



EFFECT OF GROWTH REGULATOR NAA AND IBA APPLICATIONS ON TOTAL PHENOLIC AND FLAVONOID COMPOUNDS EXTRACTED FROM *IN VITRO* PRODUCED CALLUS OF CHICORY PLANT (*Cichorium intybus* L.)

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Received 29 July, 2019

Accepted 26 August, 2019

ABSTRACT

This research study was carried out in the plant tissue culture laboratory of the Agricultural Botany Department, Faculty of Agriculture, Ain Shams Univ., Shoubra El-khaima, Cairo, Egypt. Experiments were executed for the duration of two consecutive years 2017 and 2018 on chicory plant. Chicory (*Cichorium intybus* L.), which belongs to Asteraceae family, is considered as an important medicinal plant due to the presence of many bioactive substances such flavonoids, phenolic compounds, alkaloids, steroids, terpenoids, including (coumarines, cichoriin, esculetin, inulin, sesquiterpene lactones, chicoric acid, caffeic acid and some vitamins). In this research *in vitro* experiments were carried out using full strength Murashige and Skoog basal medium (MS) supplemented with different combinations of two plant growth regulators; Indole-3-butyric acid (IBA) including two concentrations (0.5 – 2.0 mg/l) and Naphthalene acetic acid (NAA) comprising four concentrations (0.5 – 2.0 – 3.0 – 5.0 mg/l). An abaxially (lower side) leaf explants (square pieces 0.5 × 0.5 cm) which were taken from 20 days old aseptic chicory seedlings were inoculated to (MS) surface. Initially, chicory seeds were aseptically germinated on half-strength MS medium, after surface sterilization by 70 % (v/v) ethanol for 60 seconds then soaking in 10 % Clorox (0.5% sodium hypochlorite NaOCl) for 10 min to produce the aseptic chicory seedlings which were the source of true leaf explants used in this research study. Total phenolic compounds and flavonoids content were extracted from six-week *C. intybus* friable callus produced under both light and dark *in vitro* culture conditions inside a growth

chamber incubation room where temperature was adjusted at 25°C ±1. Total phenolic compounds and flavonoids were determined by spectrophotometric methods. The highest values for their contents were from chicory calli when MS callus induction medium was supplemented with 2 mg/l NAA under total dark condition when compared with the other remaining growth regulator treatment combinations and alternative light regime conditions.

Keywords: *Cichorium intybus*, NAA, IBA, Callus, Phenolic compounds, Flavonoids

INTRODUCTION

Most plant species in the plant kingdom particularly higher plants can produce bioactive compounds, which can play a serious role in human health (**Castro et al 2016**). Medicinal plants are the main best source of industrial and bioactive medicinal materials which are referred to sometimes as plant secondary metabolites that can be used in food, pharmaceutical, and cosmetic industries (**Eshun & He, 2004 and Khan & Abourashed, 2011**). Plant secondary metabolites can be used without harm as food additives (flavors, fragrances, sweeteners, colorants and food preservatives), nutraceuticals, and pharmaceuticals (**Smetanska, 2008**).

Tissue culture methods have gained more attention nowadays as an applicable safe and reliable technology to produce plant metabolites especially secondary metabolites (**Zhong, 2001**). This is because tissue culture techniques have many advantages for production of plant metabolites by offering a defined production system with continu-

ous supply of metabolites, typical quality and quantity in short culture periods free of diseases and are not exposed to seasonal, geographical and environmental factors. Furthermore, new metabolites are produced *in vitro* by tissue cultures which aren't usually produced inside mother plant grown in the field (Sasson, 1992). Plant secondary metabolites can be produced *in vitro* by callus, cell suspension, organ and hairy roots cultures (Bourgaud et al 2001).

