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Screening Fenugreek genotypes for high callus induction and growth

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Abstract

Fenugreek (*Trigonella foenume*-graecum) is a medicinal herb that has anti-diabetic properties. 4-hydroxyisoleucine, an important metabolite of this plant, is a good candidate for improving hepatic insulin resistance in type 2 diabetic patients. In vitro production of metabolites mainly relies on the ability of the tissue culture system to produce more and viable cells with maximum production rate. Callus induction is the first step of establishing plant cell based bioreactors which needs potential genotypes with higher and faster callus production. To find such genotypes, screening them is indispensable. In this research, we screened 21 Iranian landraces of fenugreek for callus induction and growth on MS medium supplemented with some plant growth regulators. The results showed different capacity of the screened genotypes in callus induction ($P<0.05$). Also, the type of explant had a significant effect on callus induction ($P<0.05$). Calli were able to produce 4-Hydroxyisulosine 67% of the amount in in vivo samples.

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Introduction

Fenugreek (*Trigonella* spp.), a vegetable as well as an important medicinal plant, includes 128 species, among which *T. eleptica* is native to Iran; however, *T. foenum-graecum* is the only cultivated one (Rezazadeh *et al.*, 2011). Fenugreek is widely distributed and being cultivated all over the world, but native to Mediterranean region (De Candolle 1986). About 32 species of *Trigonella* have been identified in Iran being cultivated in different parts of the country (Fazli and Hardman 1968). The most important medicinal properties are related to the secondary metabolites such as Diosuginin, Trigonellin and 4-hydroxyisoleucine which are potentially producible via tissue culture technology (Tabassum *et al* 2016; Ciura, J *et al* 2015; Rezaeian, S. 2011). Previous studies have reported an anti-diabetic effect of 4-hydroxyisoleucine influencing both insulin secretion and resistance (Fazli and Hardman 1971; Asli *et al*., 2016; Basu *et al.* 2015). More recently, 4-hydroxyisoleucine has been artificially synthesized, which via isoleucine deoxygenize (IDO) gene expression in bacteria (Kodera *et al.* 2009; Ogawa *et al.* 2011; Hibi *et al.* 2011; Shi, F *et al* 2015). But they hadn't efficient product (Smirnov, Sokolov *et al.* 2012). Plant tissue culture has many applications including micropropagation, plant transformation, secondary metabolite production and so forth (Chakrabarty *et al*., 2005; Chakrabarty Gravot *et al* 2001). Callus induction is an essential step for establishing a plant cell bioreactor and metabolic compound production which is affected by plant growth regulators, type of explant and genotype (Huang and McDonald, 2012). Many investigations on fenugreek tissue culture have used hypocotyl, cotyledon, leaf and root as explant for callus induction (Aasim *et al*., 2009; El Nour *et al.* 2015; Shahabzadeh *et al.* 2013). In Fenugreek, hypocotyl segments were the most responsive explant to callus induction (Zhang *et al*., 2003). Plant species and genotype as well as hormone level have also been verified as important affecters in callus formation and development (Soskov *et al.*, 1996; Deshpande and Bhalsing 2014; Ortuno *et al.*, 2004; Lahouti *et al.* 2014; El-Nour *et al.* 2013; Mavahib *et al.* 2016; Provorov *et al.*, 1996). In this report, we have screened different genotypes of fenugreek cultivated in Iran based on their response to different hormonal combinations and explant types.

Materials and methods

Plant Resources

Twenty one genotypes of *T. foenum-graecum* from different parts of Iran were selected and used for screening as described in Table (1).

Decontamination and establishment

Uniform seeds were disinfected through washing with water with a few drops of twin 20% for 5 minutes. After removing supernatant, the seeds were treated with sodium hypochlorite 2.5% for 10 minutes. Then, under the laminar flow hood, seeds were sterilized and washed with 70% ethanol for 3 minutes. Finally, the seeds were washed three times with sterile water, and some of them were placed on MS ½ medium for germination and production of leaf and cotyledon explants and some kept in sterile water (16 h) for getting embryonic axis explants (Afshari, E. *et al*; 2011).

Table 1. Fenugreek genotypes used in this research and geographic regions in Iran from which they have been collected.

