pISSN 2302-1616, eISSN 2580-2909
Vol 11, No. 1, June 2023, pp. 1-13
Available online http://journal.uin-alauddin.ac.id/index.php/biogenesis
DOI https://doi.org/10.24252/bio.v11i1. 32129

Modification of media compositions for micropropagation of *Acorus calamus* L.

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ABSTRACT. Modification of medium composition is a critical step in tissue culture to find out the best medium formulation for micropropagation. Furthermore, reduction of some nutrients as well as sugar and plant growth regulator concentrations are an effort often done to make the production of seedlings more economic. The aim of the study was to modify media composition by reduction of macronutrients and sugar concentrations in combination with BAP for micropropagation of *Acorus calamus*. Experiments of Calamus micropropagation was established by culturing single shoots to MS medium with 100, 50, 33.3 and 25% macronutrients added with sugar at 30, 20, and 10 g/l in combination with 0, 0.5, and 1 mg/l BAP. Each medium treatment had nine replicates. Growth of culture was determined every week until 6 weeks of culture. Growth variable recorded was shoots numbers, leaf length, leaf numbers and root numbers. At six weeks of culture, chlorophyll content was also observed. The results showed that reduction of macronutrients and sugar did not influence shoot and leaf numbers in MS medium but required 0.5-1 mg/l BAP. Rooting is best in medium with no addition of BAP. Chlorophyll content decreased in the medium containing BAP in all reduced macronutrients and sugar concentrations. All plantlets survived in the field. Reduction of macronutrients and sugar can be applied for Calamus shoot culture. Cytokinin BAP is critical for shoot growth but this could be omitted for rooting. Therefore, this finding offered more choices in micropropagation of Calamus with low-cost production.

Keywords: Benzyl amino purine; Calamus (*Acorus calamus* L.); macronutrients; reduction sugar; sweet flag

Article History: Received 7 October 2022; Received in revised form 21 February 2023; Accepted 16 May 2023; Available online 30 June 2023.
How to Cite This Article: Wulansari A, Ermayanti TM, Hafiizh EA, Hapsari BW, Maulana E. 2023. Modification of media compositions for micropropagation of *Acorus calamus* L. *Biogenesis: Jurnal Ilmiah Biologi*. vol 11(1): 1–13. doi: https://doi.org/10.24252/bio.v11i1.32129.

INTRODUCTION

The Calamus or Sweet Flag (*Acorus calamus* L.) is a medicinal plant belonging to the Acoraceae family (Sharma *et al*., 2014). In Indonesia it is known by the local name Jeringau or Dringo. This plant is also called Vacha in Sanskrit (Sharma *et al*., 2020). Acorus is widely used in traditional medicine in Ayurvedic, Indian (Babar *et al*., 2020), China (Shu *et al*., 2018), Iran (Gholipour *et al*., 2021) and Indonesia (Widyastuti *et al*., 2019). One hundred and forty-five compounds have been isolated and identified from this plant, including phenylpropanoids, sesquiterpenoids as well as monoterpenes (Sharma *et al*., 2020). Several studies have reported that extracts and active compounds have the potential to treat metabolic and neurological disorders, as anti-depressant, anti-inflammatory, anti-hypertensive, anti-obesity, neuroprotective, cardioprotective, and immunomodulatory (Nasir, 2021). The essential oil extracted from the rhizomes and roots can also be used as antifungal, antibacterial and insecticide (Khan *et al*., 2016). Calamus grows wild in tropical forests, cultivation has not been widely carried out, so the supply of rhizomes and roots as medicinal raw materials is limited. Its genetic diversity in several ethnic groups spread over 20 different geographical locations in Indonesia shows a high value of similarity index from 76.7 to 100%, therefore, conservation and plant breeding are needed. The fact that plants have a high similarity index are usually susceptible to biotic and abiotic stresses that can cause large population losses (Subositi *et al*., 2015).

Calamus, semi-aquatic, a grass-like monocot plant, is generally propagated through rhizomes (Sharma *et al*., 2014). Propagation through seeds is not possible because this species is triploid plants that rarely flower, thus do not produce seeds (Imam *et al*., 2013). *In vitro* propagation through tissue

culture techniques is an alternative solution to mass produce, disease-free and genetically uniform plants. Explants that are often used in Calamus tissue culture are pieces of rhizome (Verma & Singh, 2012; Khan *et al*., 2016; Babar *et al*., 2020; Gholipour *et al*., 2021). Modification of the composition of the culture media is needed to obtain the best medium for mass micropropagation. Several studies reported that growth regulators from the cytokinin group such as benzyl amino purine (BAP) showed the best for shoot proliferation on Calamus micropropagation with the optimal concentration ranged from 1 to 2 mg/l (Sharma *et al*., 2014; Subramani *et al*., 2014; Quraishi *et al*., 2017; Tikendra *et al*., 2022). Experiments on *in vitro* growth of Calamus shoots on simple fertilizer media have also been carried out, but have not shown optimal growth (Hapsari *et al*., 2019). Reduction of medium composition, macronutrients and sugar concentrations are reported in many plant species in order to increase growth as well as to lower the cost production of plantlets. In *Salix viminalis* cultured in bioreactors, shoots successfully proliferated without sucrose, whereas shoots did not proliferate well if sucrose concentration was 0.5% or lower. More roots were formed when sucrose was added to the medium (Gago *et al*., 2021). Sugar plays important roles in plant growth development such as in apical shoot development (Van den Ende, 2014). Reduction of sugar concentration was also provided on kiwifruit *Actinidia deliciosa* (Gago *et al*., 2014), *Stevia rebaudiana* (Rantau *et al*., 2017), *Dahlia* sp. (Rudiyanto *et al*., 2017) and *Psidium guajava* (Rantau *et al*., 2019).

There has never been any study on altering the composition of *in vitro* media for Calamus micropropagation, therefore, aim of this study was to modify media composition by reduction of macronutrients and sugar concentrations in combination with BAP for micropropagation of *A. calamus*. Modification of media composition is required to provide Calamus plantlets effectively. In addition, this study is considered as a possibility that can reduce production costs efficiently without compromising crop quality, when in some circumstances the unit cost per plant becomes cost-prohibitive.