Chicory, (*Cichorium intybus* L.) is a plant member of the Aster family, (Asteraceae formerly known as the Compositae Family), that also involves Artichoke, Cornflower, Dandelion, Calendula, Sow thistle and Echinacea (Bais and Ravishankar, 2001). It is indigenous to Europe, Western Asia, Egypt, North America, and Italy easily identified by its light sky-blue flowers (Clapham et al 1962). *Cichorium intybus* L. has many common English names including blue daisy, blue sailors, blue weed, coffee-weed, common chicory, succory, bunk (Mitich, 1993). Historically, chicory was grown by the ancient Egyptians as a medical plant, leafy vegetable, and for animal feed (Greive, 1971). Moreover, chicory is all well known in Arabic and Islamic culture and is named as "hindiba" and "seris" in the Arabic language (Sari, 2007).

Medical importance of the chicory plant is attributed to its containment of many important metabolites such as flavonoids, phenolic compounds, alkaloids, steroids, terpenoids, including (coumarines cichoriin, esculetin, inulin, sesquiterpene lactones, chicoric acid, caffeic acid and vitamins), (Gonzalez, 1977; Rees & Harborne, 1985 and Abbas et al 2015). Knowingly, all parts of chicory plant were usually used from ancient times until now to treat different symptoms and ailments like liver disorders, kidney, digestive, urinary disorders, gallstones, cholesterol, diabetes, jaundice, tumors, wound healing and as a blood purifier etc. (Duke, 1983 and Street et al 2013). Chicory *in vitro* callus cultures can provide the drug and food industry with many of in demand secondary metabolites under controlled conditions in a continuous manner as was mentioned by Malarz et al (2005). Therefore, the objective of this research study was to produce some secondary metabolites (total phenolic and flavonoids compounds) from chicory plant (*Cichorium intybus* L.) through *in vitro* callus culture.

MATERIALS AND METHODS

Location and Duration

This research study was carried out in the plant tissue culture laboratory of the Agricultural Botany Department, Faculty of Agriculture, Ain Shams University, Shoubra El-khaima, Cairo, Egypt. Experiments were performed for the duration of two consecutive years 2017 and 2018.

Plant Materials

Chicory seeds were collected from a plant well defined morphologically and grown in the open field in the Province of Al-Qalyubiya in June 2016. The collected seeds were incubated in a sealed jar and kept inside a fridge (4:5°C). These seeds were used to produce aseptic seedlings as was described by Nandagopal and Ranjitha Kumari (2006).

Surface Sterilization of Initial Explants

Seeds were surface cleaned by washing in running tap water several times. Then, they were washed in a solution of liquid detergent 1 % (v/v) for 5 minutes, subsequently rinsed for several times by distilled water. After thorough washing, the seeds were taken into the laminar flow cabinet and soaked for 60 seconds in 70 % (v/v) ethanol in small petri dishes then sterilized by soaking in 10% Clorox (0.5% sodium hypochlorite NaOCl) for 10 min (Babaei et al 2013). Thereafter, the sterile seeds were rinsed 3-5 times in autoclaved distilled water. They were aseptically germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for 20 days (Malarz et al 2005 and Abdin & Ilah, 2007).

Callus Induction

Full-strength MS basal medium (Caisson Labs company, USA) supplemented with combinations of Naphthalene acetic acid (NAA) concentrations (0.5 – 2.0 – 3.0 – 5.0 mg/l) and Indole-3-butyric acid (IBA) concentrations (0.5 – 2.0 mg/l) was used for callus induction. Then, the MS basal medium provided with 30 g/l sucrose was adjusted to PH 5.7 and solidified with 0.7% (w/v) agar (B&V

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srl-parma company, Italy). The medium was dispensed into glass jars (100 ml) containing 25 ml of medium (Table 1), then the media were autoclaved at 121°C for 20 minutes for sterilization. Explants used in this research study for *in vitro* callus production were true leaves taken from 20 days old sterile seedlings resulting from *in vitro* seed germination (Rehman et al 2003). Then, these leaves were cut into square pieces (0.5 × 0.5 cm) under aseptic conditions inside the laminar flow cabinet. The leaf explants were abaxial inoculated separately in 5 explants per jar on the surface of medium. Finally, media were incubated for six weeks under continuous light or total dark conditions at 25°C ±1 (Nandagopal and Kumari, 2007).

