogenesis are in stem elongation and leaf differentiation [3].

Auxin has been recognized as a controlling factor in rooting induction since a long time [4]. Of the several types of synthetic auxin, indole butyric acid (IBA) was more effective for producing roots of woody plants than the others [5].

II. MATERIALS AND METHODS

Shoot multiplication

The objectives of this step study were to examine the effects of BAP and GA3 supplementing into MS medium on shoot multiplication of *C. pubescens*and and to determine the concentration of BAP and GA3 capable of proliferating shoot maximally.

Laterally shoots of 2 to 3 cm long of stem branch (diameter 1 cm) were obtained from selected plants 5 years old tree growing in Sikunang village, Dieng. These trees were selected on the basis of their healthy, number and characteristics of fruits. The shoot explants were washed with soft detergent and tap water, followed by 3-4 rinses with distilled water. To prevent browning, the explants were soaked in 150 ppm ascorbic acid solution, and then soaked in 70% alcohol for 3 minutes. The surface disinfectant of explants was accomplished by dipping explants in 6% (v/v) solution of commercial bleach for 15-20 minutes with 0.1% tween 20. Afterwards, the explants were rinsed five times with autoclaved distilled water under a laminar air flow (LAF) cabinet which had been sterilized by UV irradiation for 30 minutes and spraying 70% alcohol. Before planting in the multiplication medium, sterile explants were grown on MS medium without growth regulators to control contamination for 4 days, and then transferred to a basal medium, that was MS medium supplemented with 2µM BAP, 0.5µM IBA and0.3µMGA3.

Effects of adding concentration of BAP and GA3 in the MS medium on growth and multiplication of *C. pubescens* shoot were carried out in a completely randomized factorial design experiment with two factors, that were concentration of BAP and GA3. Each of the experimental unit consisted of two shoots, cultured in one culture vial (Fig. 1b). Four replicates were prepared for each combination of the BAP and GA3 concentration.

The explants were, aseptically, placed on agar-solidified MS medium containing 3% sucrose varied in plant growth regulators combinations. MS medium were supplemented with a combination of BAP and GA3 at concentrations of 1.0, 2.0, 3.0, and 4.0 µM and 0.1, 0.2, 0.3 and 0.4 µM, respectively. All media were adjusted to pH 5.8 at 25°C with KOH and HCl and were autoclaved for 20 minutes at 121°C (15 PSI nominal steam pressure) after the addition of growth regulators as required. As much as 25 ml medium was poured into each of the culture vial. The cultures were incubated in a 24 h light at 15 ± 2°C and were illuminated with fluorescent light at 1000 lux. The explants were cultured in each for a total of three months period, and were transferred onto fresh medium every month.

Shoot multiplication responses recorded were percentage of explants growing shoots, number of axillary shoots formed from explant, shoot length (cm), and number of leaves per shoot. Data were statistically analyzed in a completely randomized design with four replicates. The data were analyzed by Analysis of Variance and Duncan's multiple ranges test (DMRT) using SAS statistical analysis program System for Windows 9.0.

Root Induction

The aims of this second step study were to examine the effect of IBA supplementing into MS medium on root induction of *C. pubescens* and to determine IBA concentration capable of inducing root maximally.

Effects of IBA supplementing into MS medium on root induction was carried out in a completely randomized design experiment with one factor, that was concentration of IBA. Each of the experimental unit consisted of one shoot, cultured in one culture vial. Eight replicates were prepared for each of the IBA concentration. To induce roots from the selected shoots, all regenerated shoots (2-3 cm in height and with 1-2 leaves) were selected and cultured on MS medium supplemented with 2 ppm, 4 ppm, 8 ppm IBA for 1 week. After cultured on root induction on MS-IBA medium, the shoots were transferred onto MS medium added by active charcoal until they develop into plantlets.