MATERIALS AND METHODS

Explants used in this research were *in vitro* shoots of Calamus (*Acorus calamus* L.) collection of the Research Group of Cellular Engineering for Plant Metabolite Improvement, Research Center for Genetic Engineering, BRIN. Four-week-old shoots cultured on MS medium (Murashige & Skoog, 1962) without growth regulators were used in this experiment. Cultures were incubated in a culture room at 25-26°C with continuous photoperiod.

Combination treatment. The experiment was performed under a completely randomized design with two factors. The first factor was modification of basic medium compositions which consisted of reduced macronutrients (100, 50, 33.3, and 25%) in combination with sucrose concentrations (10, 20, and 30 g/l), while the second factor was growth regulator concentrations (Benzyl Amino Purine (BAP) at 0; 0.5 and 1 mg/l) (Jirakiattikul *et al*., 2013; Mustafa *et al*., 2013; Chin *et al*., 2021). The total number of combination treatments was 36 combinations. Details of each combination are presented in Table 1. Each treatment had three culture bottles as replicates with each bottle containing 3 explants, therefore, total number of experimental units was 324. All cultures were incubated in a culture room at 25-26°C with continuous photoperiod.

Growth observation. The growth variables observed were shoots numbers, leaf length, leaf numbers and root numbers conducted every week until six weeks of culture. Shoots number was determined by counting the new shoots formed. Leaf length was measured from the root base on the media surface to the tip of the longest leaf. Leaf number and root number were determined by counting the number of whole leaves and roots in each shoot.

Chlorophyll content. Analysis of chlorophyll content was conducted at six weeks of culture according to (Arnon, 1949). Fifty mg of leaf samples were ground in 2 ml of 80% acetone, then centrifuged. The extraction was repeated to obtain the final volume of 10 ml. The extract was put into a cuvette and its absorbance was measured using a spectrophotometer at 645 and 663 nm. Chlorophyll contents was performed by following equation (Koch, 1883):

$$\text{Chlorophyll A} = (0.0127 \times D663 - 0.00269 \times D645)\ df$$
$$\text{Chlorophyll B} = (0.0229 \times D645 - 0.00468 \times D663)\ df$$
$$\text{Total chlorophyll} = \text{Chlorophyll A} + \text{Chlorophyll B}$$
$$df\ (\text{dilution factor}) = d/e \times b/c \times 1/a \times 1000$$

Note:
d = extract volume after crushing
e = convertion from liter to milliliter
b = initial extract volume
c = volume of extract taken from initial extract
a = sample weight
1000 = convertion from gram to milligram

Table 1. Medium code for the experimental design of *Acorus calamus* micropropagation

Basal medium modification		BAP (mg/l)	Medium Code
Macronutrients (%)	Sugar (g/l)		
100	10	0	A.1.1
		0.5	A.1.2
		1	A.1.3
	20	0	A.2.1
		0.5	A.2.2
		1	A.2.3
	30	0	A.3.1
		0.5	A.3.2
		1	A.3.3
50	10	0	B.1.1
		0.5	B.1.2
		1	B.1.3
	20	0	B.2.1
		0.5	B.2.2
		1	B.2.3
	30	0	B.3.1
		0.5	B.3.2
		1	B.3.3
33.3	10	0	C.1.1
		0.5	C.1.2
		1	C.1.3
	20	0	C.2.1
		0.5	C.2.2
		1	C.2.3
	30	0	C.3.1
		0.5	C.3.2
		1	C.3.3
25	10	0	D.1.1
		0.5	D.1.2
		1	D.1.3
	20	0	D.2.1
		0.5	D.2.2
		1	D.2.3
	30	0	D.3.1
		0.5	D.3.2
		1	D.3.3

Acclimatization. Six-week-old plantlets were acclimatized in a greenhouse. The acclimatization medium was a mixture of soil, compost, roasted husk, and sand (1:1:1:1). All plantlets were taken out from the bottle cultures, and then they were cleaned from the remaining culture media with water**.** Plantlets were then planted in plastic pots and covered with transparent plastic for two weeks, then

placed in a greenhouse. After the new leaves grew, the plastic cover was removed. Numbers of survival plantlets were recorded after nine weeks of planting.

Data analysis. Data were analyzed using analysis of variance (ANOVA) to determine the effect between treatments. The significantly different variables were tested using Duncan's multiple range test (DMRT) at α 1 and 5% levels using DSAASTAT V.1.1 (open-source software). In addition, Principal Component Analysis (PCA) of shoots numbers, leaf length, leaf numbers and root numbers were also done according to Statistical Software for Excel (XLSTAT) ver. 21.

RESULTS AND DISCUSSION

The number of Calamus shoots from week 0 to week 6 cultured on basic media containing 100, 50, 33.3 and 25% macronutrients with 10, 20 and 30 g/l sugar in combination with 0, 0.5 and 1 mg/l BAP is shown in Fig. 1.

