Elicitors Derived from Hazel (Corylus avellana L.) Cell Suspension Culture Enhance Growth and Paclitaxel Production of Epicoccum nigrum

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The microbial fermentation is considered as the potential source for large-scale production of paclitaxel. Since co-cultivation/mixed fermentation strategy has been reported as a yield enhancement strategy for paclitaxel production, investigation of fungal endophyte response to plant culture medium, plant cell extract (CE) and medium filtrate (MF) of plant cell suspension culture in terms of growth and paclitaxel production is interesting. In this study, 35 endophytic fungi were isolated from Taxus baccata and Corylus avellana grown in Iran. The analysis of high-performance liquid chromatography and mass spectrometry showed that one isolate (YEF2) produced paclitaxel. The isolate YEF2 was identified as Epicoccum nigrum by sequencing of ITS1-5.8S-ITS2 rDNA region and actin gene. YEF2 was slow-growing in Murashige and Skoog medium, but the synergistic interaction of gibberellic acid (GA₃) and CE of C. avellana enhanced the growth of YEF2. The highest total yield of paclitaxel (314.7 μg/l; 11.5-folds) of E. nigrum strain YEF2 was obtained by using 28% (v/v) filter sterilized CE of C. avellana and 2 μg ml⁻¹ GA₃ that was significantly higher than the control. In this study, the effects of the plant cell extract on growth and paclitaxel production of paclitaxel producing endophytic fungus were studied for the first time.

Paclitaxel, a main impressive chemotherapeutic agent against a wide range of cancers¹ was originally extracted from the bark of Taxus brevifolia, yew native to the North-Western Pacific area and its chemical structure was elucidated in 1971². Paclitaxel stabilizes microtubules to depolymerization, thus arrest the division of actively growing tumor cells at G1 or M phases³. Unfortunately, yew trees are slow-growing and large amounts of bark are required for paclitaxel production⁴. Owing to overexploitation, many species are now endangered and on the brink of extinction⁴. Therefore, search for alternative sources of the drug is prompted. In 1993, the first paclitaxel-producing fungus was isolated from the Pacific yew⁶. This initial discovery was followed by the plethora of different endophytic fungi reported producing paclitaxel⁷,⁸. A microbial fermentation process would be the most favorable means of paclitaxel supply. Microorganisms are fast growing and their genetic manipulation is relatively easy and can be scaled-up to an industrial level. Therefore, microbial fermentation is considered as the potential source for large-scale production of paclitaxel⁹. Low productivity of paclitaxel in endophytic fungi is a drawback for commercial production. Despite the various genera of endophytes capable of producing paclitaxel⁹, there have been no major breakthroughs regarding to commercial production of paclitaxel by fungal fermentation. The problems including the inconsistent production of fungal paclitaxel by repeated sub-culturing on defined artificial media⁹ have raised doubts about the commercial possibility of endophytic fungi as sustainable production platforms. Venugopalan et al.¹¹ stated that optimization of culture parameter including the exogenous addition of elicitors to in vitro cultivation of endophytic fungi improves production of secondary metabolites. It is reported that adding the host plant extracts to the fungal culture was successful in some cases¹². Host plants affect metabolic processes of the endophytes¹³ and reciprocally transcription of rate-limiting genes in plant paclitaxel biosynthetic pathway is increased by fungal endophytes¹⁴. Indeed, during the long period of co-evolution, a

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friendly relationship has been gradually organized between each endophytic fungus and its host plant, so that host plant provides plentiful nourishment for endophytes and fascinates their inhabitation leading to the survival of these endophytes. The endophytes, in turn, synthesize several bioactive compounds for protecting the host plants against biotic and abiotic stresses and boosting their growth15,16. In some cases, endophytic fungi have gained the capability of producing identical or similar bioactive compounds as those produced by their host plants. It seems the co-cultivation or the mixed fermentation can mimic the natural habitat of endophytes. Co-cultivation/mixed fermentation strategy has been reported as a yield enhancement strategy for paclitaxel production17. It is stated that the addition of Catharanthus roseus extract and ethanol in the medium enhanced the camptothecin production in the suspension culture of Fusarium solani18. It assumes the addition of plant cell extract to suspension culture of paclitaxel-producing endophytic fungi or the co-cultivation of these endophytes with plant cells can provide the required stimulus to the fungal endophyte in the axenic culture for enhanced and sustainable production of paclitaxel.

In addition to Taxus spp., hazel (Corylus avellana) has also been described as a paclitaxel-producing species through bioprospection among angiosperms19–21. The major advantage of producing taxanes through hazel cell suspension culture (CSC) is that hazel is widely available, grows more quickly in vivo, and is easier to cultivate in vitro than yew22.

In the light of importance in vitro cultures of C. avellana as a promising and cheaper source for paclitaxel production23, it seems that the co-cultivation of C. avellana cells with paclitaxel-producing endophytic fungus can be promising for enhancing paclitaxel production. Therefore, investigation of fungal endophyte response to plant culture medium, cell extract (CE) and medium filtrate (MF) of C. avellana CSC in terms of growth and paclitaxel production is crucial.

The objectives of this study were (a) to isolate endophytic fungi from Taxus baccata and C. avellana grown in Iran, (b) to screen and identify paclitaxel producing isolates and (c) to investigate the response of paclitaxel-producing endophytic fungus (growth and paclitaxel production) to CE and MF of C. avellana CSC as well as GA3.