Root induction responses recorded were time of root emergence, root length (cm), and root number. Data were statistically analyzed in a completely randomized design with four replicates. The data were analyzed by Analysis of Variance and DMRT using SAS statistical analysis program System for Windows 9.0

III. RESULTS AND DISCUSSION

Shoot multiplication

BAP concentrations in MS medium greatly influenced multiplication of *C. pubescens* shoot explants. This cytokinin type at 2-4 µM concentration resulted in good responses of explants for shoot induction (87.5-100%). The maximum shoot number per explant, shoot length and leaf number per shoot were observed at 2 µM BAP. The medium supplemented with BAP more than 2 uM inhibited shoot multiplications (Tab. 1). The inhibition of shoot multiplication was in line to the result obtained in *Phaseolus angularis* that its bud growth and shoot multiplication were stimulated by reducing the BAP concentrations from 5.0 to 2.5 µM after 3 weeks [6].

Cytokinins have been proved to overcome apical dominance, release lateral buds from dormancy and promote shoot formation [3]. Stimulation of multiple shoot formation by BAP has been reported in several plant species, including *Ocimum basilicum* [7], *Galax urceolata* [8], and *Aloe vera* [9].

TABLE 1
EFFECT OF PLANT GROWTH REGULATORS (BAP AND GA3 ALONE) ADDED INTO MS MEDIUM ON SHOOT MULTIPLICATION OF *C. pubescens*

PGR	Percentage of explants growing shoots (%)	Shoots number/ explant	Shoot length (cm)	Leaves number / shoot
B1	62.5 b ± 21.6	1.32bc ± 0.56	3.05 b ±0.55	2.4 c ±0.44
B2	100.0 a ± 0	2.32 a ± 1.67	3.42 a ±0.39	3.8 a ±1.35
B3	87.5 a ± 21.6	1.57 b ± 0.57	2.90 bc ±0.43	2.0 d ±0.64
B2	87.5 a ± 21.6	1.57 b ± 0.57	2.82 bc ±0.37	2.1 d ±0.54
G1	87.5 a ± 21.6	1.50 b ± 0.53	2.55 c ±0.45	2.0 d ±0.17
G2	87.5 a ± 21.6	1.65 b ± 0.52	3.07 b ±0.13	2.6 c ±0.21
G3	100.0 a ± 0	2.15 a ± 0.53	3.07 b ±0.52	2.9 b ±1.34
G4	62.5 b ±21.6	1.50 b ± 0.84	2.90 bc ±0.51	2.5 c ±1.51

- Data represent mean ± standard error of two explants per treatment in four replicated experiments. Means within a single column

followed by the same letter are not significantly different according to DMRT at p = 0.05

- PGR (plant growth regulators) treatment: B1, B2, B3 and B4: concentration of BAP 1 µM, 2 µM, 3 µM and 4 µM; G1, G2, G3 and G4: concentration of GA3 0.1 µM, 0.2 µM, 0.3 µM and 0.4 µM respectively

Supplemented of GA3 into MS medium had also significant effect on shoot multiplication of *C. pubescens*. The GA3 at 0.1-0.3µM concentration resulted in good response of explants for shoot multiplication (87.5-100%). The maximum shoots number per explant and leaves number per shoot were observed at 0.3 µM GA3 (Tab. 1). Gibberelins have showed a broad spectrum of physiological effect in plants. Because of their role in cell wall formation and cell expansion they involved in dormancy breakage [10], lateral branching and young leaf growth [11]. This result was in accord with finding from previous study on *Lotus corniculatus* L. [12].

The lower and higher GA3 concentrations than 0.3 µM in MS medium were ineffective to induce shoot multiplication (Tab. 1). As a phytohormone, GA3 promotes the growth optimally at a relatively low concentration, so at below and above of the optimum concentration, the growth will be inhibited [3].

Of the 16 combinations of BAP and GA3 tested, the combination of BAP 2 µM + GA3 0.3 µM and BAP 2 µM + GA3 0.4 µM-treated explants achieved highest regeneration than those other combinations. The two combinations of treatments yielded maximum regeneration (100%) and maximum number of multiple shoots (3.0-5.3 shoots per explant). Shoots developed in this medium also were longest (3.6-3.9 cm) and generated maximum number of leaves (4.7- 5.3 (Tab. 2, Fig. 1c).

