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Formation of Catechin in Callus Cultures and Micropropagation of *Rheum ribes* L.

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Abstract: The aim of this project was to study of the effects of different nutritional factors on the production of catechin in *Rheum ribes* L. and micropropagation of this species in agar-solidified medium. Different parts of *Rheum ribes* sterile seedlings were cultured on MS medium that supplemented with IBA (1 mg L^{-1}) and BA (1 mg L^{-1}) for generation of callus. The concentration of sucrose, vitamins and myo-inositol and ratio of NO_3^- to NH_4^+ in the medium was changed and content of catechin was determined by HPLC. Myo-inositol 100 mg L^{-1} in the medium that supplemented with 0.5 mg L^{-1} IBA and 0.5 mg L^{-1} BAP. Rooting medium was ½ MS salts + 0.5 mg L^{-1} IBA. Regenerated plants were transferred to clay pots a mixture of soil, vermiculite and sand and maintained under 50-70% plants for the first 7 days.

Key words: Plant growth regulators, rhubarb, sucrose, vitamins, myo-inositol

INTRODUCTION

Catechins, a group of flavones, were recently noted as antitumour agents[1], as antioxidants[2] and radical scavengers[3].The major catechins found in grape seeds (*Vitis vinifera*) were (+)-catechin, (-)-epicatechin and as minor components, procyanidin B_1, B_2, B_3, B_4 and C_1[4,5]. The major components detected in soluble instant tea (*Camellia sinensis*) were (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin-3-O-gallate and (-)-epigallocatechin-3-gallate accompanied by a minor component (+)-catechin[6].

In general dedifferentiated plant cells such as callus are known to reduce or stop production of secondary metabolites, which the parent plant produces *in vivo*[7]. Nevertheless, *in vitro* productions of (-)-epicatechin-3-O-gallate accompanied by (+)-catechin and (-)-epicatechin have been reported in *Fagopyrum esculentum* calli and cultured hairy roots[8] and cultured cells of *Polygonum hydropiper*[9].

There are a few reports concerning secondary metabolites formation in *Rheum* sp.[10]. In the case of *Rheum palmatum* Rai[11] reported that the total contents of anthraquinones was much lower in cultures (0.3%) than in parent plant (2-5%). Kurosaki *et al.*[12] reported production of chrysophanol and emodin stimulated in culturd rhubarb tissue by treatment of cells with ethylene generating reagent: 2- chloroethylphosphonic acid. *In vitro* production of *Rheum emodi*[13] has been done using agar-solidified medium and with liquid culture producers[14]. Lassus and Voipio[15] micropropagated *Rheum rhabarbarum* with special reference to weaning stage and subsequent growth. Farzami Sepehr and Ghorbanli[16] studied effects of different nutritional factors on formation of anthraquinons in callus of *Rheum ribes* (Polygonaceae), this plant is the source of one of the most important crude drugs in Asiatic regions[17]. Rhubarb roots are used an original laxative medicine and an antipsoriatic drugs in Iran[18].

MATERIALS AND METHODS

Cultured callus: The seeds of *Rheum ribes* L. (Polygonaceae) were collected from Dosdarreh in Shahrestanak (2330-2600 m) around Tehran city on summer of 2002. Sterilization of seeds was done in 3 steps: in the first step seeds were immersed in Benomil solution (0.3%) for 1 h, then rinsed for 1 h in rising water. In the second step, the seeds were transferred to ethanol for 1 min and sodium hypochlorite solution (50%) for 10 min. In the third step the seeds were rinsed 3 times in sterile distilled water. The seeds were germinated on solid MS medium.

Callus cultures were raised from 5 days old seedling hypocotyls on Murashige and Skoog medium (19), supplemented with 30 g L^{-1} sucrose, 50 g L^{-1} myo-inositol, 8 g L^{-1} agars, BA (1 mg L^{-1}) and IBA (1 mg L^{-1}). The initial pH of medium was adjusted to 5.8 before autoclaving. The cultures were incubated at 25±2°C, 16 h light/8 h dark with irradiance of 50 μM (photon) m^{-2} s^{-1}. Maintains of cultures was carried out by periodic subculture at 2 week intervals. After three successive

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subcultures on the basic medium, callus from 6 containers, each container holding 4 calluses, were removed and after determination of their fresh weights, transferred to different media. Twenty four calluses were used for each treatment. The different media contained changes in sucrose, myo-inositol, vitamins contents (full concentration of vitamins solution included: Nicotinic acid 100 mg L^{-1}, pyridoxine HCl 100 mg L^{-1}, thiamine HCl, 100 mg L^{-1}, glycine 400 mg L^{-1}) and ratio of NO_3^- to NH_4^+ in the medium. After transferring the calluses to various media, subculturing was done at 2-week intervals and after 42 days the fresh mass and (+) – catechin contents of callus was determined.

