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Folia Forestalia Polonica, Series A – Forestry, 2018, Vol. 60 (4), 261–268

METHODOLOGICAL ARTICLE

DOI: 10.2478/ffp-2018-0027

The effect of coconut extract on callus growth and ultrasound waves on production of betulin and betulinic acid in in-vitro culture conditions of *Betula pendula* Roth species

Vahide Payamnoor[1] ✉, *Razieh Jafari Hajati*[2], *Negar Khodadai*[1]

[1] Gorgan University of Agricultural Sciences and Natural Resources, Faculty of Forest Sciences, Gorgan, Iran, phone: 0098-9113735812, e-mail: mnoori56@gmail.com

[2] Shahed University, Traditional Clinical Trial Research Center, Tehran, Iran

Abstract

To determine the effect of coconut extract on callogenesis of *Betula pendula*, Roth stem barks were cultured in NT (Nagata and Takebe) basic culture media in two individual experiments: i) cultivation explant in different treatments of coconut extracts combined with 1 mg l^{-1} 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and ii) callogenesis in NT media containing 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP (6-Benzylaminopurine) and then cultivation under the first experiment treatments. The first experiment demonstrated that not all concentrations of coconut extracts lead to callus induction individually, but callus induction increased 84% in a culture containing 5% coconut extract plus 1 mg l^{-1} 2, 4-D. Based on the results of the second experiment, this treatment also significantly increased the wet and dry weights of the produced calluses. The possibility of increasing the betulinic acid and betulin by ultrasound was also studied. Samples cultivated in the selected culture medium were exposed to ultrasound waves in two forms of 1) one exposure and 2) twice exposure (repetition with 24 hr interval) in steps of 20, 60, 100, and 160 sec, and one treatment as the control. Betulin and the betulinic acid amount were determined using HPLC (High-Performance Liquid Chromatography). The maximum betulinic acid and betulin were obtained in 100 sec in once exposure (2.3 mg g^{-1} DW) and 160 sec in the twice exposure (0.75 mg g^{-1} DW) to ultrasound. The results showed that betulinic acid content was more than betulin.

Key words

betulin, betulinic acid, *Betula pendula* Roth, callus induction

Introduction

Results of *in-vitro* experiments have shown that the *Betula pendula* Roth bark extracts can have immunological effects on the human body that is a promising fact to repair tissue (Freysdottir et al. 2011). These effects are because of various second metabolites such as terpenes, phenols, hydrocarbons, polyphenols, tannins, steroids, and methyl salicylate in it. Triterpenes have the maximum attribution of *Betula pendula* Roth bark (Abyshev

Received 20 May 2018 / Accepted 10 August 2018

et al. 2007). Betulin and betulinic acid are two main triterpenoids in *B. pendula* Roth (Krasutsky 2006) that can treat many chronic and dangerous diseases such as Malaria, HIV, hepatitis, neuroblastoma, and some cancers including lung, intestine, ovarian, breast, and prostate cancer (Freysdottir et al. 2011).

B. pendula Roth species are at risk of extinction in Iran habitats and their bioavailability is declined. Therefore, they not only cannot be picked up, but also must be protected. In such conditions, induction and extraction of useful in-vitro secondary metabolites can be helpful. In this regard, the constitutional components of cultures and used hormones are very important because the constitutional components of culture are the most important factor in the growth and morphogenesis in plant tissues culture (Sandoval Prando et al. 2014).

Using natural products such as coconut extract, hydrolysed casein, and yeast extract has been popular since the invention and evolution of tissue culture. Endospermic products, especially coconut extract, have cytokinin activity. These natural products reduce nitrogen resources and a spectrum of sophisticated chemical compounds that are able to motivate growth and organogenesis (Mamghani et al. 2007). In addition, coconut extract can be considered as a good supplement for having other compounds besides plant growth hormones (Agutampudi and Jayavardena 2009) such as sugars, vitamins, minerals, and amino acids (Namdeo et al. 2006).