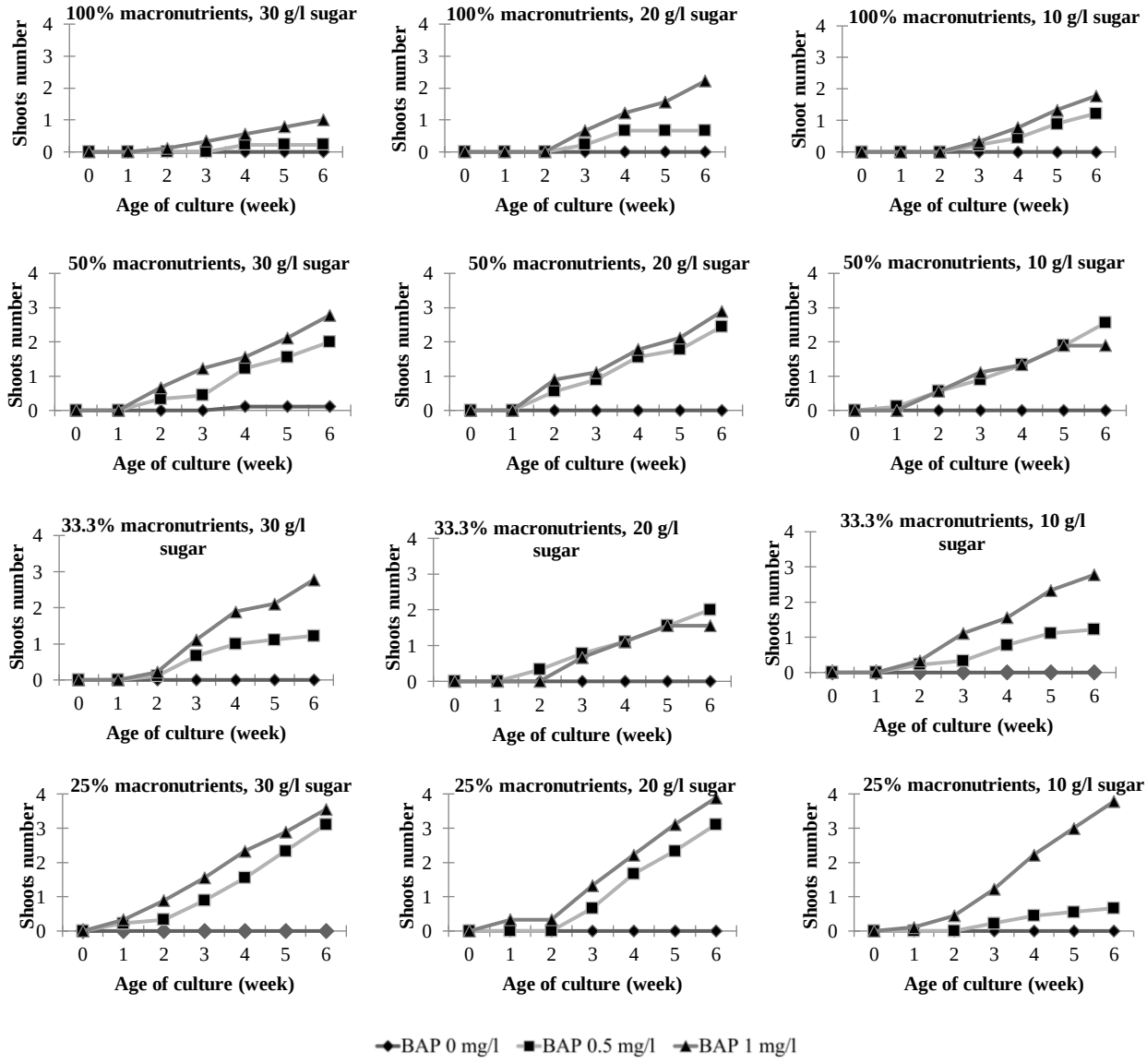

Fig. 1. Shoots number of *Acorus calamus* cultured for 0-6 weeks on MS modified media containing 10-30 g/l sugar in combination with 0-1 mg/l BAP

All media without BAP did not form new shoots, but shoots were formed in all media containing BAP. New shoots started to grow after the third week on media with 100% macronutrients. Reduction of macronutrients concentration to 50, 33.3 and 25% accelerated the formation of new shoots in the

second week of culture. The number of shoots continued to increase up to 6 weeks of culture. Reducing sugar concentration from 30 g/l to 20 and to 10 g/l with increasing BAP concentration also increased the number of shoots. Analysis of variance showed that the concentration of macronutrients and sugar added to MS media significantly affected to the number of shoots. Similarly, the concentration of BAP also gave significant effect to the number of shoots. These two factors had a significant interaction with the number of shoots (Table 2).

Table 2. ANOVA analysis of growth variables of *Acorus calamus* after 6 weeks cultured on the medium treatments

Growth variable	Base medium composition	BAP level	Basic medium composition x BAP	Coefficient of variations (%)
Shoot numbers	**	**	**	58.28
Leaf length	ns	**	*	23.09
Leaf numbers	**	**	**	33.03
Root numbers	**	**	**	57.62

Notes: *: significant at α: 5%; **: significant at α 1%; ns: not significant

The average number of Calamus shoots after 6 weeks of culture is shown in Table 3. The highest number of shoots was produced in 25% macronutrients MS medium containing 10 and 20 g/l sugar in combination with 1 mg/l BAP. Reduction of macronutrients to 25%, sugar to 10 g/l in combination with 1 mg/l BAP induced new shoots ranging from 3 to 6 shoots. These results indicate that BAP has an important role in the formation of new shoots. Without the addition of BAP, new shoots are not formed. Similar response also occurred in axillary shoot proliferation in potato culture, media containing BAP induced new shoot formation compared to media without BAP (Kazemiani *et al*., 2018), node culture of bamboo (Chavan *et al*., 2021), shoot culture of *Rhus coriaria* (Amiri & Mohammadi, 2021), shoot culture of *Salvia tomentosa* (Martini *et al*., 2022) as well as in bulb culture of *Amorphophallus muelleri* (Ibrahim *et al*., 2022). Benzyl Amino Purine (BAP), a cytokinin growth regulator, is derivatives of adenine, synthesized and translocate through the xylem (Sipayung *et al*., 2018). The role of cytokinins in tissue culture is critical. It is required for cell division and differentiation. The multiplication of adventitious shoots due to the addition of BAP to the culture media is one of the important factors that determine the efficiency of a micropropagation system (Reddy *et al*., 2014). In addition, BAP also breaks the dominance so that apical proliferation of axillary buds is induced (Al Gethami & El Sayed, 2020).

Our finding showed that all media combinations showed that leaf length began to increase at 1 week of culture. Fig. 2 shows that from 1 to 6 weeks, all combinations of media without BAP gave higher leaf length compared to that in media with BAP. Media without BAP with 100% macronutrients with sugar 30 and 20 g/l showed higher leaf length than that in media with reduced sugar to 10 g/l. The concentration of macronutrients and sugars had no significant effect on leaf length, but BAP concentrations effected significantly on leaf length as shown in Table 2. Both factors showed significant interaction on leaf length. The longest leaves 6 weeks after culture are presented in Table 3. Shoot cultured on media with macronutrients from 100 to 25% containing 30 and 20 g/l sugar produced the longest leaves.