TABLE 2
EFFECT OF GROWTH REGULATORS (BAP AND GA3 COMBINATION) ADDED INTO MS MEDIUM ON SHOOT MULTIPLICATION OF *C. pubescens*

PGR	Growing shoot (%)	Shoot number / explant	Shoot length (cm)	Leaves number
B1G1	50 b ± 17.7	1.0 c ± 0.71	2.2 c ± 1.18	1.7 c ± 0.43
B1G2	50 b ± 0.0	1.0 c ± 0.71	3.0ab ± 0.37	2.3bc ± 0.43
B1G3	100a ± 0.0	2.3 b ± 0.83	3.3 a ± 0.39	2.3bc ± 0.83
B1G4	50 a ± 17.7	1.0 c ± 0.00	3.7 a ± 0.36	3.0 b ± 0.71
B2G1	100a ± 0.0	1.7 b ± 0.43	3.0ab ± 0.35	2.0 c ± 0.71
B2G2	100a ± 0.0	1.3 c ± 0.43	3.0ab ± 0.46	2.7 b ± 0.43
B2G3	**100a** ± 0.0	**5.3 a** ± 0.43	**3.9 a** ± 0.15	**5.3 a** ± 0.83
B2G4	**100a** ± 0.0	**3.0 a** ± 0.71	**3.6 a** ± 0.28	**4.7 a** ± 0.83
B3G1	100a ± 0.0	1.0 c ± 0.00	2.0 c ± 0.21	2.0 c ± 0.71
B3G2	100a ± 0.0	2.3 b ± 1.09	3.0ab ± 0.41	2.7 b ± 0.69
B3G3	100a ± 0.0	2.0 b ± 0.71	2.6 b ± 0.46	2.3bc ± 0.83
B3G4	50 b ± 17.7	1.0 c ± 0.00	2.0 c ± 0.82	1.0 d ± 0.71
B4G1	100a ± 0.0	2.3 b ± 0.43	3.0ab ± 0.62	2.3bc ± 0.83
B4G2	100a ± 0.0	2.0 b ± 0.71	3.3 a ± 0.46	2.7 b ± 0.83
B4G3	100a ± 0.0	1.0 c ± 0.00	2.7 b ± 0.45	2.0 c ± 0.71
B4G4	50 b ± 17.7	1.0 c ± 0.00	2.3 c ± 0.38	1.3cd ± 0.43

- Data represent mean ± standard error of two explants per treatment in four replicated experiments. Means within a single column followed by the same letter are not significantly different according to DMRT at p= 0.05
- PGR (plant growth regulators) treatment: B1, B2, B3 and B4: concentration of BAP 1 µM, 2 µM, 3 µM and 4 µM; G1, G2, G3 and G4: concentration of GA3 0.1 µM, 0.2 µM, 0.3 µM and 0.4 µM respectively

Fig.1 The habitus of *Carica pubescens* and *in vitro* shoot multiplication. (a) A *C. pubescens* plant grown at Dieng. Bar = 15 cm (b) Two explants after cultured on multiplication medium for 1 month. Bar = 0.7 cm. (c) High rate of shoot multiplication on MS medium supplemented with BAP 2 µM + GA3 0,3 µM after cultured 3 months. Bar = 0.5 cm.

Previous finding have also indicated the role of BAP and GA3 in *Rosa* shoot multiplication. The highest multiplication rate of *R. canina* and *R. rubiginosa* (4.1 shoots per one explant) and *R. dumalis* (2.9 shoots per one explant) was obtained when shoots were multiplied on an MS medium supplemented with 1 µM BA and 1.5 µM GA3 [13].

Although it was difficult to quantify healthy of shoot but we have observed that shoots obtained from explant cultured in MS medium supplemented with the optimally concentrations of cytokinin and gibberelin (BAP 2 µM + GA3 0.3 µM or BAP 2 µM + GA3 0.4 µM) were generally healthier (better vigor) than those obtained from the other concentrations. This indicates that multiplication response depends upon the type and concentration of plant growth regulator used.

Root Induction

Elongated shoots (3–4 cm) were excised and placed on full-strength MS medium supplemented with various concentrations of IBA for induction of roots. These media gave developed roots within 16–30 days. Application of IBA at 2 ppm resulted fastest rooting than the higher concentration (Tab. 3). Supplementing of IBA at 2 ppm into MS medium might have triggered the early anticlinal cell division and root primordia formation than higher concentration [14].

