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EFFECT OF PHENYLALANINE AMINO ACID ON FLAVONOID PRODUCTION BY APPLE CALLUS CULTURE

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Abstract. Apple (Malus domestica) is a fruit with many health benefits because it contains phytochemicals and flavonoids. Flavonoids are phenolic compounds with potential as antioxidants, which can neutralize free radicals that enter the human body. Flavonoid production can be done through callus culture. Adding phenylalanine amino acids to apple callus can increase the flavonoid content. The research aimed to study Phenylalanine amino acids' effect on apple callus's flavonoid content. The total content of flavonoids in the callus of 3 apple varieties, namely Granny Smith, Red Delicious and Manalagi, in MS + 2,4-D 0.5 ppm + BAP 1 ppm added phenylalanine (0-1000 ppm) higher than total flavonoids fresh skinless apples. Green callus has more potential to produce flavonoids compared to brown callus. Suitable media for producing flavonoids in vitro are MS + 2,4-D 0.5 ppm + BAP 1 ppm + phenylalanine 500 ppm for Granny Smith and Manalagi apple callus, which produce total flavonoid 1.51 (QE) mg/g DW and 0.78 (QE) mg/g DW, MS + 2,4-D 0.5 ppm + BAP 1 ppm + without Phenylalanine for Red Delicious callus produce total flavonoid 1.46 9(QE) mg/g DW

1 Introduction

Apples are often used as essential ingredients of medicines in the medical world because they contain phytochemicals and flavonoids [1]. Flavonoids are natural products produced by plants and serve to protect plants from environmental biotic and abiotic stresses. Flavonoids are phenolic compounds with potentials such as antioxidants that can neutralize free radicals in the human body. Flavonoids in the human body prevent cancer, bone loss, anti-inflammatory, antibiotics, and others [2].

Flavonoids, namely Quercetin, are the most abundant flavonols in apple plants. Quercetin, or 3,3,4,5,7-pentahydroxyflavone, is a secondary metabolite compound in apples, onions, broccoli, and others [3].

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About 99.2% of shallot flesh is made up of quercetin glucosides, and 0.8% is made up of quercetin aglycone. The composition of dried shallot skin is nearly the reverse, with 16.7% quercetin glucosides and 83.3% quercetin aglycone [4]. Instead of their aglycone forms, quercetin, kaempferol, and myricetin are primarily consumed as flavanol glycosides. Quercetin, on the other hand, is an aglycone without any sugar bonded to it. It is insoluble in cold water, barely in hot water, and highly soluble in lipids and alcohol. In addition, Quercetin is believed to prevent degenerative diseases in the body and prevent the oxidation process of LDL (Low-Density Lipoproteins) by capturing free radicals while chelating transition metal ions, which are harmful to the human body, such as Chromium (Cr), Manganese (Mn), Iron (Fe), Copper (Cu), and others [2]. The content of Quercetin in apples (Malus domestica) and the skin is around 4.01 mg / 100 g [5], and apple skin has potent antioxidant activity [6]. Producing flavonoids by extracting fresh apples in Indonesia requires expensive costs because Indonesia is unsuitable for developing apple crops. Until now, the apple that was developed in Indonesia has limited types, namely Manalagi, Rome Beauty, Anna and Wanglin; in addition, they only grow in specific areas such as Batu and Nongkojajar. Therefore, it is necessary to develop technology to produce flavonoids through callus culture in vitro.

An in vitro approach to commercially producing secondary metabolites has been applied to several secondary metabolites because the production process does not depend on the season, has controlled environmental conditions, and the products produced are standard. Flavonoids derived from cell culture are more straightforward to separate in the form of intact polymers from flavonoids in complex plant tissues, and overall concentration can be significantly increased in callus cultivation through a controlled microenvironment and the addition of precursors to trigger metabolite production [7]. In order to increase the synthesis of secondary metabolites, several organic compounds can be added to culture media [8]. One of them is the amino acid phenylalanine. Phenylalanine is a precursor that produces isoflavones and flavonoids through the phenylpropanate pathway. Adding Phenylalanine in culture media will increase targeted compound levels [9].