Table 1. Summary of *in vitro* callus induction treatments and their abbreviated codes

No.	Code	Specific treatments definition
1	T1	NAA 0.5mg/l + IBA 0.5mg/l
2	T2	NAA 3mg/l + IBA 2mg/l
3	T3	NAA 5mg/l + IBA 2mg/l
4	T4	NAA 2mg/l
5	T5	NAA 3mg/l

Note: two different types of synthetic auxin, Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) were used.

Chemical Analyses

From samples of the six-week *C. intybus* friable calli produced *in vitro*, internal chemical components were determined for the following:

- Total phenolic compounds by a spectrophotometric method according to Singleton and Rossi (1965).
- Total flavonoids according to the method described by Nagy and Grancai (1996).

Experimental Design and Statistical Analysis

All experiments were arranged in a completely randomized design with five replicates for each treatment. Each replicate contained one jar and each jar contained five explants. Experiments included two factors: first factor was induction chemical treatments and, second factor was light condition during induction continuous light and dark. Duncan's multiple-range test at probability level of 5% was employed for mean comparisons according to Duncan (1955).

RESULTS AND DISCUSSION

Friable calli formation were observed after 7 days from the inoculation of chicory leaf explants onto callus induction media (Malarz et al 2005). Six-week calli were collected and extracted to determine phenolic compounds and flavonoids.

Result data presented in Table (2) show that the differences among treatments were significant in general. Regarding the effect of auxin treatments, the differences were not significant, except with T4 (NAA 2mg/l) which gave a higher value (0.262 and 0.292 mg/g) when compared to T3 (NAA 5mg/l + IBA 2mg/l) which was lower (0.193 and 0.215 mg/g) in the years 2017 and 2018, in respect order. As for the effect of light conditions, the differences were not significant between light and dark. As for the effect of the interaction between auxin and light condition treatments, results revealed significant differences amongst treatments, the highest value being T4 (NAA 2mg/l) under dark conditions (0.269 and 0.299 mg/g) for both years 2017 and 2018 respectively when compared with the remaining combinations.

Result data displayed in Table (3) indicate that the differences among treatments in total flavonoids content were significant in general. Regarding the effect of auxin treatments only, the differences were also significant, but the higher value was T4 (NAA 2mg/l). With regards the effect of light conditions, the differences were not significant. Finally, the effect of the interaction between auxin treatments and light conditions treatments revealed significant differences, and the highest value was T4 (NAA 2mg/l) under dark conditions for both two years.

Results obtained here agree with Kumari et al (2007) who used MS medium supplemented with 2 mg/l NAA to produce secondary metabolites like esculin from chicory callus. Boeuf et al (2001) also obtained friable callus from *C. intybus* mature root explants by using Heller's medium (Heller, 1953) supplemented with NAA concentrations (2 – 0.2 mg/l) *in vitro* culture and measured proteins and peroxidase activity enzyme. However, green compact callus was developed on MS medium fortified with 0.1–2.0 mg IBA and NAA in combination with 0.5 mg/l BAP in leaf, cotyledon, hypocotyl, and root of *C. intybus* explants as tested by Velayutham et al (2006). Shilpa and Lakshmi (2019) reported that total phenol content and chlorogenic acid could be extracted from *C. intybus* calli that were produced from chicory young leaf explants inoculated on MS medium with varied combinations of NAA and kinetin (KIN).