**Results**

**Isolation, screening and identification of the paclitaxel-producing endophytic fungus.** A total of 35 isolates was separated from T. baccata and C. avellana. Paclitaxel was extracted from culture filtrates and mycelia of fungi and then analyzed by HPLC. The HPLC analysis of these isolates showed that the peak positions of two strain (YEF2 and HEF12) were identical to that of standard paclitaxel (retention time = 4.59 ± 0.05 min) (Fig. S1), indicating these fungal isolates may produce paclitaxel. Analysis of paclitaxel production of these two isolates in six passages showed that only production of strain YEF2 was stable. This strain was isolated from T. baccata bud. Further confirmation for the identity of the paclitaxel was gained by LC-MS/MS. Figure 1 shows representative mass spectra of paclitaxel from strain YEF2. The selected ions for the paclitaxel standard are an (M + Na+) ion with mass-to-charge ratio (m/z) 876, an (M + H+) ion with m/z 854 and an (M + NH4+) ion with m/z 871 and asterisks indicate fragments ions of mass spectroscopy of paclitaxel that is necessary for identifying paclitaxel.

![Figure 1. Mass spectrometric analysis of fungal paclitaxel sample of strain YEF2. The arrows show an (M + Na+) ion with mass-to-charge ratio (m/z) 876, an (M + H+) ion with m/z 854 and an (M + NH4+) ion with m/z 871 and asterisks indicate fragments ions of mass spectroscopy of paclitaxel that is necessary for identifying paclitaxel.](image-url)
higher than the control (supplemented with 2 μg/mL GA₃) in terms of FW (218.1 g/l) (5.3-folds), DW (9 g/l) (2.9-folds), intracellular (30.1 μg/l) (4.3-folds), extracellular (224.0 μg/l) (12.7-folds) and total yield of paclitaxel (221.4 μg/l) (8.2-folds) as compared to the control (Fig. 2). The significant interaction effect of elicitor type (CE or MF) and sterilization method (Filter sterilized or autoclaved) indicated that elicitor type affected measured traits differently depending on used sterilization technique. Because of the significant interaction effect of elicitor type and sterilization technique, the effects of elicitor type were analyzed on each sterilization method. The means comparison indicated that FCE was more effective than ACE for the increase of FW (254.1 g/l) (6.2-folds), DW (9.6 g/l) (3.1-folds), intracellular (33.8 μg/l) (3.6-folds), extracellular (224.0 μg/l) (12.7-folds) and total yield of paclitaxel (257.8 μg/l) (9.6-folds) (Fig. 3). The interaction effect of elicitor type x concentration level indicated that the effect of elicitors was level-dependent (Fig. 4) and the means comparison showed that the highest FW (384.4 g/l) (9.9-folds), DW (12.6 g/l) (4.1-folds), intracellular (40.3 μg/l) (4.3-folds), extracellular (274.4 μg/l) (15.3-folds) and total yield of paclitaxel (314.7 μg/l) (11.5-folds) of E. nigrum strain YEF₂ were obtained with 28% (v/v) FCE and 2 μg/mL GA₃ that were significantly higher than the control (supplemented with 2 μg/mL GA₃ and 28% (v/v) filter sterilized water) with a mean of 38.9 g/l, 3.1 g/l, 9.3 μg/l, 17.9 μg/l and 27.3 μg/l, respectively (Table 1). The results of ANOVA indicated that CE and MF of C. avellana CSC increased intracellular (per liter of medium), extracellular and total yield of paclitaxel, whereas no significant increase in intracellular yield of paclitaxel per gram dry weight of mycelia was observed. Total yield of paclitaxel (dependent variable (Y)) was regressed against FW and DW. The regressions of total paclitaxel against FW and DW for FCE, ACE, AMF were positive and significant (Table 2). Additionally, the fitted models for ACE and FCE exhibited high R-Squared. Meanwhile, AMF increased FW (79.7 g/l) (1.9-folds), and DW (4.9 g/l) (1.6-folds) of E. nigrum more than FMF (Fig. 5b). However, FMF was more effective for paclitaxel production than the AMF (Fig. 5a). Also, The regressions of total paclitaxel against FW and DW for FMF were not significant (Table 2).

Synergistic interaction of gibberellic acid and CE of C. avellana CSC enhanced the growth of E. nigrum. E. nigrum is slow-growing in MS medium supplemented with GA₃ (Fig. S2c). The addition of C. avellana CE to MS medium at the time of culture did not increase the growth of E. nigrum (Fig. S2b), whereas this strain was relatively fast-growing in MS supplemented with GA₃ and CE of C. avellana (Fig. S2a). The best GA₃ concentration for C. avellana cell growth was shown to be 2 μg/ml (data not shown). So, in all next experiments, 2 μg/ml GA₃ was added to the medium at the time of culture. At the first, three concentrations (3%, 5% and 7% (v/v)) of C. avellana CE were added to culture medium and a slight increase in growth was obtained by adding 3% and 5% CE of C. avellana (Fig. S3). Therefore, three concentrations (7%, 14%, and 28%) were selected for next experiment.

The CE and MF of C. avellana CSC enhanced growth and total yield of paclitaxel of E. nigrum strain YEF₂. Paclitaxel in culture medium containing the elicitors derived from C. avellana CSC with no inoculums was analyzed (Table S2) and paclitaxel in all treatments was modified based on paclitaxel of the culture medium containing the elicitors with no inoculums. The results of induction of growth and paclitaxel production in E. nigrum using CE and MF of C. avellana CSC showed that the fresh weight (FW), dry weight (DW), intracellular paclitaxel (μg/l), extracellular paclitaxel and total paclitaxel of the fungus significantly affected by CE and MF of C. avellana CSC. The main effects of treatments and their interactions (reciprocal and trilateral effects) on mentioned traits were highly significant. Considering the treatments individually, CE was performed better as CSC. The main effects of treatments and their interactions (reciprocal and trilateral effects) on C. avellana MF of CE was performed better as CSC. The main effects of treatments and their interactions (reciprocal and trilateral effects) on C. avellana MF of CE was performed better as CSC. The main effects of treatments and their interactions (reciprocal and trilateral effects) on C. avellana MF of CE was performed better as CSC. The main effects of treatments and their interactions (reciprocal and trilateral effects) on C. avellana MF of CE was performed better as CSC. The main effects of treatments and their interactions (reciprocal and trilateral effects) on C. avellana MF of CE was performed better as CSC.