Several studies related to the use of the amino acid phenylalanine in the production of flavonoids in vitro. Adding phenylalanine 2-5 mg/l did not significantly increase the fresh Weight of Hydrocotyle bonariensis callus. Phenylalanine concentration of 3 mg/l produces flavonoids 11.43 mg/g dw or 23% higher than controls. Phenylalanine concentrations higher by five mg/l only produce flavonoids by 9.06 mg/l [10]. Phenylalanine concentration of 500-750 mg/l in Maytenus emarginata callus culture produced a total flavonoid of 1.82-2.02 mg/100g dw while control (without Phenylalanine) was 1.61 mg/100g dw [11]. Phenylalanine (50mg/100ml) increased the yield of total Quercetin only 1 to 2 folds compared to control (control = 0.23 mg/g dry wt; fed = 0.38mg/g dry wt). The total phenolics and flavonoid content of the Murraya callus cultures showed higher antioxidant activity than stem and leaf parts in vivo due to phenolics and flavonoid content. The total flavonoids of the ethanolic extracts of M Callus-stem-leaf coenigii were 50.0, 35.1 and 30.5 g routine equivalent (RE)/100g of extract, respectively. The IC50 (inhibition concentration 50) of the ethanolic extracts of M. Callus stem leaf was 47-78 and 116 μg/ml, respectively. Among them, callus showed higher antioxidant activity in vitro due to phenolics and flavonoid content [12]. Therefore, the study aimed to study the effect of the amino acid phenylalanine on callus flavonoid production in vitro of three apple varieties.

2 Methods

The investigation employed two components and a factorial, fully randomised design. Four phenylalanine concentration levels—0, 500, 750, and 1000 ppm—are the first factor. Three different apple varieties—Granny Smith, Red Delicious, and Manalagi—are the second factor.

Callus induction used cotyledon explants of old apple seeds using MS + 0.1 ppm BAP + 1 ppm 2.4-D + 0.5 ppm IAA media. Apple callus formed aseptically placed on MS medium (Murashige and Skoog, 1962) supplemented with 0.5 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) and one ppm of 6-Benzylaminopurine (BAP) and Phenylalanine according to treatment. The initial callus weighed between 0.27 and 0.29 grams. After using HCl or NaOH to bring the medium's pH down to 5.7, it was autoclaved for 15 minutes at 121 °C. The explants were cultured at 25 ± 2° C for 16 hours with a photoperiod of 1200 lux. The callus Every four weeks, tissues were subculture onto new media. At nine weeks, the callus is harvested. Following planting (WAP). Apples' overall flavonoid content and antioxidant capacity without Three skin types were examined for comparison. The variables that were observed were fresh callus weight, total flavonoid content, callus dry weight, growth index, and antioxidant activity.

Growth index (GI) were calculated=

$$\frac{\text{Final fresh Weight of callus} - \text{Initial fresh Weight of callus}}{\text{Initial fresh Weight of callus}} \text{ X } 100\%$$

Callus dry weight is obtained by drying the callus in an oven at 40-500 C for 24 hours. The dry callus, as much as 0.5 mg, is mashed to powder with mortar and extracted. Callus was extracted using ethanol solvent—filtration of the extract using Whatman paper No. 1.

Total Flavonoid Analysis of the Colorimetric Method
Total flavonoid analysis used the colourimetric method, and the ingredients used were apple callus extract and apple flesh. This process is done by observing the absorbance value (quersetin standard at various concentrations and callus extract samples) at a wavelength of 510 nm, by substituting the absorbance value (y) of the callus extract sample to the quercetin standard regression equation (y = ax + b), so we get the concentration value (x) and is read as mg equivalent of quercetin / g callus extract.

Antioxidant Activity Testing DPPH Method (1,1-diphenyl-2-picrylhydra-zil)
This is an analysis of antioxidant activity by the DPPH method (1,1-diphenyl-2-picrylhydra-zil) with the calculation of inhibition percentage. Analysis of antioxidant activity, which observed the absorbance value of DPPH and the absorbance value of callus extract given DPPH at a wavelength of 517 nm in a UV-Vis spectrophotometer.