The results showed that addition of BAP decreased the average leaf length. The longest leaves were obtained on Calamus shoots grown on media containing 33.3% macronutrients with 20 g/l sugar, but without BAP (Table 3). The ANOVA showed that the variable leaf length was not affected by the composition of the basic media (concentration of macronutrients and sugars), but it was strongly influenced by the concentration of BAP (Table 2). The response of *in vitro* shoots to the addition of cytokinin group PGR such as BAP varies depending on the species or genotype and physiological conditions. Banana var. Graine Naine shoot culture showed various responses to BAP concentrations. The higher BAP up to 9.4 mg/l did not correspond to the increase in shoot height. On the other hand, low BAP (0.5 mg/l) was not followed by a decrease in shoot height. The highest *in vitro* shoots of banana var. Graine Naine was at 2 mg/l BAP (Reddy *et al*., 2014). A similar response was also shown by Eucalyptus hybrid plants (*Eucalyptus urophylla* x *Eucalyptus grandis*) having optimal growth on

media containing BAP in combination with NAA. Higher concentration of BAP and NAA produced stunted axillary shoots (Nazirah *et al*., 2021). Optimization of BAP concentration is needed to obtain the best growth response.

Table 3. Growth and chlorophyll content of *Acorus calamus* after 6 weeks cultured on MS modified medium containing 10-30 g/l sugar in combination with 0-1 mg/l BAP (Medium code is shown in Table 1).

Medium code	Shoots number	Leaf length (cm)	Leaf number	Root number	Chlorophyll (mg/g)		
					A	B	Total
A.1.1	0.0 ^{l}	6.3 ^{efgh}	3.4 ^{o}	2.1 ^{jklmn}	0.8	0.4	1.2
A.1.2	1.2 ^{hij}	6.0 ^{efgh}	5.6 ^{lmno}	0.2 ^{op}	0.7	0.3	1.0
A.1.3	1.8 ^{fghi}	6.0 ^{efgh}	8.3 ^{ijklm}	0.0 ^{p}	0.4	0.2	0.6
A.2.1	0.0 ^{l}	9.0 ^{abc}	4.7 ^{no}	4.4 ^{defgh}	1.2	0.5	1.7
A.2.2	0.7 ^{jkl}	4.7 ^{ghij}	5.7 ^{lmno}	1.0 ^{mnop}	0.7	0.3	1.0
A.2.3	2.2 ^{cdefg}	6.3 ^{efg}	9.3 ^{hijk}	0.3 ^{nop}	0.6	0.2	0.8
A.3.1	0.0 ^{l}	9.3 ^{abc}	4.3 ^{no}	4.1 ^{defgh}	1.2	0.4	1.6
A.3.2	0.2 ^{kl}	6.2 ^{fghij}	5.2 ^{mno}	1.3 ^{lmnop}	0.6	0.3	0.9
A.3.3	1.0 ^{ijk}	4.7 ^{ij}	7.3 ^{jklmn}	0.0 ^{p}	0.4	0.2	0.6
B.1.1	0.0 ^{l}	7.7 ^{def}	5.0 ^{no}	4.4 ^{defgh}	1.0	0.4	1.3
B.1.2	2.6 ^{bcde}	6.4 ^{fghi}	13.5 ^{abcdef}	0.8 ^{mnop}	0.5	0.2	0.7
B.1.3	1.9 ^{efghi}	4.8 ^{gh}	9.2 ^{hijk}	0.0 ^{p}	0.3	0.1	0.5
B.2.1	0.0 ^{l}	7.8 ^{cdef}	6.6 ^{klmno}	5.1 ^{bcdef}	0.5	0.2	0.8
B.2.2	2.3 ^{cdefg}	6.3 ^{fghij}	11.3 ^{bcdefghi}	2.1 ^{jklmn}	0.5	0.2	0.7
B.2.3	2.9 ^{bcd}	5.0 ^{fgh}	13.8 ^{abc}	1.2 ^{lmnop}	0.3	0.1	0.4
B.3.1	0.1 ^{l}	8.9 ^{abcd}	5.4 ^{lmno}	5.8 ^{bcde}	0.7	0.3	1.0
B.3.2	2.0 ^{defgh}	6.4 ^{fghi}	11.3 ^{bcdefghi}	3.2 ^{ghijk}	0.4	0.2	0.6
B.3.3	2.6 ^{bcde}	5.2 ^{fgh}	14.6 ^{ab}	1.4 ^{klmnop}	0.2	0.2	0.3
C.1.1	0.0 ^{l}	7.1 ^{efg}	4.6 ^{no}	4.1 ^{defgh}	1.0	0.3	1.3
C.1.2	1.2 ^{hij}	5.6 ^{ghij}	8.6 ^{ijkl}	1.8 ^{klmnop}	0.4	0.2	0.6
C.1.3	2.8 ^{bcde}	5.3 ^{ghij}	13.6 ^{abcd}	1.2 ^{lmnop}	0.4	0.2	0.6
C.2.1	0.0 ^{l}	9.4 ^{abc}	5.6 ^{lmno}	5.8 ^{bcd}	0.9	0.4	1.3
C.2.2	2.0 ^{defgh}	6.4 ^{fghi}	12.2 ^{bcdefgh}	4.8 ^{cdefgh}	0.4	0.2	0.6
C.2.3	1.7 ^{ghij}	5.8 ^{ghij}	12.1 ^{bcdefgh}	2.9 ^{hijkl}	0.3	0.1	0.5
C.3.1	0.0 ^{l}	9.4 ^{abc}	5.0 ^{no}	4.1 ^{defgh}	1.0	0.4	1.4
C.3.2	1.2 ^{hij}	6.3 ^{fghij}	11.2 ^{cdefghi}	6.6 ^{b}	0.6	0.2	0.8
C.3.3	2.8 ^{bcde}	5.1 ^{hij}	15.4 ^{a}	0.4 ^{nop}	0.3	0.1	0.4
D.1.1	0.0 ^{l}	8.2 ^{bcde}	5.1 ^{mno}	3.5 ^{fghij}	1.0	0.4	1.4
D.1.2	1.0 ^{jkl}	5.3 ^{ghij}	9.0 ^{jklmn}	1.2 ^{klmnop}	0.3	0.1	0.4
D.1.3	3.8 ^{a}	4.7 ^{ij}	13.6 ^{abcde}	0.6 ^{mnop}	0.1	0.1	0.2
D.2.1	0.0 ^{l}	10.1 ^{a}	5.9 ^{lmno}	8.1 ^{a}	0.7	0.3	1.0
D.2.2	3.1 ^{abc}	5.0 ^{fgh}	9.8 ^{ghij}	5.0 ^{bcdeg}	0.3	0.2	0.5
D.2.3	3.9 ^{a}	4.6 ^{gh}	12.8 ^{abcdefg}	0.3 ^{nop}	0.1	0.1	0.2
D.3.1	0.0 ^{l}	9.1 ^{abcd}	6.0 ^{lmno}	6.2 ^{bc}	0.9	0.4	1.3
D.3.2	3.1 ^{abc}	5.9 ^{ghij}	10.3 ^{defghij}	4.0 ^{efghi}	0.4	0.2	0.6
D.3.3	3.7 ^{ab}	4.5 ^{h}	11.4 ^{bcdefghi}	2.4 ^{ijklm}	0.2	0.1	0.3