Numerous efforts have been done since past on plant tissue culturing for using completely known elements to prepare culture and avoid using natural raw extracts. Despite the high costs involved in preparing synthetized cultures, the desired results are not obtained by using mineral cultures with specific chemical elements. Sandval Prando et al. (2014) used the *in vitro* culture of *Corylus avellana* L. plant on coconut extract with the hormone. Their results showed that addition of 20% coconut water increases germination. Results of Manghani et al. (2007) showed that callus weight of *Ulmus parvifolia* Jacq. plant on Murashige and Skoog medium with coconut extract increased by 31.7% more than treatments without coconut extract.

Plant secondary metabolites in nature are produced as a defensive mechanism against attack by pathogens. Moreover, they indicated the reactions of the plants when facing pathogenic compounds. Elicitors produce signals that stimulate secondary metabolites (Rao and Ravishankar 2002). Ultrasonic waves are as abiotic elicitor as the modern non-thermal technology, physical elicitor, and a safe and secured energy resource with no chemical undesirable effects in addition to stimulating secondary metabolites synthesis (Atrashi et al. 2011). Using ultrasonic waves in biotechnology increases the activity of cells and enzymes (Tumova et al. 2014). Ultrasonic waves increase secondary metabolites synthesis based on the plant species (Atrashi et al. 2011). Increasing the permeability of the cell membrane by ultrasonic waves, called sonoporation, can remove intracellular products such as medicinal secondary metabolites from the cultured plant cells. This research aims at increasing the callus of *B. pendula* Roth growth with different compounds of coconut extract and ultrasonic effect evaluation on increasing induced betulin and betulinic acid in cells.

Material and methods

Preparing plant samples: All stem samples with 1 cm diameter were collected at the same height from Cheshmeh Shahi habitant in Alborz Province of Iran (longitude 22°51' and latitude 56°35') in spring and was transferred in 4°C to the laboratory. First, the samples were under pre-sterilized treatments (washing with running water, putting in a dishwashing liquid for 3 min, and disinfecting it with 4 g.l of Benomyl solution for 45 min), followed by sterilization treatments (3 times washing with distilled sterile water, 7 min in 0.1% mercury chloride, and then 3 times washing with distilled sterile water). The internal bark was separated from the stem and cut into 5–10 mm pieces.

First experiment: Sub-bark sample was cultured in the first experiment to induce callus in NT (Nagata and Takebe 1971) medium with pH 5.7, supplemented with 3% sucrose, 0.8% agar, and treatments of Table 1. NT compositions and growth regulator: 2,4-D and BAP (2,4-Dichlorophenoxyacetic acid and 6-Benzylaminopurine) were purchased from Merck KGaA, Darmstadt, Germany. Coconut was purchased from a local market in Gorgan, Iran, and was used for its extract. The cultures were grown in 25±2°C of growth room with a 16 h photoperiod from cool white fluorescent lamps (Osram Luminox, Munich, Germany) at a light intensity

of 43-µmol $m^{-2}s^{-1}$. Initial callus induction percentage was examined after one month. Samples were subcultured to complete their growth period. After 3 months and enough callus growth, 3 replications for each treatment was harvested. The wet weights were measured for each treatment. Then, they were dried in an oven at 50±2°C for 2 days and dry weights were calculated.

Table 1. Various concentrations of coconut extract combined with 1 mg l^{-1} 2, 4-D used in the NT medium

	Treatments
1	Free hormone
2	5% coconut extract
3	10% coconut extract
4	15% coconut extract
5	20% coconut extract
6	5% coconut extract and 1 mg l^{-1} 2,4-D
7	10% coconut extract and 1 mg l^{-1} 2, 4-D
8	15% coconut extract and 1 mg l^{-1} 2, 4-D
9	20% coconut extract and 1 mg l^{-1} 2, 4-D

Second experiment: One-month callus was cultured in NT medium supplemented with hormonal compounds of 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP was transferred to NT medium with compounds of Table 1 and their growth was examined such as the first step at the end of 3 months.

Ultrasonic exposed treatments: Calluses were selected from a culture with treatment that has the maximum growth and callus weight increase. About 1000 mg of callus was separated for each replication, put in an ultrasonic bath (Liarre Starsonic 35, Italy), and exposed to ultrasonic waves. Exposure to ultrasonic waves was done twice for samples with 1) once exposure and 2) twice exposure with a 24 hr interval from the first exposure. Treatments were exposed to ultrasonic waves for 20, 60, 100, and 160 sec and one treatment as the control in triplicates (Lin, 2001). The temperature was kept at a constant temperature of 25°C. Output power was constant and waves' frequency was 38.5 kHz. Three replications were taken from each treatment. Wet weight was measured and dried in an oven at 50±2°C for 2 days.