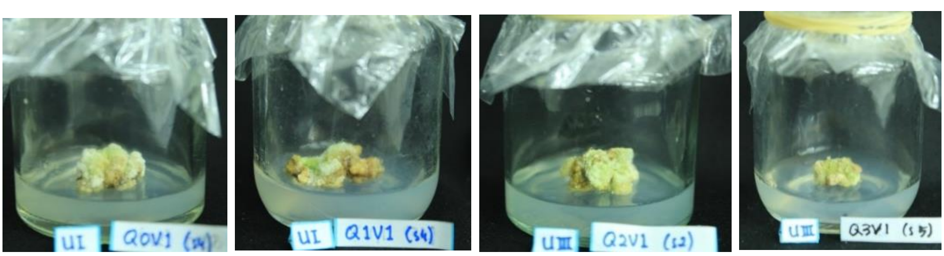
Calculation formula (Inhibition percentage):

$$\frac{\text{Absorbance of DPPH} + (\text{Absorbance of DPPH} + \text{Absorbance of sample})}{\text{DPPH absorbance}} \text{ x } 100\%$$

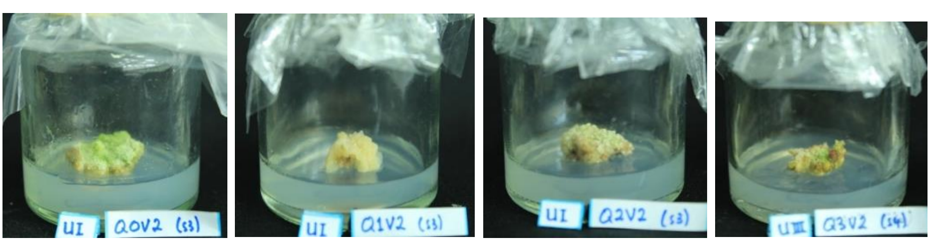
Further, after knowing the percentage of inhibition, determine the equation y = ax + b with linear regression calculations (x = concentration (µg / ml), y = percentage of inhibition (%). Antioxidant activity is expressed by 50% inhibitory concentration (IC50), the sample concentration that can reduce the DPPH radical by 50%. IC50 values were obtained from the value of x after replacing y = 50.

3 Result

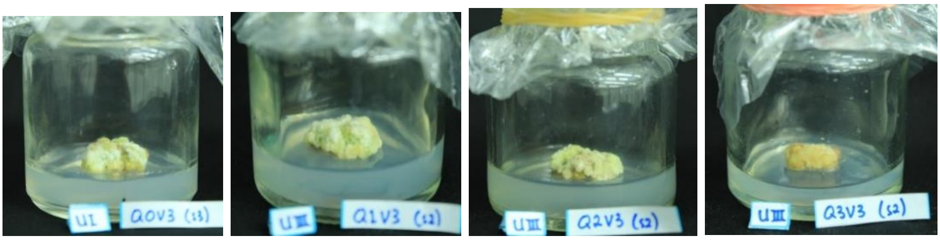
Based on the analysis of the various interactions between apple varieties and phenylalanine concentrations it significantly affected fresh Weight at ages 4 and 9 WAP and dry Weight of callus at 9 WAP. Fresh Weight of callus Granny Smith varieties aged 4 WAP decreased with the addition of Phenylalanine. However, at age 9 WAP, Phenylalanine added a fresh weight of callus that was not different from the control. Adding 500 ppm phenylalanine can increase the fresh Weight of Granny Smith variety callus apples by 24.4% aged 9 WAP compared to control (without Phenylalanine) (Table 1). The fresh Weight of callus varieties of Red Delicious decreased with the addition of Phenylalanine (500-1,000 ppm) compared to controls at 4 and 9 WAP, although the decrease was not significantly different. The fresh callus weight with the addition of 500 ppm phenylalanine decreased by 50% compared to the control at 9 WAP. The fresh Weight of callus apple variety Manalagi showed no significant difference between control and phenylalanine supplementation at age 4 WAP. The fresh Weight of callus apples of Manalagi varieties without adding Phenylalanine tends to be higher than that of Phenylalanine (Table 1). Callus growth with adding 0-750 ppm Phenylalanine is faster, or the appearance of the callus is larger than the callus with the addition of 1000 ppm phenylalanine. This occurs in Granny Smith, Red Delicious, and Manalagi callus. Fig. 1 shows the callus of 3 apple varieties at the age of 8 WAP.