Row	Genotype number	Geographic region	Row	Genotype number	Geographic region
1	GF1	Hamadan	12	GF12	Dastgerd, Esfahan
2	GF2	Eghlid, Fars	13	GF13	Khozestan, Shoshtar
3	GF3	Yasuj	14	GF14	Yazd1
4	GF4	Kermanshah	15	GF15	Zanjan
5	GF5	Khozestan, Dezful	16	GF16	Khomeinyshahr, Esfahan
6	GF6	Yazd	17	GF17	Zarghan, Fars
7	GF7	Yazd, Abarkooh	18	GF18	Jiroft, Kerman
8	GF8	Bandar Abbas	19	GF19	Inported Seed
9	GF9	Rahnan, Esfahan	20	GF20	Esfahan
10	GF10	Ardestan, Esfahan	21	GF21	Kerman
11	GF11	Khoram Abad			

Hormonal treatments and experimental design

In this study, we performed a factorial experiment based on completely randomized design putting genotype, medium and type of explant as factors each replicated three times to study callus induction rate and parameters in genotypes of fenugreek. Three different culture media including; MS1: MS + (2 mg/L NAA + 0.5 mg/L BAP); MS2: MS + (2 mg/L 2,4-D + 0.5 mg/L BAP); MS3: MS + (2 mg/L IBA + 0.5 mg/L BAP) were used to examine the response of three

types of explant from each genotype: leaf, cotyledon, and embryonic axis. Leaf and cotyledon was prepared from 15-day old plants and embryonic axis from the hydrated seeds. Thirty days after putting them on the mentioned media, callus fresh and dry weight, dry to fresh callus weight ratio (in percent) and chlorophyll content of the calli were measured. Chlorophyll content was measured with a SPAD chlorophyll meter. We used IBM SPSS software (IBM Corporation, USA) for analyzing the data. Mean comparison was done using Duncan's new multiple range test (DNMRT) with $P<0.05$.

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Extraction and determination of 4-hydroxyisoleucine content

One hundred mg powder of dried calli and leaves of the GF21 genotype was macerated with the ratio of 1:4 w/v using ethanol 50% with shaking at 37 °C for 24 h two times. To determine free amino acid content in each extract, spectrophotometry was done using ninhydrin as the reagent. The concentration of amino acids was confirmed in 570 nm (Hardman and Abu-Al-Futuh 1979).

The presence of amino acids was confirmed in Ethanol extract. 4-hydroxyisoleucine in extract were analyzed by HPLC Agilent G1311A, Quaternary pump, Standard Auto-sampler, FLD Detector and Reverse phase column C18 5 micron 150*4.6 mm and used OPA derivatization that was described by (Hajimehdipoor, Sadat-Ebrahimi *et al.* 2010).

Results

The results of ANOVA (Table 2) showed that callus fresh and dry weight, ratio of dry to fresh weight and chlorophyll content were influenced by genotypes, hormones, explants. All two- and three-way interactions were also significant ($p<0.01$).

Callus fresh weight

The results showed that callus fresh weight was affected by genotype, hormone, explants and their interactions (Table 2). The mean comparison using DNMRT at $P<0.05$ indicated that the maximum CFW obtained in GF15 and GF14 genotypes as much as 8.72 and 8.23 g, respectively and the minimum CFW obtained in GF5 genotype was 4.98 g (Fig. 1). In this study, callus induction was observed in all hormonal treatments; however, maximum and minimum CFW were observed in the combination of IBA 2mg/L and 2-4-D 2mg/L, a mount of 8.14 g and 5.21 g, respectively (Fig. 8). The embryonic axis explants produced maximum callus amount of CFW 8.02 grams. Cotyledon explants produced minimum callus amount of CFW 4.6 grams (Fig. 7). The callus fresh weight was influenced by interaction genotype and hormones. The genotype GF14 and IBA 2mg/L had the highest CFW amount 12.3 gr, and the genotype GF18 with the 2-4-D 2mg/L all of them produced 3 gram. Mean comparison shown interactions between genotype and explants that genotype GF14 produced maximum CFW amount 11.65gr with embryo axis; genotype GF10 produce minimum CFW amount 3.64 g with embryo axis. Effects of hormonal treatment and explants shown that the highest CFW was observed in IBA 2mg/L on embryonic axis amount 11.22 gram. The lowest callus was obtained in 2-4-D 2mg/L hormone on embryonic axis explants amount 4.13 gram (Fig. 4).

The results shown that interaction was between genotype, hormone and explants; the maximum CFW were obtained amount of 6.22 gram in GF8, 14 on the embryonic axis explants with 2mg/L IBA hormone. On the other hand, the minimum CFW were obtained amount of 2.63 gram in GF21 on the embryonic axis explants with 2mg/L NAA hormone.