Micropropagation: After production of callus and subculturing at 2-week intervals, for multiple shoot formation and root regeneration calluses were transferred to different MS mediums that supplemented with different concentration of IBA and BA. The conditions for growth and regeneration were constant.

The extraction of catechin: Each (0.5 g) of materials of calli, leaves, stems and roots of *Rheum ribes* was ground mechanically with liquid nitrogen and extracted with 2 mL acetone (2 mL ×3). Each extract was, after filtration through a filter paper (Wattman No. 2), dissolved in 2 mL H_2O after evaporation and dryness at 30°C and their lower phases were collected after separation by addition of 2 mL n-hexan (2 mL ×3). Then, the EtOAc phases were separated and collected by adding 2 mL EtOAc into lower phases and dissolved in 1 mL MeOH: 0.1%, H_3PO_4 = 15:85 after concentration and dryness (2 mL ×3) and were analyzed by HPLC filtration through a Millipore filter (0.45 μm).

HPLC analysis of (+) – catechin: The HPLC system: A crystal 200 liquid chromatography isocratic pump, Unicame UV detector model 4225, Unicame computer integrator model 4851, GTO 6A, column, fluofix 120NØ 406 mm×25 cm, particle size 5 μm was obtained from Unicame (England), The moving bed, MeOH: 0.1%, H_3PO_4 = 15:85, detection of wavelength: 280 nm, velocity of flow 0.5 cm^3 min^{-1}, injection volumes: 5 μL, chart speed: 10 mm min^{-1}.

The standard solution (100 μL L^{-1}) of (+) – catechin was prepared in mobile phase. The standard solutions of 0.01-100 mL L^{-1} were prepared by dilution of stock with mobile phase from which a 5 mL aliquot was injected[19]. An external standard method was used for quantitative determination. The calibration curve was established for (+)- catechin using peak area.

Statistical analysis: The results were analysed with SPSS (version 10.05) statistical package. All the experimental values reported in this article are the means of at least three individual experiments.

RESULTS AND DISCUSSION

Determinations of (+)-catechin content in different parts of *Rheum ribes* were shown higher accumulation of this material in stem (Table 1). To determine the optimal amount of sucrose needed for catechin production, three media containing different concentration of sucrose were used. The maximum content of (+)-catechin was obtained in medium with 3% sucrose (Table 2).

Increasing the concentration of vitamins did not promote catechin production and the highest concentration of (+) – catechin was obtained in free vitamin medium (Table 3).

The addition of myo-inositol decreased production of catechin. The highest catechin yield was achieved at level of 100 mg L^{-1} (Table 4) and increasing levels of myo-inositol suppressed catechin formation in calluses.

For study of the effect of nitrogen source on catechin formation, the ratio of ammonium to nitrate was differed in the basal medium. When the ratio of ammonium: Nitrate

Table 1: Comparison of catechin contents between 3 parts of rhubarb

Different parts of rhubarb	(+)-Catechin mg/100 g FW
Stem	5.06±0.08
Leaf	4.85±0.25
Root	1.25±0.01

Values are Mean±SE from 6 replicated,
Catechin contents are not significant between stem and leaf ($p<0.05$)
FW: Fresh Weight

Table 2: Effect of sucrose content on catechin production callus cultures of rhubarb

Sucrose (%)	(+)-Catechin mg/100 g FW
3	8.64±1.00
6	5.32±0.05
9	3.48±0.00

Murashige and Skoog medium, 1 mg L^{-1} IBA+1mg L^{-1} BA
Values are Mean±SE from 6 replicated
All the values are significantly different ($p<0.05$), FW: Fresh Weight

Table 3: Effect of vitamin concentration on catechin production in cultured callus of rhubarb

Basal medium[a]	(+)-Catechin mg/100 g FW
Vitamin free medium	7.32±0.03
0.25 strength	6.12±0.11
0.5 strength	6.03±0.28
Full concentration	5.94±0.83
Double concentration	5.42±1.23

a: Murashige and Skoog medium, 1mg L^{-1} IBA + 1mg L^{-1} BA + sucrose (3%) + myo-inositol (100 mg L^{-1}), Values are Mean±SE from 6 replicated, Differences between catechin contents in different mediums with different values of vitamins are not significant but between catechin contents of free vitamin medium with others is significant ($p<0.05$), FW: Fresh Weight

Table 4: Effects of myo-inositiol concentration on catechin production in cultured callus of rhubarb