Table 2. Effect of auxin type and concentration on total phenolic compounds extracted from friable callus produced in dark and light by *in vitro* culture from leaf explants of Chicory (*Cichorium intybus* L.) in two years (2017 and 2018)

2017				
Auxin treatments	Treatment code	Total phenolic compounds (mg/g)		
		Light	Dark	mean
NAA 0.5mg/l + IBA 0.5mg/l	T1	0.224 ABCD	0.236 ABC	0.230 AB
NAA 3mg/l + IBA 2mg/l	T2	0.211 BCD	0.222 BCD	0.217 AB
NAA 5mg/l + IBA 2mg/l	T3	0.188 D	0.198 CD	0.193 B
NAA 2mg/l	T4	0.256 AB	0.269 A	0.262 A
NAA 3mg/l	T5	0.216 BCD	0.227 ABCD	0.222 AB
	Mean	0.219 A	0.231 A	
2018				
Auxin treatments	Treatment code	Total phenolic compounds (mg/g)		
		Light	Dark	mean
NAA 0.5mg/l + IBA 0.5mg/l	T1	0.243 A	0.256 A	0.255 AB
NAA 3mg/l + IBA 2mg/l	T2	0.235 CD	0.247 BCD	0.241 AB
NAA 5mg/l + IBA 2mg/l	T3	0.209 D	0.220 CD	0.215 B
NAA 2mg/l	T4	0.284 AB	0.299 A	0.292 A
NAA 3mg/l	T5	0.240 BCD	0.253 ABCD	0.246 AB
	Mean	0.243 A	0.256 A	

Values in the same column followed by the same letter(s) are not statistically different according to Duncan's multiple range test (Duncan, 1955).

Table 3. Effect of auxin type and concentration on total flavonoids extracted from friable callus produced in dark and light by *in vitro* culture from leaf explants of Chicory (*Cichorium intybus* L.) in two years (2017 and 2018)

2017				
Auxin treatments	Treatment code	Total flavonoids (mg/g)		
		Light	Dark	mean
NAA 0.5mg/l + IBA 0.5mg/l	T1	0.157 ABC	0.165 ABC	0.161 AB
NAA 3mg/l + IBA 2mg/l	T2	0.148 ABC	0.156 ABC	0.152 AB
NAA 5mg/l + IBA 2mg/l	T3	0.132 C	0.139 BC	0.135 B
NAA 2mg/l	T4	0.179 AB	0.188 A	0.184 A
NAA 3mg/l	T5	0.151 ABC	0.159 ABC	0.155 AB
	Mean	0.153 A	0.161 A	
2018				
Auxin treatments	Treatment code	Total flavonoids (mg/g)		
		Light	Dark	mean
NAA 0.5mg/l + IBA 0.5mg/l	T1	0.174 ABC	0.183 ABC	0.179 AB
NAA 3mg/l + IBA 2mg/l	T2	0.164 ABC	0.173 ABC	0.169 B
NAA 5mg/l + IBA 2mg/l	T3	0.147 C	0.154 BC	0.151 B
NAA 2mg/l	T4	0.199 AB	0.209 A	0.204 A
NAA 3mg/l	T5	0.168 ABC	0.177 ABC	0.173 AB
	Mean	0.170 A	0.179 A	

Values in the same column followed by the same letter(s) are not statistically different according to Duncan's multiple range test (Duncan, 1955).

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Al Khateeb et al (2012) found that MS medium with NAA plus 6-Benzylaminopurine (BAP) (0.5: 2.0 mg/l) can produce callus from chicory young leaf explants and was able to obtain high amount of phenolic compounds from it.

Other growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) plus kinetin (1mg/l + 0.3 mg/l in order) added to MS medium were used by **Malarz et al (2005)** to initiate chicory callus *in vitro* from leaf explants and isolate three new and one known furofuran lignans. In addition, 2, 4-D (0.5-1.0 mg/l) alone or in combination with BAP (0.5-1.0 mg/l) were added to MS medium to induct callus from chicory leaf, stem and bud explants and led to increase total phenolics and flavonoids content (**Verma et al 2012**).