Table 2. Analysis of variance (ANOVA) of the measured traits affected by different treatments.

	Mean of squares				
Source of Variation	Degree of Freedom	CFW	CDW	DW/FW	CHI
Replication	2	2.72 ns	0.63 ns	0.075 ns	3.6ns
Genotype (G)	20	30.73**	2.35**	2.59*	40.17**
Hormonal treatment (H)	2	452.46**	40.40**	43.13**	97.23**
Explant (E)	2	155.33**	52.19**	85.35**	110.63**
G x H	62	34.17**	4.25**	3.28**	76.16**
G x E	62	24.61**	0.67**	4.12**	69.21**
H x E	8	284.59**	34.57**	45.56**	853.25**
G x H x E	187	29.23**	6880.5**	3.69**	62.56**
Error	566	6844.75	361.55	694.23	11863.6

* P<0.05, ** p<0.01; ns: Nonsignificant; CFW: callus fresh weight; CDW: callus dry weight,

DW/FW: callus dry weight/fresh weight x 100; CHI: chlorophyll index

Callus dry weight

Analysis of variances showed that callus dry weight was significantly influenced by genotype, hormonal treatment, explants and interaction two and three factors (Table 2). The mean comparison with Duncan's (P<0.05) indicate that the maximum CDW obtained in GF11 and GF8 genotypes amount 380.7 and 369.6 mg, respectively, and the minimum CDW obtained in GF4 genotypes amount 227.4 milligram (Fig. 2) in this study Maximum and minimum CDW were observed in the combination of NAA 2mg/L and 2-4-D 2mg/L, a mount of 371.3 mg and 240.2 mg, respectively (Fig. 8). The embryonic axis explants produced maximum dry callus amount of 341.3 mg. Cotyledon explants produced minimum dry callus amount of 260.1 mg (Fig. 7). The callus dry weight was influenced by the interaction of genotype and hormonal treatments. The GF8 genotype and 2mg/L NAA hormone had the highest CDW amount 565mg, and GF16 genotype on 2mg/L 2-4-D hormonal had the lowest CDW amount 160mg.

Mean comparison showed interactions between genotype and explants that GF11 genotype produced maximum CDW amount 516 mg with embryo axis and GF10 genotype produce minimum CDW amount 136 mg with embryo axis. Effects of hormonal treatment and explants shown that the highest CDW was observed in NAA 2mg/L on embryonic axis amount 481.2 mg. The lowest CDW was obtained in 2mg/L 2-4-D hormone on cotyledon explants amount 218.02 mg (Fig. 5). The results shown that interaction were between genotype, hormone and explants; the maximum CDW was obtained amount of 931.1 mg in GF14 and GF11 on the embryonic axis explants with 2mg/L IBA hormone. On the other hand, the minimum CDW was obtained amount of 52.2 mg in GF21 on the leaf explants with 2mg/L IBA hormone.

Relative percentage of dry to fresh weight callus

The result of Analysis variances showed that Relative percentage of dry to fresh weight callus was significantly influenced by genotype, hormonal treatment, explants and interaction two and three factors (Table 2). The mean comparison with Duncan's (P<0.05) indicate that the maximum DW/FW obtained in GF18 and GF8 genotypes amount 5.06 and 4.81g respectively, and the minimum DW/FW obtained in GF14 genotypes amount 3.69 g (Fig. 1). in this study Maximum and minimum DW/FW were observed in the combination of NAA 2mg/L and IBA 2mg/L, a mount of 5.06 g and 3.8 g respectively (Fig. 8). The embryonic axis explants produced maximum DW/FW callus amount of 5.13g. Cotyledon explants produced minimum DW/FW callus amount of 3.94g (Fig. 7). The Relative percentage of dry to fresh weight callus was influenced by the interaction of genotype and hormonal treatments. The GF15 genotype and 2mg/L NAA hormone had the highest DW/FW amount of 9.13g, and GF8 genotype on 2mg/L IBA hormonal had the lowest DW/FW amount 3.1g. Mean comparison showed interactions between genotype and explants that GF15 genotype produced maximum DW/FW amount 9.54g with embryo axis and GF13 genotype produce minimum DW/FW amount 3.59g with cotyledon explant.

Effects of hormonal treatment and explants shown that the highest DW/FW was observed in 2-4-D 2mg/L on embryonic axis amount 6g. The lowest DW/FW was obtained in 2mg/L IBA hormone on cotyledon explants amount 3.23g (Fig. 4). The results shown that interaction were between genotype, hormone and explants; the maximum DW/FW was obtained amount of 9.8g in GF15 on the embryonic axis explants with 2mg/L NAA hormone. On the other hand, the minimum DW/FW was obtained amount of 1.4g in GF10 on the embryonic axis explants with 2mg/L IBA hormone.