Note: The value followed by the same letter in the same column are not significantly different according to Duncan's multiple range test at $\alpha = 5\%$

Our results showed that in contrast to leaf length, the number of leaves of Calamus was influenced by the composition of the base medium and the concentration of BAP. From the first week to 6 weeks after culture, the number of leaves still increased (Fig. 3). Reducing the concentration of macronutrients to 50, 33.3 and 25% increased the number of leaves compared to the number of leaves on media with 100% macronutrients. On the other hand, an increase in BAP concentration was also followed by an increase in the number of leaves. Our results showed that in contrast to leaf length, the number of leaves of Calamus was influenced by the composition of the base medium and the concentration of BAP. From the first week to 6 weeks after culture, the number of leaves still increased (Fig. 3). Reducing the concentration of macronutrients to 50, 33.3 and 25% increased the number of leaves compared to the number of leaves on media with 100% macronutrients. On the

other hand, an increase in BAP concentration was also followed by an increase in the number of leaves. A similar response was also shown by shoot tip culture of Iranian traditional medicine plants *Rhus coriaria* L. using BAP for shoots proliferation and shoot elongation. BAP is important in cell expansion, it can metabolize immediately in plant tissue (Amiri & Mohammadi, 2021). Reduction of sugar concentration in Calamus culture did not influence the growth of shoots. The previous report on *Prunus africana*, sucrose at 15 and 30 g/l was favourable for shoot development compared to no addition of sucrose or sucrose at higher than 30 g/l (Komakech *et al*., 2020).

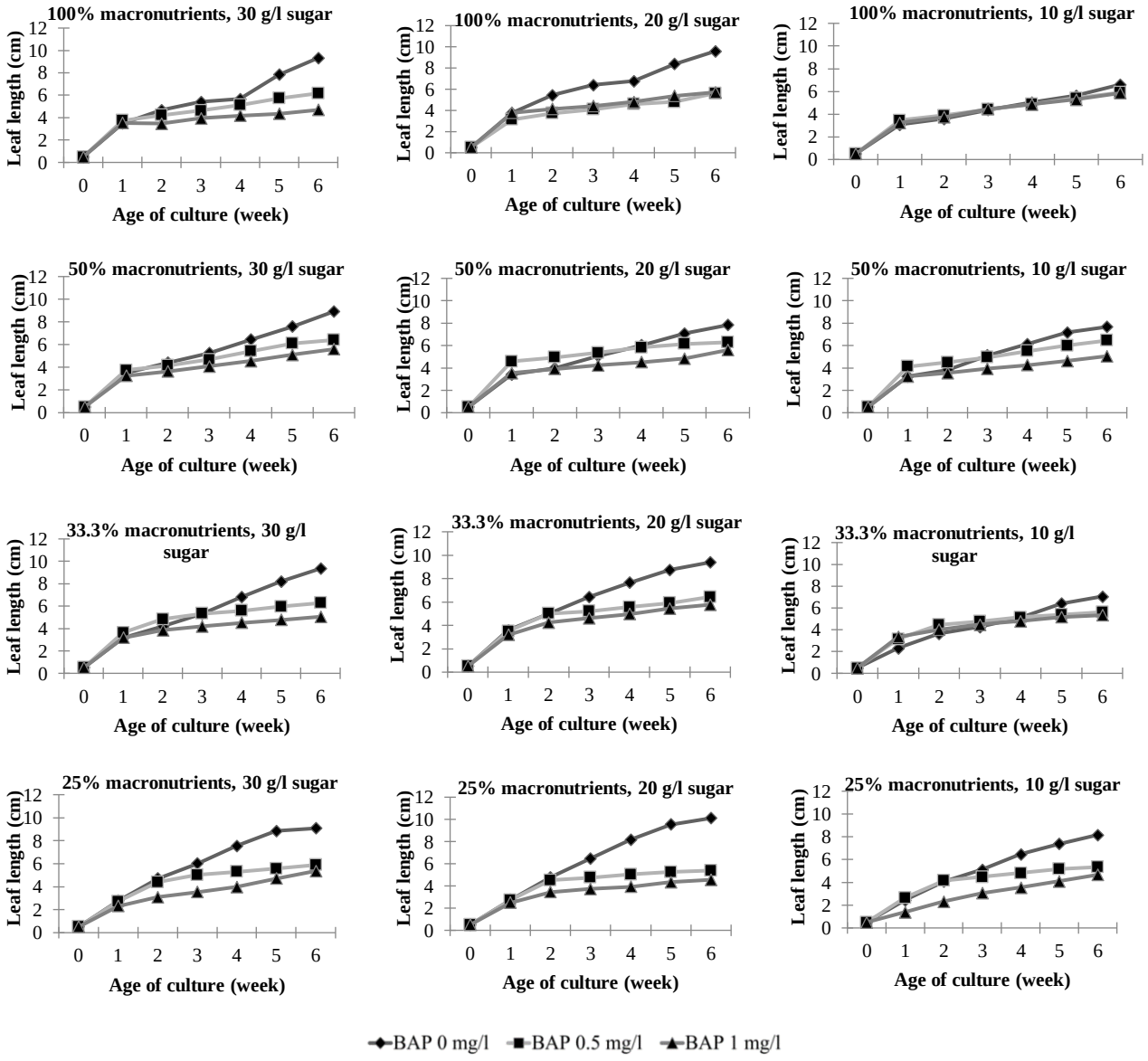

Fig. 2. Leaf length of *Acorus calamus* cultured for 0-6 weeks on MS modified media containing 10-30 g/l sugar in combination with 0-1 mg/l BAP