Granny Smith 0 ppm 500 ppm 750 ppm 1000 ppm



Red Delicious 0 ppm 500 ppm 750 ppm 1000 ppm



Manalagi 0 ppm **500 ppm** **750 ppm** **1000 ppm**

Fig 1. Apple callus aged 8 WAP on media containing Phenylalanine

Table 1. Effects of Treatments on Callus Fresh and Dry Weight in Callus Culture *Malus domestica*

Treatments	Fresh Weight (g)						Dry Weight (g)	
	0 Weeks		4 Weeks		9 Weeks		9 Weeks	
Granny Smith + 0 ppm Phenylalanine	0.27	a	0.67	b	0.86	ab	0.12	ab
Granny Smith + 500 ppm Phenylalanine	0.28	a	0.48	ab	1.07	ab	0.15	ab
Granny Smith + 750 ppm Phenylalanine	0.28	a	0.43	ab	0.88	ab	0.13	ab
Granny Smith + 1000 ppm Phenylalanine	0.27	a	0.37	a	0.44	a	0.06	a
Red Delicious + 0 ppm Phenylalanine	0.28	a	0.58	ab	1.13	b	0.21	b
Red Delicious + 500 ppm Phenylalanine	0.28	a	0.36	a	0.57	ab	0.12	ab
Red Delicious + 750 ppm Phenylalanine	0.28	a	0.43	ab	0.75	ab	0.16	ab
Red Delicious + 1000 ppm Phenylalanine	0.28	a	0.41	a	0.58	ab	0.06	a
Manalagi + 0 ppm Phenilalanine	0.27	a	0.47	ab	1.16	b	0.17	ab
Manalagi + 500 ppm Phenilalanine	0.26	a	0.48	ab	0.92	ab	0.14	ab
Manalagi + 750 ppm Phenilalanine	0.28	a	0.50	ab	1.05	ab	0.12	ab
Manalagi + 1000 ppm Phenilalanine	0.27	a	0.39	a	0.60	ab	0.06	a
HSD α 5%	0.02		0.23		0.75		0.11	

Means with the same letter are not significantly different by HSD (Honestly Significance Difference) range test (α= 0.05).

The dry Weight of callus apples for three varieties shows the same thing as in the fresh Weight of callus; the good result is the dry Weight of callus varieties of Granny Smith with the addition of 500 ppm phenylalanine while for the dry Weight of callus varieties Red Delicious and Manalagi on media without the addition of Phenylalanine. Based on growth indices calculation at 9 WAP, the highest values were Granny Smith apple callus with the addition of 500 ppm phenylalanine at 289.1%, Red Delicious apple callus without Phenylalanine at 306.0%, and Manalagi apple callus without Phenylalanine at 332.3% compared to the beginning of planting (Fig 2.). The growth of apple callus in vitro depends on the variety and composition of the planting media. The addition of Phenylalanine 500-1,000 ppm showed no difference in fresh Weight and dry Weight at 9 WAP except in the Red Delicious apple callus; the addition of 1,000 ppm phenylalanine significantly reduced callus dry weight compared to those without Phenylalanine. (Table1.).

Callus growth is influenced by several factors: the plant growth regulator, precursors, and the kind of plant. The callus culture of Kepel (*Stelechocarpus burial*) with mesocarp explants produced 2.52 g fresh weight callus after 9 weeks using Picloram 7.5 mg/L [13]. In the callus culture of *Artemisia annua* MS media containing 0.5 mg/L NAA and 0.5 mg/L BAP produced 5.83 mg/L dry weight callus [14]. Phenylalanine (2 mg/l) produced maximum biomass (3.14 ± 0.11 g FW/culture) followed by 3, 4, and 5 mg/l in the callus culture of *Hydrocotyle bonariensis* [15]. The apple callus culture research results showed that callus growth in each variety did not show any difference in fresh Weight and dry Weight of callus with the addition of Phenylalanine. The callus produced was 1.13 g FW for Red Delicious and 1.16 g FW for Manalagi, while for Granny Smith, 1.07 g FW. The results of apple callus culture were lower than the growth of *S. burahol* callus [13] and *H. bonariensis* callus [15] but higher than *A. annua* callus culture [14].