Chlorophyll Index

In the present study total chlorophyll was analyzed in callus. The results shown that, Chlorophyll index was affected by genotype, hormone, explants and interacting two and three factors (Table 2). The mean comparison with Duncan's ($P<0.05$) indicate that, the maximum Chlorophyll index obtained in GF21 and GF8 genotypes amount 22.8 and 20.5 respectively, and the minimum Chlorophyll index obtained in GF2 genotypes amount of 17.32 (Fig. 3). In this study Maximum and minimum Chlorophyll index were observed in the combination of NAA 2mg/L and 2-4-D 2mg/L, a mount of 22.58 and 17.8 respectively (Fig. 8). The leaf explants produced maximum Chlorophyll index amount of 21.9. Embryonic axis explants produced minimum Chlorophyll index amount of 17.2 (Fig. 7). The Chlorophyll index was influenced by interaction genotype and hormones. The genotype GF21 and NAA 2mg/L had the highest Chlorophyll index, and the genotype GF19 with the 2-4-D 2mg/L produced amount of 15.7. Mean comparison shown interactions between genotype and explants that genotype GF21 produced maximum Chlorophyll index amount 25.3 with embryo axis; genotype GF2 produce minimum Chlorophyll index amount 13.8 with embryo axis. Effects of hormonal treatment and explants shown that the highest Chlorophyll index was observed in NAA 2mg/L on cotyledon amount 24.8. The lowest Chlorophyll index obtained in IBA 2mg/L hormone on embryonic axis explants amount 15.37 (Fig. 6).

The results shown that interaction was between genotype, hormone and explants; the maximum Chlorophyll index were obtained amount of 24.1 in GF21 on the embryonic axis explants with 2mg/L NAA hormone. On the other hand, the minimum Chlorophyll index were obtained amount of 11.46 in GF3 on the embryonic axis explants with 2mg/L IBA hormone.

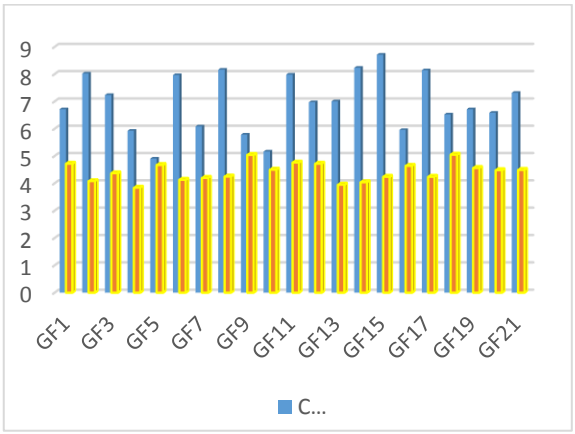

Fig. 1. Mean comparison of the effect genotype on CFW and DW/FW (gram).

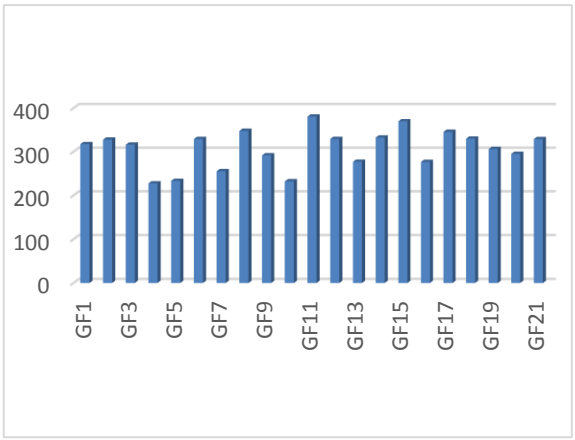

Fig. 2. Mean comparison of the effect genotype on CDW (mg).

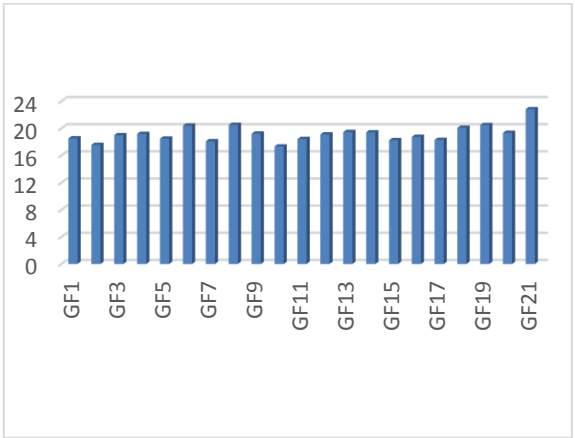

Fig. 3. Mean comparison of the effect genotype on CHI.