Evaluating the amounts of betulin and betulinic acid: To measure betulin and betulinic acid, dried cells were extracted from each treatment in methanol solvent (grade HPLC – High-Performance Liquid Chromatography). Samples were filtered through a 0.45 µM L^{-1} filter membrane. We analysed betulin and betulinic acid contents though the quantitative HPLC (Hitachi L-2450 series, Tokyo, Japan). For this purpose, we used a C18 reversed-phase column with dimensions of 150 mm × 4.6 mm and a pore size of 5-µm. The mobile phase was acetonitrile-water (86:14, v/v). We quantified betulin and betulinic acid using UV detection at λ = 210 nm. The flow rate and the injection volume was 1.0 mL min^{-1} and 20 µL, respectively. Standard curves from pure betulin and bulimic acid (Sigma-Aldrich, St. Louis, MO) were prepared in methanol compared to the callus extract.

Statistical analysis: Experiments were conducted as a fully randomized design. Data were analysed using SPSS software. The mean effect of various treatments was compared in 5% *P*-value by LSD test.

Results

First experiment: Studying callus induction of the transferred bark samples to NT medium with the mentioned compounds in Table 1 show that callus induction did not occur in control treatments and in cultures that only used various concentrations of coconut extract; but when coconut extract was mixed with 2, 4-D, the callus induction appeared. Results of analysis of variance (ANOVA) for callus induction percentage, wet weight, and dry weight of the obtained callus in NT culture with coconut extract concentrations and 2, 4-D showed a significant difference in 1% *P*-value.

Based on Table 2, the maximum callus induction (84%) was related to a 5% coconut extract plus 1 mg l^{-1} 2, 4-D, and the maximum wet weight (1,200 mg) and dry weight (100 mg) of callus were seen in the same compound of coconut extract, which is significantly different from the other treatments.

Second experiment: One-month calluses (grown in NT medium supplemented with 1.5 mgl^{-1} 2, 4-D and 0.5 mgl^{-1} BAP) were used in this experiment to grow in the NT medium containing the mentioned compounds (Tab. 1). The results of ANOVA showed a 1% *P*-value difference between wet and dry weights of calluses in different treatments.

Table 2. Mean analysis of callus induction percentage, wet weight, and dry weight of calluses under NT medium and various concentrations of coconut and 1 mg l^{-1} 2,4-D (the same letters are not significantly different)

Treatments	Callus induction percentage	Wet weight (mg)	Dry weight (mg)
5% coconut extract and 1 mg l^{-1} 2,4-D	84 ± 10 a	1200 ± 260 a	100 ± 10 a
10% coconut extract and 1 mg l^{-1} 2, 4-D	65 ± 6 b	573 ± 64 d	50 ± 8 c
15% coconut extract and 1 mg l^{-1} 2, 4-D	55 ± 5 bc	1033 ± 152 b	97 ± 15 ab
20% coconut extract and 1 mg l^{-1} 2, 4-D	50 ± 6 c	673 ± 64 c	60 ± 10 b

Table 3 compares wet and dry weights of calluses in various coconut extract compounds, where the maximum wet weight (9413 mg) and dry weight (543 mg) were observed after three months in combination with 5% coconut extract plus 1 mg l^{-1} 2,4-D, which is significantly different from the other treatments at 5% *P*-value. Then, 10 and 15% coconut extracts plus 1 mg l^{-1} 2, 4-D showed a significantly higher average biomass than the other treatments. Calluses growth in the second experiment was higher compared to the first experiments in the same treatment.