Figure 2. Influence of cell extract (CE) and medium filtrate (MF) of Corylus avellana cell suspension culture on paclitaxel production (a) and growth (b) of Epicoccum nigrum strain YEF₂ grown in MS medium supplemented with 0.2 mg/L BAP, 2 mg/L GA₃ and 2 mg/L 2,4-D. The controls for CE and MF were water and MS medium supplemented with 0.2 mg/L BAP, 2 mg/L GA₃ and 2 mg/L 2,4-D, respectively. Average values are given, error bars are represented by vertical lines.
Figure 3. Influence of autoclaved cell extract (ACE) and filter sterilized cell extract (FCE) of *Corylus avellana* cell suspension culture on paclitaxel production (a) and growth (b) of *Epicoccum nigrum* strain YEF2 grown in MS medium supplemented with 0.2 mg/L BAP, 2 mg/L GA₃ and 2 mg/L 2,4-D. The controls for ACE and FCE were autoclaved water and filter sterilized water, respectively. Average values are given, error bars are represented by vertical lines.

Figure 4. *Epicoccum nigrum* strain YEF2 grown in MS medium supplemented with 0.2 mg/L BAP, 2 mg/L GA₃, 2 mg/L 2,4-D and different concentration of filter sterilized cell extract of *Corylus avellana* cell suspension culture (a to d, 0%, 7%, 14% and 28% (v/v), respectively).

| Elicitors (% (v/v)) | Fresh weight (g/L) | Dry weight (g/L) | Extracellular paclitaxel (μg/L) | Intracellular paclitaxel (μg/g) | Intracellular paclitaxel (μg/L) | Total yield of paclitaxel (μg/L) | Extracellular paclitaxel portion (%) |
|--------------------|---------------------|------------------|-------------------------------|-------------------------------|-------------------------------|---------------------------------|-----------------------------------|
| Control (without elicitor) | 43.3 ± 0.0 | 3.11 ± 0.03 | 18.2 ± 0.6 | 2.88 ± 0.17 | 8.97 ± 0.60 | 27.2 ± 1.0 | 67.0 ± 1.4 |
| ACE (7%) | 105.6 ± 2.9 | 6.25 ± 0.04 | 109.7 ± 6.5 | 3.05 ± 0.34 | 19.10 ± 2.22 | 128.8 ± 8.8 | 85.3 ± 0.7 |
| ACE (14%) | 137.8 ± 2.2 | 8.07 ± 0.06 | 146.1 ± 7.0 | 3.33 ± 0.34 | 26.93 ± 2.86 | 173.0 ± 8.35 | 84.5 ± 1.4 |
| ACE (28%) | 303.3 ± 1.9 | 11.22 ± 0.13 | 219.8 ± 12.3 | 2.98 ± 0.36 | 33.57 ± 4.40 | 253.4 ± 15.3 | 86.8 ± 1.2 |
| FCE (7%) | 137.8 ± 1.1 | 7.17 ± 0.15 | 161.2 ± 10.4 | 3.79 ± 0.47 | 27.18 ± 3.37 | 188.4 ± 9.9 | 85.5 ± 2.0 |
| FCE (14%) | 240.0 ± 3.8 | 8.86 ± 0.22 | 236.6 ± 14.4 | 3.80 ± 0.44 | 33.79 ± 4.31 | 270.4 ± 18.4 | 87.6 ± 0.89 |
| FCE (28%) | 384.4 ± 4.8 | 12.64 ± 0.21 | 274.4 ± 11.0 | 3.18 ± 0.42 | 40.30 ± 5.40 | 314.7 ± 15.2 | 87.3 ± 1.2 |
| AMF (7%) | 64.6 ± 2.7 | 4.05 ± 0.05 | 76.4 ± 8.2 | 3.10 ± 0.37 | 12.60 ± 1.67 | 89.0 ± 9.32 | 85.8 ± 1.4 |
| AMF (14%) | 72.2 ± 2.2 | 4.33 ± 0.04 | 76.9 ± 8.7 | 3.16 ± 0.36 | 13.72 ± 1.66 | 90.6 ± 10.3 | 84.9 ± 0.7 |
| AMF (28%) | 102.2 ± 4.8 | 6.28 ± 0.59 | 96.7 ± 3.4 | 3.08 ± 0.39 | 19.81 ± 4.22 | 116.5 ± 7.5 | 83.3 ± 2.6 |
| FMF (7%) | 51.1 ± 1.1 | 3.06 ± 0.01 | 224.6 ± 11.9 | 3.23 ± 0.40 | 9.90 ± 1.22 | 234.5 ± 13.07 | 95.8 ± 0.3 |
| FMF (14%) | 60.0 ± 1.9 | 3.58 ± 0.04 | 225.3 ± 8.9 | 3.09 ± 0.43 | 11.12 ± 1.66 | 236.4 ± 10.4 | 95.3 ± 0.5 |
| FMF (28%) | 66.7 ± 3.3 | 4.05 ± 0.03 | 227.7 ± 9.8 | 3.22 ± 0.36 | 13.10 ± 1.59 | 240.8 ± 11.3 | 94.6 ± 0.4 |