In this study, synthetic auxins IBA and NAA were used in aseptic culture systems to induce and stimulate callus formation. Their use in inducing callus were established quite a long time ago. The common practice of using synthetic auxins such as IBA or NAA as substitutes for naturally 3-indole acetic acid (IAA) was shown to be more effective for certain purposes such as inducing callus (**Bonga, 1982**). The promoting effects of auxins could be interpreted by their mechanisms to cause cell enlargement, cell elongation and loosening cell walls within a few minutes after application by modification of cell membranes (**Wareing and Phillips 1970 and Kevers et al 1981**).

It is worth mentioning that, Phenolics are secondary plant metabolites that are ubiquitously present in plants and their products (**Razali et al 2008**). Flavonoids are one of the largest classes of plant phenolics. Plant phenolics are biosynthesized by several different routes and thus constitute a heterogeneous group from a metabolic point of view. Two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway. In higher plants, most phenolics are derived at least in part from phenylalanine, a product of the shikimic acid pathway. Phenylalanine is an intermediate in the biosynthesis of most plant phenolics. Synthetic auxins such as NAA and IBA are also derived from phenylalanine, tyrosine, or tryptophan as aromatic amino acids (**Taiz and Zeiger, 2002**).

Physiologically, auxins are known to trigger their effects through affecting some steps in protein synthesis or by affecting enzyme activity (**Wareing & Phillips, 1970 and Leopold & Kriedemann, 1975**). Synthetic auxins like IBA and NAA produce effects similar to those which IAA

(the natural auxin) itself elicits (**Wareing and Phillips, 1970**). One possibility to interpret results obtained here could be that auxins applied exogenously may have exerted an effect by affecting phenylalanine ammonia-lyase (PAL) which is an enzyme considered as a precursor for the various phenolic and flavonoid compounds by initially forming cinnamic acid in the process (**Dey & Harborne, 1997; Heldt, 1997 and Taiz & Zeiger, 2002**).

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تأثير معاملات منظمات النمو NAA و IBA على مركبات الفينولات والفلافونات الكلية
المستخلصة من الكالس الناتج معملياً من نبات الشيكوريا *Cichorium intybus*

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Received 29 July, 2019

Accepted 26 August, 2019

الأوراق الحقيقية لبادرات الشيكوريا عمر 20 يوم
المستتبتة معملياً من البذرة على بيئة مورايشيغ وسكوج
نصف القوة والتي تم تعقيمها باستخدام كلوروكس
10% لمدة 10 دقائق. تم تقدير الفينولات والفلافونات
الكلية في الكالس المفكك (عمر 6 أسابيع) الناتج تحت
ظروف من الإضاءة والإظلام. أوضحت النتائج أن
أعلى قيمة بالنسبة لمحتوى الكالس من المركبات
الفينولية والفلافونية الكلية كانت عند استخدام NAA
بتركيز 2 ملجم/لتر تحت ظروف الإظلام.

الكلمات الدالة: شيكوريا, *Cichorium intybus*,
كالس, نقتالين حمض الخليك, إندول حمض البيوتريك,
فينولات, فلافونات

الموجز

يعتبر نبات الشيكوريا *Cichorium intybus*
التابع للعائلة النجمية من النباتات الطبية المهمة بسبب
إحتوائها على العديد من المركبات الفعالة خاصة
الفينولات والفلافونات. وفي هذه الدراسة أجريت بعض
التجارب في معمل زراعة الأنسجة بقسم النبات الزراعي
كلية الزراعة - جامعة عين شمس في الفترة من
2017 إلى 2018. تمت التجارب باستخدام بيئة
مورايشيغ وسكوج MS كاملة القوة مضافاً إليها نوعين
من منظمات النمو وهما نقتالين حمض الخليك NAA
وإندول حمض البيوتريك IBA بتركيزات (0.5 - 2.0
- 3.0 - 5.0 ملجم/لتر) و (0.5 - 2.0 ملجم/لتر)
على الترتيب، وذلك لزراعة منفصلات نباتية من