Table 3. The mean analysis of wet weight and dry weights of calluses under various concentrations of coconut and 1 mg l^{-1} 2,4-D

Treatments	Wet weight (mg)	Dry weight (mg)
Control	1750 ± 50 f	116 ± 5 g
5% coconut extract	4333 ± 208 bcd	297 ± 15 c
10% coconut extract	2434 ± 200 e	190 ± 10 e
15% coconut extract	1593 ± 110 fg	130 ± 10 f
20% coconut extract	1700 ± 100 f	123 ± 15 fg
5% coconut extract and 1mg l^{-1} 2,4-D	9413 ± 241 a	543 ± 47 a
10% coconut extract and 1 mg l^{-1} 2, 4-D	5833 ± 2608 b	350 ± 10 b
15% coconut extract and 1 mg l^{-1} 2, 4-D	5150 ± 50 c	296 ± 15 c
20% coconut extract and 1 mg l^{-1} 2, 4-D	3566 ± 152 d	230 ± 10 d

Callus growth under various treatments is shown in Figure 1. As can be seen, the coconut extracts added with 1 mg l^{-1} 2, 4-D hormone show organogenesis with root genesis in all the treatements except the treatment with 5% coconut extract and 1mg l^{-1} 2, 4-D.

Measurement of betulin and betulinic acid: Calibration curve was drawn and calculated by injection at various concentrations from standards. The betulinic acid peak appeared 15–17 min and its standard calibration formula was $y = 25830x + 532039$ ($R^2 = 0.9903$). Betulin peak appeared 25–27 min and its standard calibration formula was $y = 29922x - 483239$ ($R^2 = 0.9951$). Betulin and betulinic acid contents were converted to mg.g dry weight based on the obtained ppm regarding the level of the area under the peak and placing it in the calibration line equation.

ANOVA results of betulin and betulinic acid in callus extracts under various treatments by ultrasonic wavelength exposure show a statistically significant difference from the ones with effective materials in 1% *P*-value in treatments level.

Table 4. The mean analysis of betulin and betulinic acid content in *B. pendula* callus under various times of ultrasonic waves' exposures

Treatments	Betulinic acid (mg g^{-1} DW)	Betulin (mg g^{-1} DW)
Control	0.7 ± 0.05 d	0.3 ± 0.002 f
20s once exposure	0.93 ± 0.06 c	0.33 ± 0.003 e
20s twice exposure	1.38 ± 0.07 b	0.54 ± 0.001b
60s once exposure	0.15 ± 0.006 f	0.4 ± 0.007 c
60s twice exposure	0.009 ± 0.005 h	0.35 ± 0.007 d
100s once exposure	2.3 ± 0.12 a	0.55 ± 0.05 b
100s twice exposure	0.32 ± 0.07 e	0.34 ± 0.006 d
160s once exposure	0.29 ± 0.07 e	0.41 ± 0.005 c
160s twice exposure	0.03 ± 0.004 g	0.75 ± 0.07 a

Table 4 compares the mean level of betulin and betulinic acid in callus extracts grown in the selected treatment (an NT medium supplemented with 5% coconut extract percentage plus 1 mg l^{-1} 2, 4-D) under ultrasonic exposure treatment during two steps. Based on the obtained results, the maximum betulinic acid was obtained in the treatment of 100 sec of once exposure in 2.3 mg g^{-1} DW than in the treatment of 20 sec in twice exposure of 1.38 mg g^{-1} DW, which is significantly different from the