Table 1. Effect of various elicitors derived from *Corylus avellana* L. cell suspension culture on growth and paclitaxel production of *Epicoccum nigrum* strain YEF2. Average values ± standard error of mean are given. Abbreviations are as follows: ACE; Autoclaved cell extract, FCE; Filter sterilized cell extract, AMF; Autoclaved medium filtrate, FMF; Filter sterilized medium filtrate.
MS medium and filter sterilized MS medium (supplemented with 0.2 mg/L BAP, 2 mg/L GA3 and 2 mg/L 2,4-D), \textit{E. nigrum} orange and later brown in late growth stage (Fig. S4). A yellow pigment was extracted from the culture of \textit{E. nigrum} and reported that it stimulates growth in cellulolytic fungi, i.e., \textit{Chaetomium globosum}, \textit{Memnoniella echinata} and \textit{Ourvularia lunata}. Therefore, investigating the ability of \textit{E. nigrum} to grow in plant culture media is essential. \textit{C. avellana} is slow-growing in MS medium supplemented with GA 3. (Fig. S2c). Since \textit{E. nigrum} strain YEF2 is very slow-growing in MS supplemented with GA 3 and CE of \textit{C. avellana} enhanced the growth of strain YEF2 in the presence of hazel CE. Indeed, GA 3 was the essential prerequisite for the growth of strain YEF2 in the presence of \textit{C. avellana} CE.

\textbf{Synergistic interaction of gibberellic acid and cell extract of \textit{C. avellana} enhanced the growth of \textit{E. nigrum}}. Probably the co-cultivation of \textit{E. nigrum} with \textit{C. avellana} cells can enhance the yield of paclitaxel. Therefore, investigating the ability of \textit{E. nigrum} to grow in plant culture media is essential. \textit{C. avellana} cell clone used in this study have cultured in MS medium. MS medium is one of the most commonly used media \textit{E. nigrum} strain YEF2 in the presence of hazel CE. Indeed, GA 3 was the essential prerequisite for the growth of strain YEF2 in the presence of \textit{C. avellana} CE.

\textbf{CE and MF of \textit{C. avellana} CSC enhanced growth and total yield of paclitaxel of \textit{E. nigrum} strain YEF2}. Resident endophytes within plants are steadily interacting with their hosts. It is found that plants would have a substantial influence on in planta metabolic processes of the endophytes\textsuperscript{25}. For example, the study of the gene cluster expression for lolitrem biogenesis in endophytic \textit{Neotyphodium lolii} resident in perennial ryegrass

| Treatment | Equation (Y = total yield of paclitaxel (µg L\(^{-1}\))) | P-value | R-squared | Correlation Coefficient |
|-----------|----------------------------------------------------------|---------|-----------|-------------------------|
| ACE       | 77.9597 + 0.587427*FW -30.6762 + 25.3427*DW             | 0.0001  | 0.89265   | 0.93489                 |
| FCE       | 125.25 + 0.505965*FW                                      | 0.0007  | 0.82590   | 0.77233                 |
| AMF       | 27.6739 + 0.891776*FW                                      | 0.0047  | 0.70457   | 0.64193                 |
| FMF       | 173.128 + 1.081166*FW                                      | 0.2078  | 21.52770  | 4.82189                 |

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Influence of autoclaved medium filtrate (AMF) and filter sterilized medium filtrate (FMF) of \textit{Corylus avellana} cell suspension culture on paclitaxel production (a) and growth (b) of \textit{E. nigrum} grown in MS medium supplemented with 0.2 mg/L BAP, 2 mg/L GA\(_3\), 2 mg/L 2,4-D. The controls for AMF and FMF Were autoclaved MS medium and filter sterilized MS medium (supplemented with 0.2 mg/L BAP, 2 mg/L GA\(_3\) and 2 mg/L 2,4-D), respectively. Average values are given, error bars are represented by vertical lines.}
\end{figure}

\textbf{Discussion}. \textit{E. nigrum as a Paclitaxel-Producing Endophytic Fungus}. \textit{E. nigrum} is a fungal species from the phylum Ascomycota and a saprophyte on crop residues\textsuperscript{36}. This species can live as an endophyte in different plants\textsuperscript{27,28}. In some studies, \textit{Epicoccum} genus was reported as a paclitaxel producing endophytic fungus\textsuperscript{10,24,29}. Also, \textit{E. nigrum} is yellow initially and then become orange and later brown in late growth stage (Fig. S4). A yellow pigment was extracted from the culture of \textit{E. nigrum} and identified as flavipin (3,4,5- trihydroxy-6-methylphthalaldehyde) which had antifungal activity\textsuperscript{32}. Also, the red pigments produced by \textit{E. nigrum} were analyzed and detected to consist of four carotenoids\textsuperscript{27,33}. These colorants produced by \textit{E. nigrum} could be used in food, pharmaceutical, textile, and cosmetic industries\textsuperscript{34}.