TABLE 3
EFFECT OF IBA CONCENTRATIONS IN MS MEDIUM ON ROOTING INDUCTION OF *C. pubescens*

IBA concen-tration (ppm)	Root emergence (days)	Root induction (%)	Root number	Root length (cm)
2	16.75 a ± 1.71	100 ± 0	4.50 a ± 0.93	7.57 a ± 1.43
4	24.88 b ± 0.92	100 ± 0	2.25 b ± 0.43	2.18 b ± 0.57
8	29.75 c ± 0.85	100 ± 0	1.50 b ± 0.50	1.27 b ± 0.36

Data represent mean ± standard error of one explant per treatment in eight replicated experiments. Means within a single column followed by the same letter are not significantly different according to DMRT at p= 0.05

All of the shoot explants capable to generate roots at a high frequency (100%) in MS medium containing 2, 4

and 8 ppm IBA, but in medium containing 2 ppm IBA the number of roots was highest (4.50) The greater IBA concentration, the lower number of roots (Tab. 3, Fig. 2). The result showed that auxin is essential to induce rooting in the *C. pubescens* microcutting as no rooting was observed in the absence of IBA (data not shown).

IBA produced the longest roots (7.57 cm) also at concentration of 2 ppm (Tab. 3, Fig. 2). The results also revealed that root length tend to reduce with higher than optimum concentration of IBA. Like other developmental processes, root cell elongation involves sequential changes in levels and/or activity of enzymes. The enzymes involved in cell enlargement processes are triggered by the auxin [3].

Indole-3-acetic acid (IAA) is the most abundant naturally or endogenous auxin and IBA is classified as a synthetic auxin [3]. Process of root formation in cuttings involves the activity of peroxidase, IAA oxidase, and phenolics [15]. Exogenous IBA induce changes in activities of peroxidase and IAA oxidase and in contents of phenolics allowing the establishment of the favourable endogenous hormone balance. The metabolism of the exogenous IBA, especially its combination with phenolic compounds, has been considered in relation to the promotion of adventitious rooting [16].

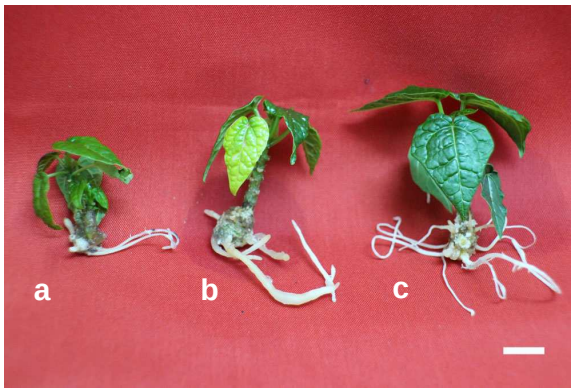

Fig 2. Formation of roots of shoots cultured in MS medium supplemented with (a) 8 ppm, (b) 4 ppm, and (c) 2 ppm IBA. Bar = 1 cm

Compared to stems, roots may require a less concentration of auxin to grow [17]. Therefore root growth is strongly inhibited by higher level of auxin because at high level, it induces the production of ethylene, a root growth inhibitor [18], and induces the higher level of degradative metabolites in tissues which blocking the regeneration process [19]. Besides these, specific exogenous IBA to endogenous IAA ratio may be important for plant development [20], and the application of exogenous IBA of 2 ppm might shift the balance to promote root development of *C. pubescens*.

The stimulatory effect of IBA of root formation has been reported in many other plant species. Exogenous IBA had a significant positive effect on the rooting responses of woody plants such as date palm [21], apple [22], and olive cultivar 'Moraiolo' [23].

IV. CONCLUSION

Supplementing BAP and GA3 into MS medium proved to be suitable plant growth regulators for multiplication of *C. pubescens*. The best shoot multiplication of *C. pubescens* was achieved in MS medium containing 2.0 µM of BAP and 0.3 µM of GA3. IBA 2 ppm were also proved to be the best rooting hormone in term of root emergence rate, root number and root length. This protocol can be used to regenerate a large number of rooting shoots as materials of *C. pubescens in vitro* conservation.

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