Reduction of macronutrient concentrations without BAP increased the number of Calamus roots from the first week to 6 weeks of culture (Fig. 4). On media without BAP but with 100% macronutrients containing 30 and 20 g/l sugar, the number of roots increased from 2 weeks to 6 weeks of culture. Reducing sugar to 10 g/l slowed root formation. Few roots formed 4 weeks after culture. In media with 50% macronutrients, roots formed faster at week 2. In this medium, the reduced sugar concentration resulted in fewer roots. Media containing 33.3% macronutrients with 30 g/l sugar showed different growth in the number of roots compared to media containing 20 and 10 g/l sugar. In media with 30 g/l sugar, the addition of 0.5 mg/l BAP increased the number of roots slowly, at

week 5 the number of roots was higher than the media without or with 1 mg/l BAP. However, media containing 20 and 10 g/l sugar showed a higher number of roots than without BAP. Media containing 25% macronutrients, 20 g/l sugar without BAP increased root growth, but the number of roots decreased in media containing lower sugars. An increase in BAP from 0.5 to 1 mg/l further inhibited root growth (Fig. 4). Commonly, auxins are required to rooting rather than cytokinins. Type of auxin also affected root growth. In *Prunus africana*, IBA was the best for rooting compared to IAA and NAA (Komakech *et al*., 2020). While in *Coleus forskohlii*, the use of IAA was good for rooting (Sivakumar *et al*., 2021).

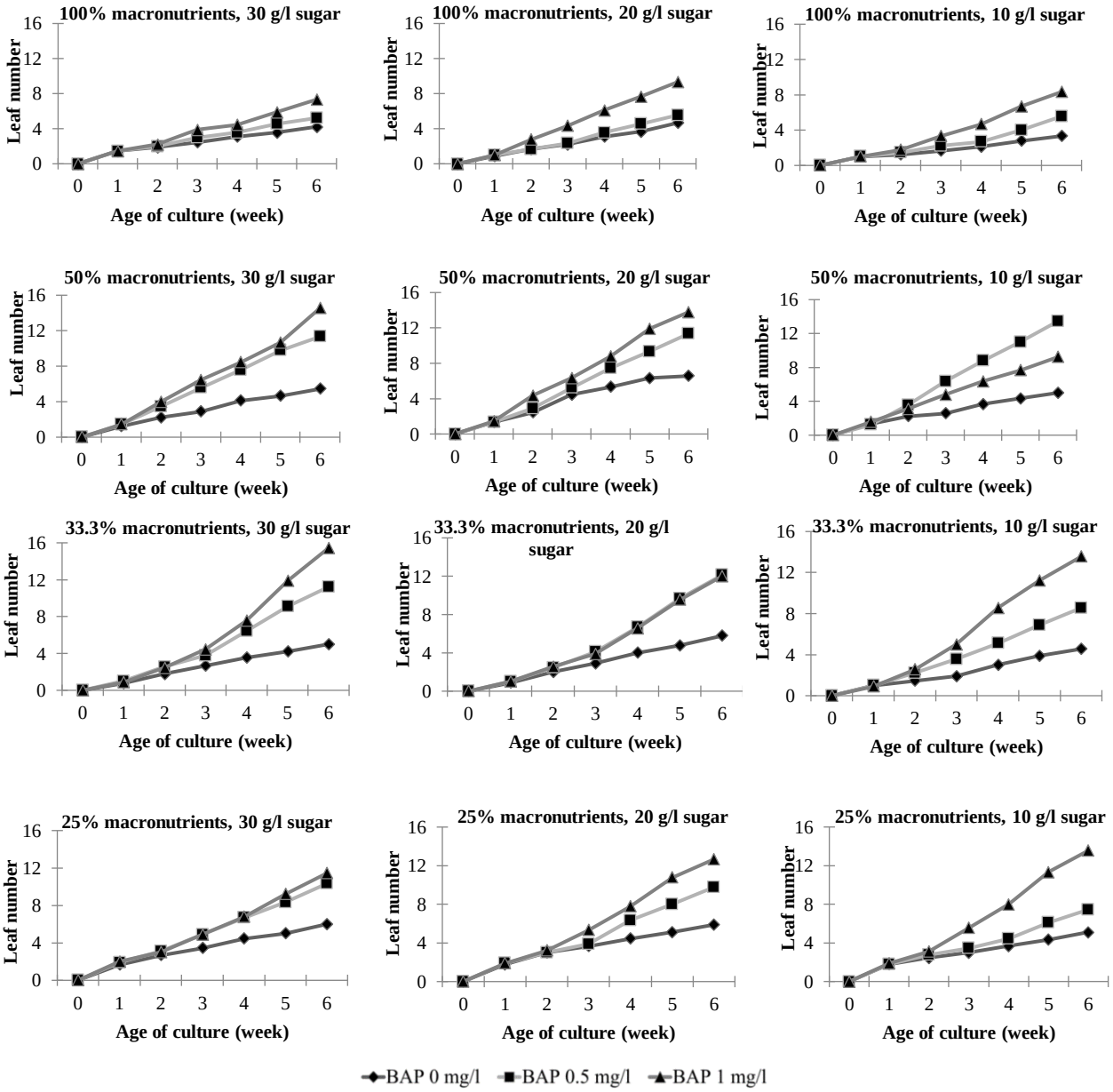

Fig. 3. Leaf number of *Acorus calamus* cultured for 0-6 weeks on MS modified media containing 10-30 g/l sugar in combination with 0-1 mg/l BAP

The ANOVA showed that the basic medium composition factors (macronutrients and sugar concentrations) and BAP concentration factors significantly affected the number of roots. Both factors also showed a significant interaction with the number of roots (Table 2). Table 3 shows the highest average Calamus root formation was 8.1 roots per plantlet on media containing 25% macronutrients, 20 g/l sugar without BAP. This media is the best medium for rooting of Calamus. In

this medium the number of roots formed ranged from 5 to 12 roots at 6 weeks of culture. The decrease in the concentration of macronutrients resulted in an increase in the number of roots. This seems to be related to a decrease in nitrogen levels, such as in the shoot culture of the Thai medicinal plant *Smilax corbularia* (Jirakiattikul *et al*., 2013). Shoot culture of ornamental plants *Chrysanthemum indicum* also showed the same response (Alsoufi *et al*., 2021).