| Treatment | Equation (Y = total yield of paclitaxel (µg L\(^{-1}\))) | P-value | R-squared | Correlation Coefficient |
|-----------|----------------------------------------------------------|---------|-----------|-------------------------|
| Against E.W. | Against D.W. | Against E.W. | Against D.W. | Against E.W. | Against D.W. |
| ACE       | 77.9597 + 0.587427*FW -30.6762 + 25.3427*DW             | 0.0001  | 0.89265   | 0.93489                 |
| FCE       | 125.25 + 0.505965*FW                                      | 0.0007  | 0.82590   | 0.77233                 |
| AMF       | 27.6739 + 0.891776*FW                                      | 0.0047  | 0.70457   | 0.64193                 |
| FMF       | 173.128 + 1.081166*FW                                      | 0.2078  | 21.52770  | 4.82189                 |
revealed that expression of these genes is high in planta, but low in vitro fungal cultures\textsuperscript{37}. So it seems that fungal paclitaxel production such as fungal growth would be affected by CE and MF of C. avellana CSC. Therefore, the effects of CE and MF of C. avellana CSC on fungal paclitaxel production were investigated. The CE was performed better as plant elicitor in terms of the growth and paclitaxel production. Indeed, The CE of C. avellana supplies plenteous nutrients and enhances the growth of strain YE\textsubscript{F}. The means comparison indicated that FCE was more effective than ACE for the increase of growth and paclitaxel production (Fig. 3). Autoclaving denatures proteins and degrades amino acids and may decrease the nutritional value of CE which resulted in decreased growth and paclitaxel production. The results of ANOVA indicated that CE and MF of C. avellana CSC increased intracellular (per liter of medium), extracellular and total yield of paclitaxel whereas no significant increase in intracellular yield of paclitaxel per gram dry weight of mycelia was observed. Indeed, the part of produced paclitaxel accumulated in mycelia and much of it secreted in the culture medium. Simple linear regression was developed to determined relationship between total yield of paclitaxel and FW and DW. The fitted models for ACE and FCE exhibited high R-Squared which showed these models explain high percent of the variability in total paclitaxel. Also, the high correlation coefficients indicate relatively strong relationship between total paclitaxel and FW and DW (Table 2). It seems that CE (autoclaved and filter sterilized) and AMF of C. avellana CSC enhanced the growth of E. nigrum strain YE\textsubscript{F}\textsubscript{2} and the increase in total paclitaxel production is due to increase biomass rather than a direct effect of the CE and AMF of C. avellana CSC. Meanwhile, AMF increased the growth of E. nigrum more than FMF (Fig. 3b). However, FMF was more effective for paclitaxel production than the AMF (Fig. 3a). Also, Regressions of total paclitaxel against FW and DW for FMF were not significant (Table 2). It is assumed that there are many precursors of paclitaxel in C. avellana CSC and used to produce paclitaxel that these precursors were degraded by autoclaving and lower growth in MS medium supplemented with FMF rather than AMF can be due to increasing paclitaxel production. The inverse relationship between cell growth and paclitaxel accumulation has been reported previously\textsuperscript{38,39}. There is one study which has reported the plant cells produced inhibitory substances in the late growth curve of cell suspension that decreased the fungal biomass\textsuperscript{37}. The presence of these inhibitors in FMF may be a drawback for the increase of YE\textsubscript{F}\textsubscript{2}, growth but autoclaving partly degraded these substances. Therefore, MS medium supplemented with AMF increased growth more than FMF. The presence of paclitaxel precursors in plant cell cultures and the decrease of the final contents of these precursors in fungus through fungi fermentation were reported\textsuperscript{37}. In this context, further studies may be useful on the initial and final contents of paclitaxel precursors and investigation of converting them to paclitaxel in fungus during fermentation by labeled precursors. Improved growth of Fusarium mairei by adding supernatants of yew cell suspension cultures of days 10 and 15 was reported\textsuperscript{37}. Also, it is showed that yew needle extract can elicit fungal paclitaxel production. However, no information has been reported regarding the influence of plant cell extract in the culture medium on growth and paclitaxel production of endophytic fungi. We studied for the first time the effects of C. avellana CE on growth and paclitaxel production of paclitaxel producing E. nigrum strain YE\textsubscript{F}\textsubscript{2}.

Conclusion

The major limitation of using endophytic fungi for industrial paclitaxel production are the low and unstable productivity. In this study, the addition of C. avellana cell extract to MS medium enhanced growth and paclitaxel production of E.nigrum strain YE\textsubscript{F}\textsubscript{2}. It is thought that addition of hazel cell extract to MS medium in E. nigrum culture simulated relatively the chemical environment of its host and resulted in increased growth and paclitaxel production. Since paclitaxel production by endophytic fungi is significantly reduced by repeated sub-culturing on defined artificial media\textsuperscript{40}, using plant cell extract in artificial media may be useful. It is essential to examine whether stable enhanced production of paclitaxel can be obtained using plant cell extract in the fungal culture and the co-cultivation of endophytic fungus with plant cells. Also, it is stated that endophytic fungi in artificial media have no access to the specialized microenvironment of the host plant which may lead to silencing of their secondary metabolite genes, and standard culture conditions may not be sufficient to trigger expression of the cryptic biosynthetic gene clusters\textsuperscript{40}. Based on achieved results, using plant cell extract in the fungal culture or the co-cultivation of fungi with plant cells can be useful for enhancement of paclitaxel production. Large-scale production and extraction of pharmaceutical compounds from the plants are high-priced and tedious. However, the endophytic fungi isolated from the medicinal plants can be easily cultured and large-scale production of the drugs is possible through the fermentation process. It seems that using of plant cell extract in fungal artificial media is promising for the stable enhanced production of paclitaxel in fungi.

Material and Methods

Fungi and plant cell culture reagents. The medium components, plant growth regulators and paclitaxel standard used in this experiment were supplied by Sigma (USA) and Merck (Germany) Chemical Companies.