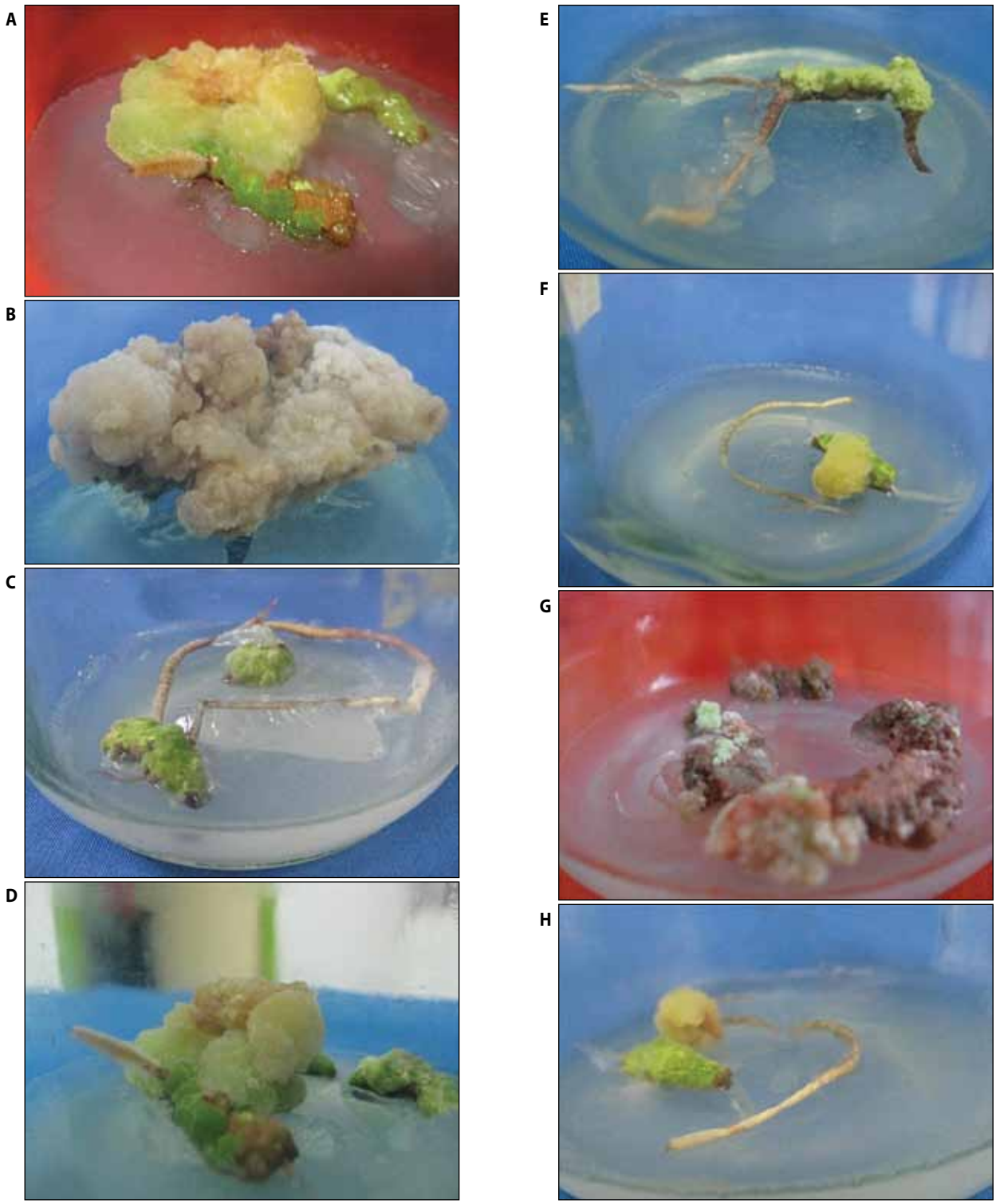

Figure 1. *B. pendula* callus induction under various coconut extract concentrations: A – 5% coconut extract, B – 5% coconut extract and mg l^{-1} 2,4-D, C – 10% coconut extract, D – 10% coconut extract and 1 mg l^{-1} 2, 4-D, E – 15% coconut extract, F – 15% coconut extract and 1 mg l^{-1} 2, 4-D, G – 20% coconut extract, and H – 20% coconut extract and 1 mg l^{-1} 2, 4-D

other exposure treatments. The minimum betulinic acid amount was observed in the 60-sec treatment of twice exposure. The maximum betulin amount (0.75 mg g^{-1} DW) was observed in 160 sec treatment of twice exposure and then in 100 sec treatment of once exposure and 20-sec treatment of twice exposure. The minimum betulin amount was observed in the control treatment.

Discussion

Callus induction with coconut extract in *B. pendula* and using ultrasonic waves to increase *B. pendula* metabolites was reported for the first time. Using coconut extract alone did not result in callus induction in *B. pendula* stem bark because coconut extract has cytokinin activity (Mamghani et al. 2007). While auxins are needed for callus induction in vitro condition in a short time, the callus induction in coconut extract treatments in combination with 2, 4-D resulted in the increased callus growth. In addition, more growth in the cultured callus was seen in combination with 2, 4-D. Previous studies showed the maximum fresh and dry weight of *B. pendula* callus in NT medium with 2.5 mg l^{-1} 2,4-D + 1 mg l^{-1} BAP compared to various concentrations of 2,4-D (Jafari et al. 2016). Baskaran et al. (2005) concluded that using low concentration of coconut (10% coconut extract) in combination with 2, 4-D led to the maximum callus induction in *Sorghum bicolor* and an increase in coconut extract while using NAA, IBA, and IAA reduced callus induction that was in agreement with the result of this research. Mamun et al. (2004) claimed that using 10% coconut extract with 2, 4-D maximizes callus induction in cane sugar plant. Based on their results, although various concentrations of coconut extract induce root in callus, only 5% coconut extract concentration plus 2, the 4-D tissue is not discernible. This combination of coconut extract induces the maximum callus (84%) and the maximum callus growth. Callus induction percentage was decreased by an increase in the coconut extract concentration. Therefore, it is expected that low concentration of coconut extract induces callus, and increasing coconut extract concentration makes organogenesis in *B. pendula*.