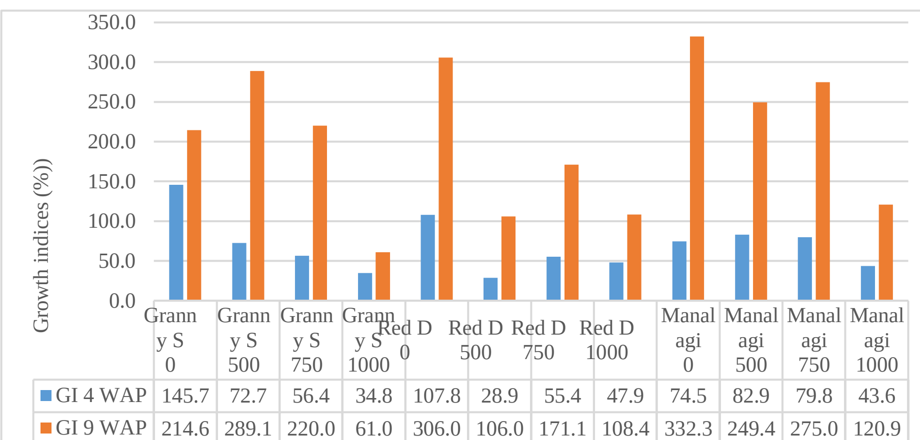


Fig 2. Effects of Treatments on Growth Indices (%) Callus Culture *Malus domestica*

Flavonoids can be produced in various in vitro systems, such as callus, cell suspension, root, or shoot cultures [16]. Compared to flavonoids found in complex plant tissues, cell-culture-derived flavonoids are more straightforward in separating them into an intact polymeric form. Additionally, by controlling the microenvironment and using precursor feeding to stimulate the production of metabolites, the concentrations of flavonoids in callus cultures can be significantly increased [17]. Several chemical substances can be added to the growth medium to improve the synthesis of secondary metabolites [18].

There are callus apples that grow brown and green. Analysis of the flavonoid content and antioxidant activity showed that the green callus had a higher flavonoid content (1.4-1.7 times) than the brown callus (Table 2), and the antioxidant activity of the green callus was more active (2.1-7.7 times) than the brown callus (Table 3). This shows that the apple callus, which has the potential to produce flavonoids with high activity, is the green callus (Tables 2 and 3). The content of dry green callus flavonoids in vitro results for all phenylalanine treatments in 3 apple varieties was higher (1.4 - 7.7 times) compared to the flavonoid content in fresh fruit without skin (Tables 2 and 4). The total flavonoid of fresh meat without Granny Smith and Red Delicious apple skin was 0.27 (QE mg/g dw), while in vitro callus was 0.76 - 1.51 (QE mg/g dw). Total flavonoids of fresh fruit flesh without apple peel were 0.17 (QE mg/g dw), and in vitro callus were 0.45 - 0.78 (QE mg/g dw) (Table 2).

The highest total flavonoids were 1.51 (QE mg/g dw) for the dry callus of Granny Smith and Manalagi apples by 0.78 (QE mg/g dw) with the addition of 500 ppm phenylalanine while for Red Delicious apples by 1.46 (QE mg/g dw) on media without Phenylalanine. The highest antioxidant activity of dry callus of Granny Smith apple based on IC 50 value (inhibition concentration 50) is 1,098.24 ppm with the addition of Phenylalanine 750 ppm, Manalagi apple is 1,665.04 ppm by adding Phenylalanine 750 ppm, and Red Delicious apple is 1,204.57 ppm without adding Phenylalanine (Table 3). Several studies have shown that Phenylalanine can increase flavonoid content in callus culture. The optimal concentration of Phenylalanine depends on the type of plant. In *Hydrocotyle bonariensis* callus culture, 3 mg/l Phenylalanine produced flavonoids 11.43 mg/g DW, which was 23% higher than without Phenylalanine [15]. Meanwhile, the optimized phenylalanine concentration for paclitaxel (Taxol) production from *Taxus chinensis* was 15 mg/l [19].