Myo-inositol (mg L^{-1})[a]	(+) – Catechin mg/100 g FW
0	8.25±0.04
100	18.26±0.25
500	15.25±1.11
1000	15.11±1.30
2000	14.11±2.30

[a]: Murashige and Skoog medium, 1 mg L^{-1} IBA + 1 mg L^{-1} BA + sucrose (3%), Values are Mean±SE from 6 replicated
Differences between catechin contents in mediums with high levels of myo-inositol(500,1000 and 2000) are not significant but between catechin contents of free myo-inositol medium, 100 (mg L^{-1}) and with others is significant ($p<0.05$), FW: Fresh Weight

Table 5: Effect of ratio of ammonium-nitrate on catechin production in cultures callus of rhubarb

NO_3:NH_4 ratio [a]	(+) – Catechin mg/100 g FW
NO_3 only	4.23±0.08
1:3	4.98±0.03
1:2	6.25±0.34
1:1	13.82±0.27
2:1	10.71±0.32
3:1	8.53±0.09
NH_4 only	7.11±0.03

[a]: Murashige and Skoog medium, 1 mg L^{-1} IBA + 1 mg L^{-1} BA + sucrose (3%) + myo-inositol (100 mg L^{-1}), Values are Mean±SE from 6 replicated
All the values are significantly different ($p<0.05$) from control,
FW: Fresh Weight

Fig. 1: Regenerated rhubarb plant

was 1:1, the catechin yield was about 100% higher than of the control culture (the media that had NO_3 or NH_4 only) (Table 5). The addition of KCl to the medium was carried to keep the amount of K^+ ions the same as in the control culture.

For multiple shoot formation, calluses were transferred to MS medium (inorganic salts and vitamins) + 100 mg L^{-1} myo-inositol + 30 g L^{-1} sucrose + 0.5 mg L^{-1} IBA + 0.5 mg L^{-1} BAP with 0.7% (W/V) agar. Rooting medium comprised ½ MS salts and vitamins + 20 g L^{-1} sucrose + 0.5 mg L^{-1} IBA with 0.7% (W/V) agar. Regenerated plants (Fig. 1) were transferred to clay pots that included a mixture of soil, vermiculite and sand (60:20:20 V/V). Plants were irrigated every third day with Asher and Edwards[20] solution and maintained fewer than 50-70% plants for the first 7 days.

The accumulation of catechin and its derivatives in aerial parts of plant has been reported in number of papers[21]. Zenk *et al.*[22] reported that 5% sucrose concentration was best for anthraquinons yield in cell suspension cultures of *Morinda ctrifolia*. The best sugar concentration (3%) for production of catechin was the same as the optimal concentration for anthraquinons formation in *Rheum ribes*[16]. The addition of increasing concentration of vitamins did not have any promoting effect on catechin production and vitamin free medium suppressed catechin formation. This observation differed from earlier report of phenolic compounds production where there was an absolute requirement for vitamins for phenolic compounds production[23]. Increasing levels of myo-inositol suppressed catechin formation. Higher catechin yield was achieved at level 100 mg L^{-1} myo-inositol and this result was in agreement with the production of anthraquinones in *Morinda citrifolia*[22] and in *Rheum ribes*[16].

Nitrate ions are the usual nitrogen source for cell suspension culture. Hagendoorn *et al.*[24] suggested that exhaustion of nitrogenous nutrients mainly NO_3 may cause a switch of cellular metabolism to reactions forming nitrogen free products. Since catechins are also nitrogen free secondary metabolites, it was of interest to investigate the influence of the ratio of NO_3-NH_4 on metabolism expression. When the ratio of ammonium-nitrate nitrogen was 1:1 the catechin yield was higher that other treatments. Results were in agreement with Donnenburg and Knorr[25], Suzuki *et al.*[26] and Farzami Sepehr and Ghorbanli[16] on anthraquinones production.

Differentiation of plants from cultured cells via shoot-root formation (*de novo* origin) or somatic embryogenesis, where applicable, can be the fastest method of shoot multiplication and cloning of plant species[26]. The best medium for regeneration of shoot and shoot multiplication was in MS medium with 0.5 mg L^{-1} IBA + 0.5 mg L^{-1} BAP, result was in agreement with a general concept proposed by Skoog and Miller[27] staining

that organ differentiation in plants is regulated by an interplay of auxin and cytokinin, but is not in agreement with Lassus and Voipio[15] on micropropagation of rhubarb. Razdan[28] believed that low concentration of salts have proven satisfactory for rooting of shoots micropropagated. We observed the best percent of rooting at half MS medium with removing of BAP from medium; this result is in agreement with Ghorbanli and Farzami Sepehr[29].

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