Mean comparison Fig.; GF1- GF21 are the genotypes used in Table (1); CFW: callus fresh weight (g); CDW: callus dry weight (mg); DW/FW: dry weight percentage to callus fresh weight(g/g ×100); CHI: Chlorophyll index

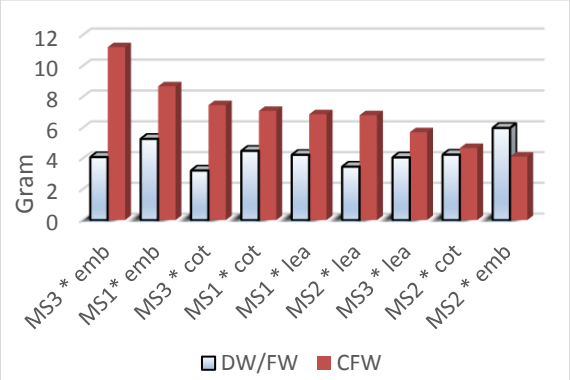

Fig. 4. Mean Comparison of the interactions between hormone composition and explant on CFW and DW/FW (gram).

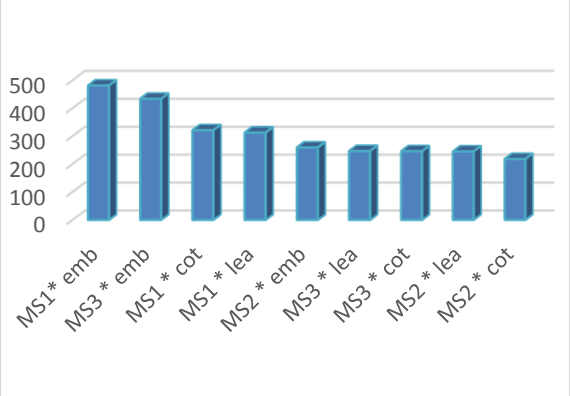

Fig. 5. Mean Comparison of the interactions between hormone composition and explant on CDW(mg).

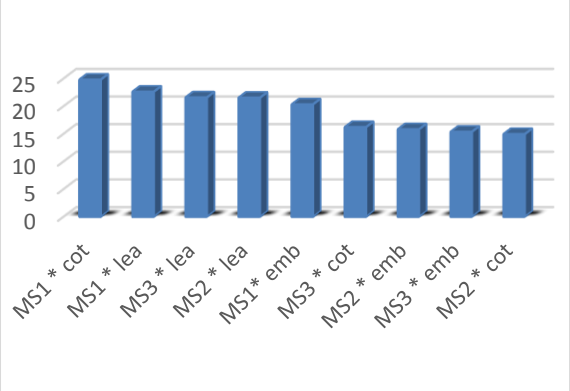

Fig. 6. Mean Comparison of the interactions between hormone composition and explant on CHI.

Mean comparison Fig.; MS1: (2mg/l NAA+0/5mg/l BAP), MS2: (2 mg/l 2-4-D+0/5mg/l BAP), MS3: (2mg/l IBA+0/5mg/l BAP); EMB: embryo axis; LEA: leaves; COT: cotyledon; CFW: callus fresh weight (g); CDW: callus dry weight(mg); DW/FW:dry weight percentage to callus fresh weight(g/g ×100); CHI: Chlorophyll index.

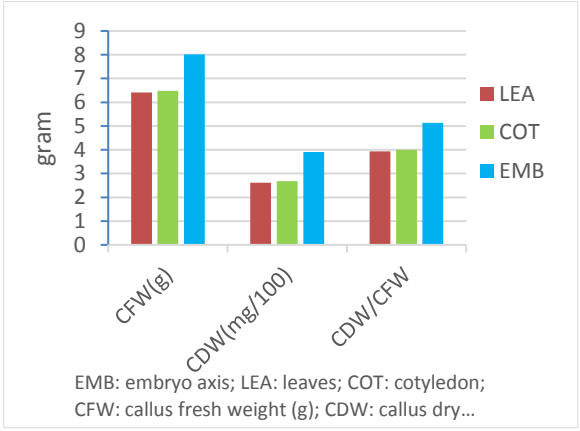

Fig. 7. Mean Comparison the effects of explant on measured traits.