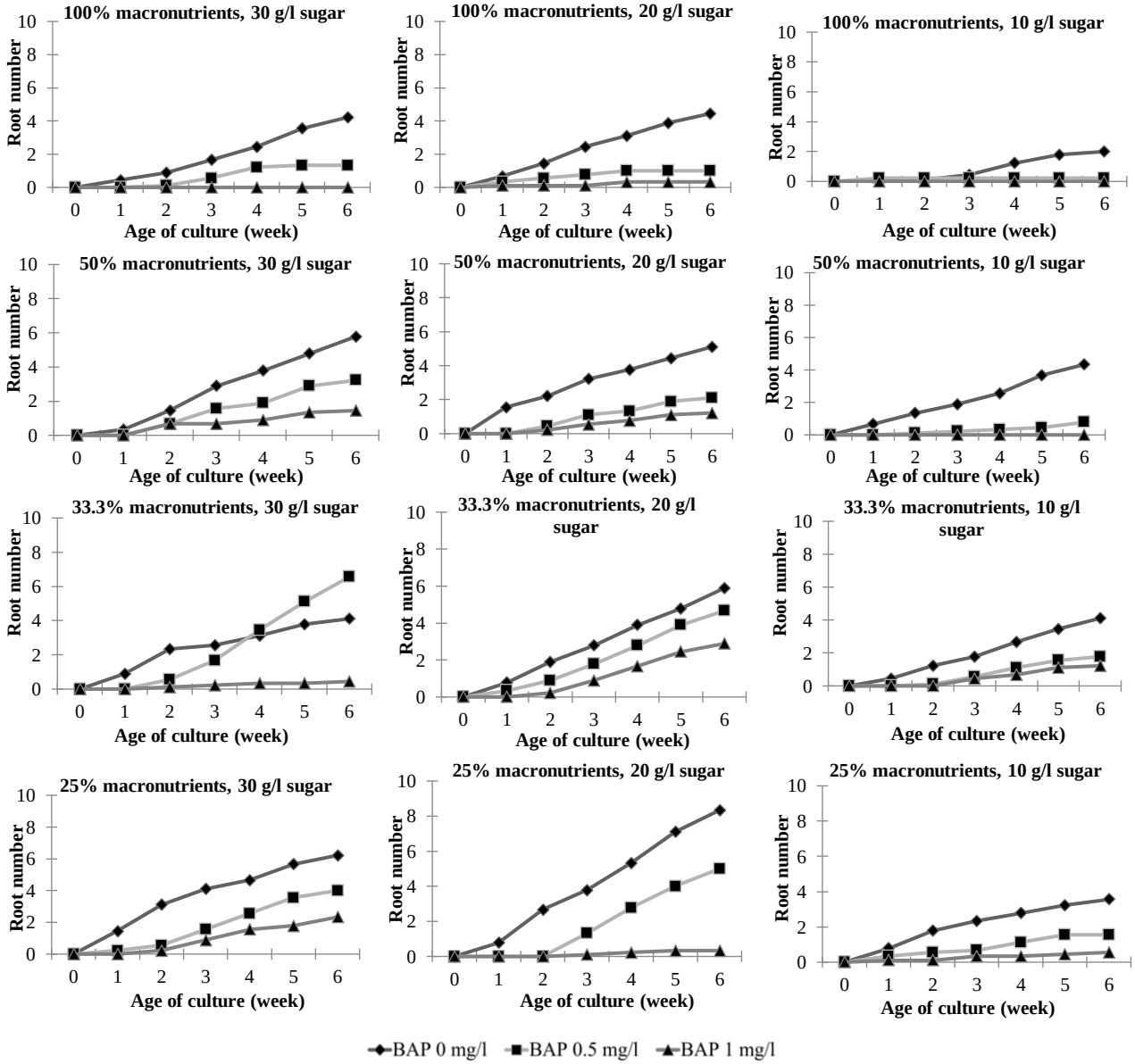

Fig. 4. Root number of *Acorus calamus* cultured for 0-6 weeks on MS modified media containing 10-30 g/l sugar in combination with 0-1 mg/l BAP

The results showed that the reduction of macronutrients and sugars did not reduce plantlet performance. At 6 weeks of culture some of the leaves began to turn yellow but did not affect the growth in the greenhouse in the acclimatization process. Although chlorophyll content reduced by reducing the level of macronutrients (Table 3), plantlets grow well after acclimatization. All plantlets had 100% survival rates during acclimatization processes. Fig. 5 shows the micropropagation of Calamus started from initiation of shoot culture to plantlets production ready for planting in the field.