Isolation of endophytic fungi from T. baccata and C. avellana. Healthy samples including the bark pieces, stem, bud and leaves were collected from T. baccata and C. avellana grown at the botanical garden of College of Agriculture and Natural Resources (36°40’01”N, 51°10’18” E at an altitude of 1321 m), University of Tehran, located in Karaj, Alborz Province of Iran, in July and September 2014. The samples were treated with 75% ethanol (v/v) for 1 min and 2.5% sodium hypochlorite (w/v) for 2 min and rinsed two times with sterilized water. In order to test the effectiveness of surface sterilization\textsuperscript{41}, 10 ml of the last rinsing water was centrifuged for 10 min at 10,000 g. The supernatant was removed and plated onto PDAC (PDA; supplemented with 250 mg/l Chloramphenicol). The surface sterilization was validated because no mycelial growth occurred. The surface disinfected small pieces (4 mm\textsuperscript{2}) of inner bark, bud and leaf segments were excised and placed on the surface of PDAC in unique Petri dishes (100 × 15 mm), incubated at 25 °C to allow the growth of endophytic fungi. Pure fungal cultures of the endophytic isolates were prepared by the hyphal tip culture\textsuperscript{42}. All fungal endophytes isolated from T. baccata and C. avellana were numbered as YE\textsuperscript{F}# and HE\textsuperscript{F}# series, respectively and stored on PDA at 4 °C.
Culture, extraction and detection of paclitaxel. Two agar plugs (5 mm diameter) containing mycelia of the fungal isolates were cultured individually in 100 ml Erlenmeyer flasks containing 30 ml potato dextrose broth (PDB) medium. Cultures were incubated at 110 rpm at 25 °C for 12 days. The fungal mycelia were separated from the broth by filtration. This filtered culture was subsequently extracted by adding two volumes of dichloromethane. The extracted solvent was evaporated using rotary evaporator (Eyela, Tokyo, Japan) to dryness at 35 °C. The dry residue was re-dissolved in 0.5 ml of absolute methanol. Intracellular paclitaxel was extracted from the mycelia with a procedure described below. Freeze-dried mycelia (100 mg) were soaked in 4 ml methanol, sonicated for 40 min. After centrifugation, the supernatant was removed and extracted with dichloromethane:water (1:1, v/v). The organic fraction was collected, dried under vacuum and resuspended in 0.5 ml methanol. All samples were filtered through 0.22 μm cellulose acetate syringe filters before further analysis with high-performance liquid chromatography (HPLC) and high-performance liquid chromatography-mass spectrometry (LC-MS/MS).

Paclitaxel in samples was analyzed by the HPLC system (Waters, USA) with a C18 analysis column (Macherey-Nagel EC 4.6 x 250 mm, 5 μm, Nucleodur). The sample (20 μl) was injected each time and detected at 230 nm using a UV detector. The mobile phase was methanol:water (80:20 v/v) at a flow rate of 1.0 ml/min. The quantification of paclitaxel was based on an external standard of genuine paclitaxel (Sigma). Electrospray mass spectrometry was done on fungal paclitaxel sample using the electrospray technique by an Alliance 2695 waters with a C18 analysis column (Eclipse Agilent 4.6 x 150 mm, 5 μm). The sample in 100% methanol was injected with a spray flow of 0.5 ml/min and a spray voltage of 4.5 kV by the loop injection method. The mobile phase was composed of acetonitrile acidified with 0.1% (v/v) formic acid (A) and water acidified with 0.1% (v/v) formic acid (B) with binary solvent-delivery gradient elution (0–5 min, 40–90% A, 60–10% B).

Molecular studies: Genomic DNA extraction, PCR and sequencing. The endophytic fungus was cultured in PD broth at 25 °C with constant shaking for 7 days. The fungal mycelia were freeze-dried and the genomic DNA was extracted as described by Safaie et al. Briefly, 50 mg of fungal mycelia were vigorously crushed in liquid nitrogen to make a fine powder. The cells were lysed in 400 μl of DNA salt solution (Tris-HCl 100 mM, EDTA pH = 7.5–8 5 mM and NaCl 1.4 mM), mixed thoroughly and incubated at 65 °C for 15 min. The samples were cooled on crushed ice for 10 min and centrifuged at 13,000 g for 10 min at 4 °C. About 300 μl of the aqueous phase was transferred into a new labeled sterile tube. 210 μl cold isopropanol was added and mixed by inverting the tubes several times. The tubes were centrifuged for 15 min at 10,000 g and the supernatant was discarded, air-dried and dissolved in 50 μl of sterile Millipore water.

The fungal internal transcribed spacer (ITS) fragments (ITS1-5.8S-ITS2) were amplified by PCR using the universal primers ITS1 and ITS4. The actin gene (ACT) was partly amplified with primer pair ACT-512F and ACT-783R (Table S1). The PCR reaction mixtures (25 μl) consisted of 1 μl genomic DNA (~100 ng), 1 μl forward and reverse primers (10 μM), and 12.5 μl Premix Taq (TaKaRa Biotechnology Ltd., Japan), and 10.5 μl PCR quality water. The PCR reaction programs were an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation (94 °C for 30 s), annealing (56 °C (ITS) and (59 °C (ACT) for 30 s), extension (72 °C for 1 min) and a final extension at 72 °C for 5 min. The PCR products were analyzed by agarose gel electrophoresis and purified using a DNA gel extraction kit (Axygen Biotechnology Ltd., China). The purified PCR product was directly sequenced using the same primers by Bioneer (Shanghai, China).

The sequences of ITS1-5.8S-ITS2 region and actin gene of the endophytic fungus were compared with the data in National Center for Biotechnology Information, USA (NCBI) using BLAST search (http://blast.ncbi.nlm.nih.gov/BLAST.cgi) to estimate the phylogenetic relationship. CLUSTAL X software (version 2.0, Conway Institute, USA) was used to generate the alignment of endophytic fungus. Phylogenetic analysis was carried out by the neighbor-joining method using MEGA software (version 4.0, Biodesign Institute, USA). The bootstrap was 1,000 replications to assess the reliable level to the nods of the tree.