The antioxidant activity of dry callus in vitro in several treatments, namely Granny Smith + 500 - 750 ppm phenylalanine, Red Delicious + 0 ppm phenylalanine and Manalagi + 0 - 1,000 ppm phenylalanine showed higher antioxidant activity (IC 50) compared to fresh fruit flesh (Table 3 and 4). DPPH test based on IC 50 value of A. annua callus is much more active than IC 50 of apple callus. IC50 in A. annua callus is 53.20-99.15 mg/l [14] while in apple callus it is 1140-2268 mg/l. *A. annua* callus produces fewer flavonoids than apple callus, but the antioxidant activity of *A. annua* flavonoids is much higher compared to apple callus

Based on the observation variables of fresh callus weight, dry Weight of callus, total flavonoids and antioxidant activity, it was found that the production of flavonoids through callus in vitro was influenced by apple varieties, the composition of the growing media in this case, the concentration of Phenylalanine. Suitable media for Granny Smith callus is MS + 2,4-D 0.5 ppm + BAP 1 ppm + Phenylalanine 500 ppm, Red Delicious apple on MS + 2,4-D 0.5 ppm + BAP 1 ppm media + phenylalanine 0 ppm and Manalagi apple on MS medium + 2,4-D 0.5 ppm + BAP 1 ppm + Phenylalanine 500 ppm.

Table 2. Effects of Treatments on Total Flavonoids in Callus Culture *Malus domestica*

Treatments	Total Flavonoids (QE) (mg/g d.w.)	
	Green Callus	Brown Callus
Granny Smith + 0 ppm Phenylalanine	0.76	0.21
Granny Smith + 500 ppm Phenylalanine	1.51	0.23
Granny Smith + 750 ppm Phenylalanine	1.15	0.20
Granny Smith + 1000 ppm Phenylalanine	0.86	0.58
Red Delicious + 0 ppm Phenylalanine	1.46	0.77
Red Delicious + 500 ppm Phenylalanine	0.91	0.35
Red Delicious + 750 ppm Phenylalanine	0.99	0.35
Red Delicious + 1000 ppm Phenylalanine	0.77	0.50
Manalagi + 0 ppm Phenilalanine	0.69	0.20
Manalagi + 500 ppm Phenilalanine	0.78	0.18
Manalagi + 750 ppm Phenilalanine	0.61	0.20
Manalagi + 1000 ppm Phenilalanine	0.45	0.32

Table 3. Effects of Treatments on Antioxidant Assay by DPPH in Callus Culture *Malus domestica*

Treatments	IC 50 (ppm)	
	Green Callus	Brown Callus
Granny Smith + 0 ppm Phenilalanine	1622.63	3500.08
Granny Smith + 500 ppm Phenilalanine	1140.55	7038.78
Granny Smith + 750 ppm Phenilalanine	1098.24	6822.59
Granny Smith + 1000 ppm Phenilalanine	2139.78	4410.53
Red Delicious + 0 ppm Phenilalanine	1204.57	2786.58
Red Delicious + 500 ppm Phenilalanine	1801.55	6526.56
Red Delicious + 750 ppm Phenilalanine	1749.60	5697.00
Red Delicious + 1000 ppm Phenilalanine	2268.40	7687.30
Manalagi + 0 ppm Phenilalanine	2047.75	6378.52
Manalagi + 500 ppm Phenilalanine	1802.96	4413.33
Manalagi + 750 ppm Phenilalanine	1665.04	12803.33
Manalagi + 1000 ppm Phenilalanine	2438.22	6240.55

Table 4. Total Flavonoids and Antioxidant Assay by DPPH in Fresh Fruit (without skin) *Malus domestica*

Varieties	Total Flavonoids (QE) (mg/g d.w.)	IC 50 (ppm)
Granny Smith	0.27	1193.55
Red Delicious	0.27	1631.01
Manalagi	0.17	2542.92

4 Conclusion

The total content of callus flavonoids in 3 apple varieties, namely Granny Smith, Red Delicious and Manalagi in MS + 2,4-D 0.5 ppm + BAP 1 ppm added Phenylalanine (0-1000 ppm) higher than total flavonoids fresh skinless apples. Green callus has more potential to produce flavonoids compared to brown callus. Suitable media for producing flavonoids in vitro are MS + 2,4-D 0.5 ppm + BAP 1 ppm + phenylalanine 500 ppm for Granny Smith and Manalagi apple callus which produces total flavonoid 1.51 (QE) mg/g DW and 0.78 (QE) mg/g DW, MS + 2,4-D 0.5 ppm + BAP 1 ppm + without Phenylalanine for Red Delicious callus produce total flavonoid 1.46 9(QE) mg/g DW ..

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