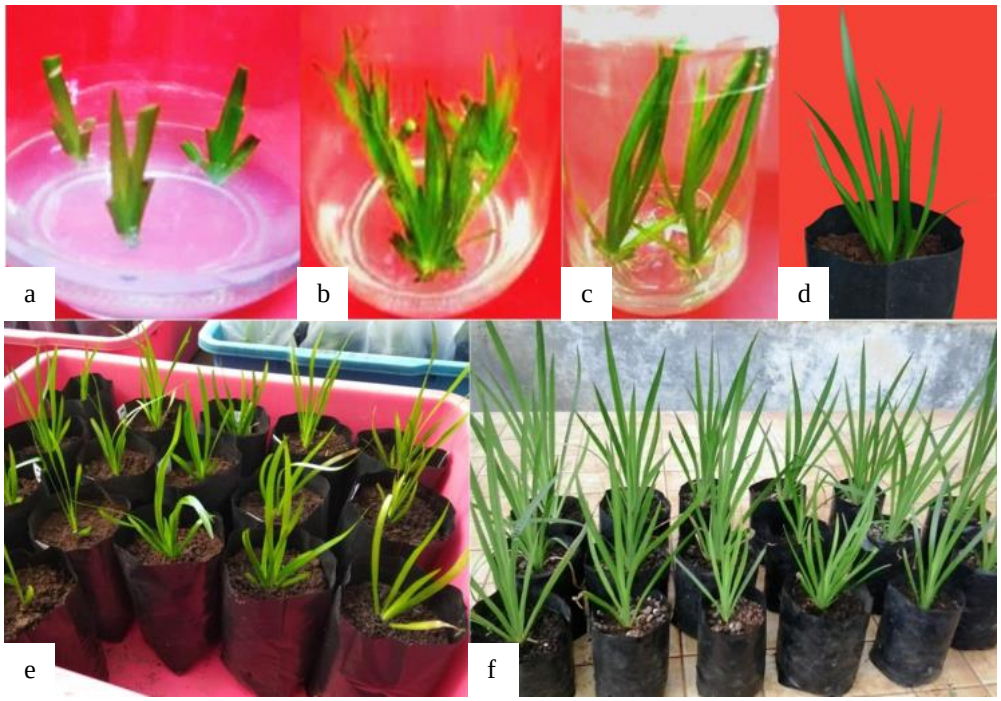

Fig. 5. Micropropagation of *Acorus calamus*. *In vitro* shoots development: a. shoots at two weeks old; b. shoots at four weeks old; c. shoots at six weeks old); acclimatization processes; d. planlets at 2 weeks after acclimatization; e. planlets at 4 weeks after acclimatization; f. seedlings ready to plant in the field

Fig. 6 represents biplot of the Principal Component Analysis (PCA) of Calamus micropropagation. The PCA produced two principal components (PC1 or F1 and PC2 or F2) based on the Eigen value >1, which has been able to explain 93.29% of total variation of growth variables. The results showed that full strength MS macronutrients was not the dominant components of shoot growth of Calamus.

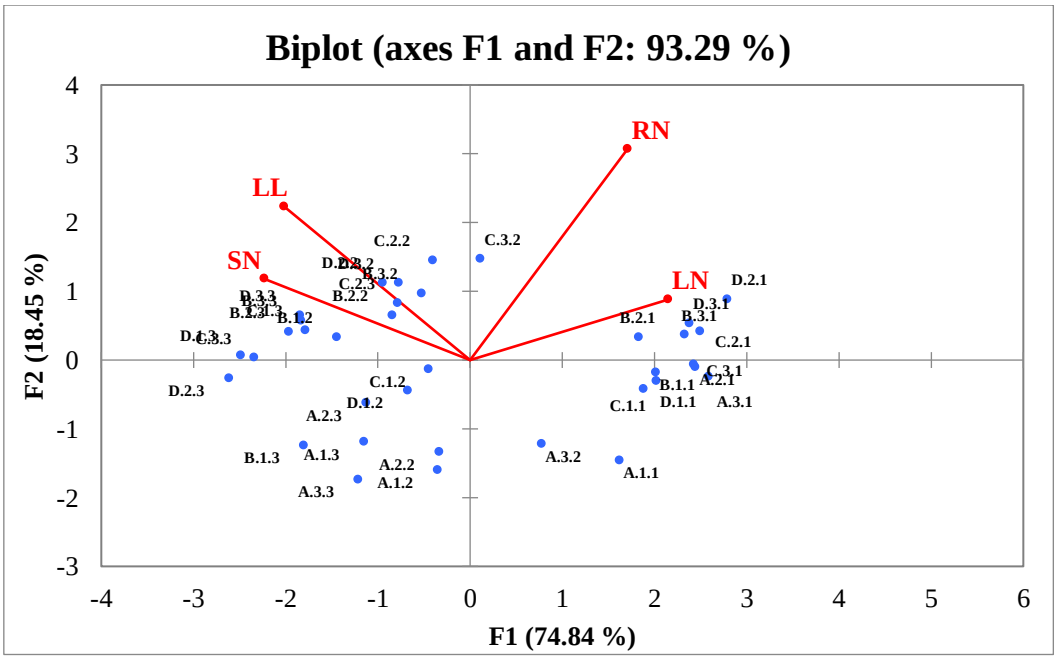

Fig. 6. Biplot of principal component analysis (PCA) of *Acorus calamus* grown *in vitro* on MS modified medium containing 10-30 g/l sugar in combination with 0-1 mg/l BAP. (SN = Shoot Numbers; LN = Leaf Numbers; LL = Leaf Length, RN = Root Numbers)

This indicated that reduction of macronutrients was not affected significantly for micropropagation of Calamus. Number of shoots was mostly affected by reduced macronutrients and sugar with addition of 1 mg/l BAP (B.1.2, B.2.3, B.3.3, C.1.3, C.3.3, D.1.3, and D.3.3), similar finding was also for number of leaves but with addition of 0.5 BAP (B.2.2, B.3.2, C.2.2, C.2.3, D.2.2, and D.3.2.), and for leaf length but with no addition of BAP (B.2.1, B.3.1, C.2.1, D.2.1, and D.3.1). Rooting was dominated by MS medium containing 33.33% macronutrients with 30 g/l sugar and 0.5 mg/l BAP (C.3.2) as well as medium containing 25% macronutrients with 20 g/l sugar (D.2.1). This result showed that each stage in micropropagation of Calamus was affected by medium compositions. The previous study was also reported on *Prunus africana*. Sucrose at 15 g/l and BAP at 1.0 mg/l supported the optimum rate of axillary shoot initiation, while sucrose at 15 g/l in combination with IAA at 1.5 mg/l was optimum for root initiation (Komakech *et al*., 2020).

CONCLUSION

This study provides a standard protocol for *Acorus calamus* micropropagation in a lower cost medium. *Acorus calamus* can be micropropagated in MS medium with reduction macronutrients to 25% and sugar to 10 g/l with addition of 0.5 and 1 mg/l BAP. Reduction of macronutrients and sugar can be applied for Calamus shoot culture. Cytokinin BAP is critical for shoot growth but this could be omitted for rooting. Therefore, this finding offered more choices in micropropagation of Calamus with low-cost production.

ACKNOWLEDGEMENTS

This work was funded by DIPA. Research Center for Biotechnology LIPI year 2019. The authors would like to thank Hoerudin as an assistant for acclimatization process and Rifatul Adabiyah for Principal Component Analysis.

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