Preparation of cell extract (CE) and medium filtrate (MF). The callus of hazel (C. avellana) was obtained from seed cotyledons on MS medium supplemented with 0.2 mg/l 6-benzylaminopurine and 2 mg/l 2,4-dichlorophenoxyacetic acid and solidified with 8 g/l agar agar. The C. avellana CSC was established with transferring 5 g callus into 250 ml flasks containing 100 ml medium and were maintained at 25 °C in darkness on gyratory shakers at 110 rpm. Suspensions were also subcultured every 15 days until the cells reached homogeneity. Then 1.5 ± 0.1 g of hazel cells (fresh mass) was transferred to 100 ml flasks containing 30 ml of the cell culture medium. The cell culture medium and the culture conditions for growing cells of C. avellana remained the same as described above. Then the fresh cells were harvested on the 21st day (the stationary growth phase) by passing CSC through a filter paper (Whatman No. 1). The cells washed several times with sterile double distilled water and dried at 60 °C. Then crushed thoroughly in liquid nitrogen. Crushed cells were soaked in water (100 mg/ml), sonicated for 20 min, mixed thoroughly and incubated at 65 °C for 30 min with continuous shaking. The hydrolysate centrifuged at 10,000 g for 15 min. After centrifugation, the supernatant (cell extract) was collected. The cell extract of hazel was divided into two parts: one part was autoclaved at 121 °C for 20 min and designated as autoclaved cell extract (ACE) and another part was filtered through 0.22 μm cellulose acetate syringe filters and designated as filter sterilized cell extract (FCE).

The spent medium was centrifuged at 12,000 g for 20 min to remove completely suspended cells. This cell-free medium was designated as medium filtrate. The medium filtrate of hazel CSC was divided into two parts: one part was autoclaved at 121 °C for 20 min and designated as autoclaved medium filtrate (AMF) and another part was filtered through 0.22 μm cellulose acetate syringe filters and designated as filter sterilized medium filtrate (FMF).
Establishment of suspension culture of paclitaxel producing endophytic fungus. The experiment was carried out with three replicates. Each replication consisted of 100 ml flask containing 30 ml MS medium supplemented with 0.2 mg/l BAP, 2 mg/l GA3 and 2 mg/l 2,4-D. The inoculum was prepared from 7-day-old cultures of strain YEF2 grown on PDA medium at 25°C. Two mycelial agar plugs (5 mm diameter) per replication were cut from the margin of the growing colony using a sterilized cork borer. Cultures were maintained at 25°C in darkness on gyratory shakers at 110 rpm.

Four plant elicitor preparations viz. ACE, FCE, AMF and FMF were added at different concentrations (7, 14, 28% (v/v)) to the medium at the time of culture. The control received an equal volume of the MS medium (for MF)/water (for CE). Since CSC of C. avellana produces paclitaxel30, elicitors derived from it may contain paclitaxel. As the control for fungal paclitaxel production, four elicitors derived from hazel CSC were added at different concentrations (7, 14, 28% (v/v)) to the culture medium with no inoculation and maintained in mentioned condition. The cultures were harvested on the 14th day and analyzed for growth and paclitaxel production.

This experiment was designed as factorial based on a Completely Random Design (CRD) to determine how the CE and MF of C. avellana CSC affected the growth and paclitaxel production of paclitaxel-producing endophytic fungus strain YEF2. The factorial arrangement of the treatments was designed and consisted of three factors containing the type of elicitor with four levels, sterilization method with two levels and elicitor concentration with three levels, given 24 treatments.

Statistical analysis. The hypothesis of normality and equal variance were met and conventional parametric statistics used for the analysis. Analysis of variance and means comparison using least significant difference (LSD) were performed by SAS (SAS 9.3, 2011) and SPSS (SPSS 15.0, 2006). Excel software (Excel, 2011) was used for making graphs. Simple linear regression of total paclitaxel against FW and DW for different treatments was developed by Statgraphics (54. Statgraphics Centurion XVII, 2015).

Availability of data and material. The dataset supporting the conclusions of this article is included in the article.