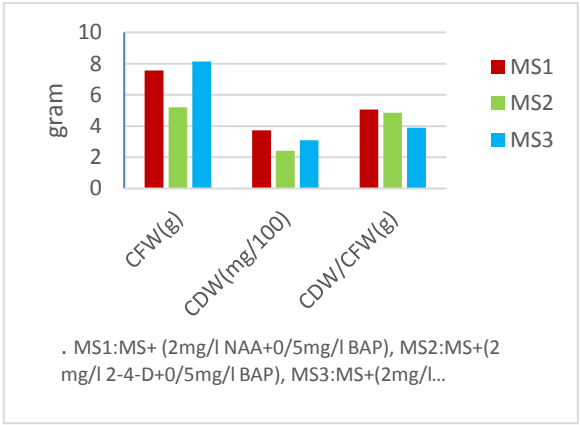

Fig. 8. Mean Comparison the effects of auxin on measured traits.

Discussion

Bioactive compound are economically important as drugs. Interest in bioactive compound can be altering the production of Secondary metabolite by means of tissue culture technology. We can compare Amount of drugs, which are artificially produced in callus and naturally occurring in plant. Fenugreek is the most important vegetable and medicinal plant. Short time and high callus induction is important for bioactive production in vitro culture. the culture medium –MS or B5- does not affect callus induction (El-Nour, M. *et al.* 2013; Afshari, E. *et al*; 2011). Previous study indicate that hypocotyl, cotyledon, leaf and root is

successful in callus induction (Agarwal, M *et al.* 2015; ElNour, Ali *et al.* 2015; Prabakaran and Ravimycin 2012; Ciura, Szeliga *et al.* 2015; Elaleem, Ahmed *et al.* 2014; El-Nour, M. *et al.* 2013; Afshari, E. *et al*; 2011)) In these study all explants have callus induction but embryonic axis explants have produced the maximum amount of CFW, CDW and CDW/CFW these results agree with previous study. In these research 0.5 mg/l BAP was used in combination with three types of auxins: NAA, 2-4-D and IBA hormone. Result show that, Maximum callus fresh weight was obtained in 2mg/L IBA + 0. 5mg/L BAP treatment, and the highest CDW, CDW / CFW were obtained with 2mg/L NAA + 0. 5mg/L BAP. These results have few different with result obtained by (Ciura, J. *et al* 2015; Elaleem, KH. *et al* 2014; ELNour, M. *et al* (2013), suggest that 1.5- 2mg/L 2-4-D is the best hormone for callus formation. In previous study don't reported genotype effects on callus production. We observed that each genotype has a special feature.

Genotype GF15 and GF14 had the maximum amount of callus fresh weight, but genotypes GF8 and GF11 had the maximum callus dry weight. Also Minimum callus dry & fresh weight observed in genotype GF10. Chlorophyll content is important for primary metabolite production. In these study combination leaf explant and 2mg/l NAA have a maximum chlorophyll index, the results obtained here agree with (Prabakaran and Ravimycin 2012). interaction between three factor Indicate that the maximum Chlorophyll index were obtained amount of 24.1 in GF21 on the embryonic axis explants with 2mg/L NAA hormone. Callus induction is controlled by the level of plant growth regulators, Explant and plant species. The results show that interaction were between genotype, hormone and explants; the maximum CDW was obtained amount of 931.1 mg in GF14, GF11 on the embryonic axis explants with 2mg/L IBA hormone.

Also maximum CFW was obtained amount of 6.22 gram in GF8, GF14 on the embryonic axis explants with 2mg/L IBA hormone. Maximum DW/FW was obtained amount of 9.8g in GF15 on the embryonic axis explants with 2mg/L NAA hormone. On the other hand, the minimum DW/FW was obtained amount of 1.4g in GF10 on the embryonic axis explants with 2mg/L IBA hormone. This information indicate that callus production is strongly controlled by fenugreek species. The result provide important knowledge on callus production, emphasizing that callus can be a good source of secondary metabolite production. 4-Hydroxyisulosine is produced in vitro culture about 67% of mother plant. 4-hydroxyisoleucine accumulation was same way with mother plant. There has been no alteration in the metabolic pathway as a result of in vitro culture. Therefore selection and study interaction between various factors of callus induction are essential for the production compounds such as diosignin, trigonellin, and 4-hydroxyisulosinein in cell bioreactor.

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