In reports of researchers, using coconut extract increased secondary metabolites. Malpathak and David (1986) used 15% coconut extract in combination with the other hormones to produce Allin in *Allium sativum* L. Khouri et al. (1986) also used 5% coconut extract in culturing *Cinchona succirubra* cell suspension culture. Coconut extracts increase diterpenoids in cell suspension culture of *Torreyan uciferavar* Radicans (Orihara et al. 2002). Therefore, increasing the secondary metabolites by coconut extract is not out of expectance.

The aim of using coconut extract in this research was to enhance callus induction and its growth and ultrasonic elicitor were used to increase betulin and betulinic acid. The maximum betulinic acid (2.3 mg g^{-1} DW) was obtained in 100s of once exposures. It can be claimed that an increase in betulin and stimulation of producing cells and enzymes of this material needs the longer exposure of ultrasonic waves. In this regard, the maximum betulin amount (0.75 mg g^{-1} DW) was obtained in 160 sec twice exposure to the ultrasonic wave. The content of betulinic acid and betulin were 0.41 and 0.2 mg g^{-1} DW, respectively, which are lower than those reported in the study of Jafari et al (2016). Increasing metabolites can be performed for increasing permeability of the treated cells' membrane in ultrasonic waves. Many researchers have used ultrasonic waves to increase the secondary metabolites. In a similar research, Tumia et al. (2014) reported that using ultrasonic waves increases genistein (0.8 mg g^{-1} DW) after 3 min in culture of cell suspension of *Genista tinctoria* L. Wu and Lin (2003) increased Taxol amount to 1.5–1.8 times more in cell culture of *Taxus chinensis* using ultrasonic in 2 min. Lin et al. (2001) used ultrasonic waves to increase the biosynthesis of Saponin secondary metabolites in *Panax ginseng* plant cell suspension culture. Atrashi et al. (2011) showed that ultrasonic waves with twice exposure and 35 sec exposure increased the Caron secondary metabolite synthesis significantly. Rezaei and Ghanati (2011) used ultrasonic waves in cell culture of hazelnut, which increased Taxol release and specific performance than the control treatment significantly.

It has been reported that betulin content is significantly more than betulinic acid in *B. pendula* bark (Zhao et al. 2007), while the biological activity of betulinic acid is higher for having more solubility (Abishu et al. 2007). Betulinic acid is more than betulin in *B. pendula* callus extracts in this research. Therefore, betulinic acid production by tissue culture and using elicitors changing it in callus is significantly important in the reduction of production costs.

Considering the sophistication of biosynthesis paths of the secondary metabolites and varieties of encoding genes of enzymes of this path, artificial production of many secondary metabolites such as betulin and betulinic acid face many difficulties. Therefore, obtaining techniques to increase the production of these metabolites in live plants tissues is considered as the great breakthrough. Ultrasonic waves can be used as a useful policy and abiotic elicitor to increase metabolites synthesis in tissue culture. This technology shows a promising future in increasing the important medicinal compounds.

Conclusion

In this study, coconut extract was used as a cytokinin in combination with an auxin (i.e., 2, 4-D) for callus induction and growth. Ultrasound treatments at some time increased the content of betulin and betulinic acid. In a future study, it is possible to study the variation of betulin by increasing the time of ultrasonic waves' exposures. The results of this study can help to increase the content of secondary metabolites in the cultivation of cell suspension and hairy root culture of *B. pendula* in the bioreactor.

Acknowledgements

This work was supported by Gorgan University of Agricultural Sciences and Natural Resource [grant numbers 93-324-61].

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