References
1. Kohler, D. R. & Goldspiel, B. R. Paclitaxel (Taxol). 14, 3–34. https://doi.org/10.1002/1975-9119.1994.tb02785 (1994).
2. Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P. & Mc Phail, A. T. Plant antitumor agents. VI. The isolation and structure of taxol. Anovel antiileukemic and antitumor agent from Taxus brevifolia. J. Am. Chem. Soc. 93, 2325–2327, https://doi.org/10.1021/ja00738a045 (1971).
3. Schiff, P. B., Fant, J. & Horwitz, S. B. Promotion of microtubule assembly in vitro by taxol. Nature 277(5689), 665–667, https://doi.org/10.1038/277665a0 (1979).
4. Cragg, G. M., Schepartz, S. A., Suffness, M. & Grever, M. R. The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. J. Nat. Prod. 56(10), 1657–1668. https://doi.org/10.1021/jp50100a001 (1993).
5. Shinwari, Z. K. & Qaisar, M. Efforts on conservation and sustainable use of medicinal plants of Pakistan. Pak. J. Bot. 43, 5–10 (2011).
6. Stierle, A., Strobel, G. A. & Stierle, D. Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew. Science 260, 214–216, https://doi.org/10.1126/science.8097061 (1993).
7. Guo, B. H. et al. An endophytic taxol-producing fungus BtI isolated from Taxus chinensis var. mairei. Afr J Biotechnol. 5(10), 875–877 (2006).
8. Zhao, K. et al. Aspergillus niger var. taxi, a new species variant of taxol-producing fungus isolated from Taxus cuspidata in China. J. Appl. Microbiol. 107(5), 2622–2627, 2009.04.30.5 (2009).
9. Flores-Bustamante, Z. R., Rivera-Orduña, F. N., Martinez-Cardenas, A. & Flores-Cotera, L. R. Microbial paclitaxel: advances and perspectives. J. Antibiot. 63, 460–467, https://doi.org/10.1038/ja.2010.83 (2010).
10. Somjiapeng, S., Medina, A. & Magan, N. Environmental stress and elicitors enhance taxol production by endophytic strains of Paraconiothyrium variable and Epicoccum nigrum. Enzyme Microb. Technol. 90, 69–75, https://doi.org/10.1016/j.enzmictec.2016.05.002 (2016).
11. Venugopalan, A., Potunuru, U. R., Dixit, M. & Srivastava, S. Effect of fermentation parameters, elicitors and precursors on camptothecin production from the endophyte Fusarium solani. Bioreour. Technol. 206, 104–111, https://doi.org/10.1016/j.biortech.2016.01.079 (2016).
12. Soliman, S. M. & Raizada, M. N. Interactions between co-habitating fungus eliciting synthesis of taxol from an endophytic fungus in host Taxus plants. Prom. Microbiol. 4, 1–14, https://doi.org/10.3389/fmicb.2013.00033 (2013).
13. Kusari, S., Hertweck, C. & Spitterer, M. Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem. Biol. 19(7), 792–798, https://doi.org/10.1016/j.chembiol.2012.06.004 (2012).
14. Soliman, S. M., Trobacher, C., Tao, R., Greenwood, J. & Raizada, M. A. Fungal Endophyte Induces Transcription of Genes Encoding a Redundant Fungicide Pathway in Its Host Plant. BMC Plant. Biol. 13(1), 93, https://doi.org/10.1186/1471-2229-13-93 (2013).
15. Rodriguez, R. I., White, J. F., Arnold, A. E. & Redman, R. S. Fungal endophytes: diversity and functional roles. New Phytol. 182, 314–330, https://doi.org/10.1111/1469-8137.2009.02773.x (2009).
16. Firakova, S., Sturidkova, M. & Muckova, M. Bioactive secondary metabolites produced by microorganisms associated with plants. Biologia 62, 251–257, https://doi.org/10.2478/s11756-007-0044-1 (2007).
17. Li, Y. C. & Tao, W. Y. Interactions of taxol-producing endophytic fungus with its host (Taxus spp.) during taxol accumulation. Cell Biol. Int. 33(1), 106–112, https://doi.org/10.1006/cjbi.2008.10.007 (2009).
18. Venugopalan, A. & Srivastava, S. Enhanced camptothecin production by ethanol addition in the suspension culture of the endophyte, Fusarium solani. Bioreour. Technol. 188, 251–257, https://doi.org/10.1016/j.biortech.2014.12.106 (2015).
19. Hoffman, A. et al. Bioprosp. Taxol in angiosperm plant extracts—Using high performance liquid chromatography thermospray mass spectrometry to detect the anticancer agent and its related metabolites in Filbert trees. Spectrosc. 13(6), 22–32 (1998).
20. Hoffman, A. & Shahidi, F. Paclitaxel and other taxanes in hazel. J. Funct. Foods 1, 33–37, https://doi.org/10.1016/j.jff.2008.09.004 (2009).
21. Service, R. F. Hazel trees offer new source of cancer drug. Science 288, 27–28, https://doi.org/10.1126/science.288.5463.27a (2000).
22. Bestoso, F. et al. In vitro cell cultures obtained from different explants of Corylus avellana produce Taxol and taxanes. Biotechnol. 6, 45–56, https://doi.org/10.1186/1422-6553-6-45 (2006).
23. Gallego, A. et al. Taxol from Corylus avellana: paving the way for a new source of this anti-cancer drug. Plant Cell Tissue Organ Cult. 129, 1–16, https://doi.org/10.1007/s11240-016-1164-5 (2017).
24. Somaijapen, S., Medina, A., Kwaśna, H., Ortiz, J. O. & Magan, N. Isolation, identification, and ecology of growth and taxol production by an endophytic strain of Paraconiothyrium variabile from English yew trees (Taxus baccata). Fungal Biol. 119(11), 1022–1031, https://doi.org/10.1016/j.funbio.2015.07.007 (2015).
25. Kerns, E. H., Volk, K. J., Hill, S. E. & Lee, M. S. Profiling taxanes in Taxus extracts using LC/MS and LC/MS/MS techniques. J. Nat. Prod. 57, 1391–1403, https://doi.org/10.1021/np0112a008 (1994).
26. Samsom, R. A., Houbbraken, J., Thirane, U., Frisvad, J. C. & Anderson, B. Food and Indoor Fungi. CRS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherland (2010).
27. Da Silva Araujo, F. D. et al. Epicolactone–Natural Product Isolated from the Sugarcane Endophytic Fungus Epicoccum nigrum. Eur. J. Org. Chem. 2012(27), 5225–5230, https://doi.org/10.1002/ejoc.201200757 (2012).
28. De Lima Favaro, L. C., Melo, F. L., Aguilar-Vildoso, C. I. & Araújo, W. L. Polyphasic analysis of intraspecific diversity in Epicoccum nigrum warrants reclassification into separate species. PROTOC ONE 6, 14828, https://doi.org/10.1371/journal.pone.0014828 (2011).
29. Caruso, M. et al. Isolation of endophytic fungi and actinomycetes taxane producers. Ann Microbiol 50, 3–13 (2000).
30. De Lima Favaro, L. C., de Souza Sebastianes, F. L. & Araújo, W. L. Epicoccum nigrum P16, a sugarcane endophyte, produces antifungal compounds and induces root growth. PLOS One 7(6), 36826, https://doi.org/10.1371/journal.pone.0036826 (2012).
31. Larena, I. et al. Biological control of postharvest brown rot (Monilinia spp.) of peaches by field applications of Epicoccum nigrum. Biol. Control 32(2), 303–310, https://doi.org/10.1016/j.biocontrol.2004.10.010 (2005).
32. Bamford, P. C., Norris, G. L. F. & Ward, G. Flavipin production by Epicoccum spp. Trans. Br. Mycol. Soc. 44(3), 354–356, https://doi.org/10.1016/S0007-1536(81)80028-4 (1981).
33. Poppen, F. H. & Griganovski-Sassu, O. Lipids produced by Epicoccum nigrum in submerged culture. Biochim. J. 106(1), 97–100, https://doi.org/10.1042/bj106097 (1968).
34. Cretu, R., Bahrim, G., Stefan, D. & Oleane, M. Evaluation of physical and chemical characteristics of yellow colorant produced by Epicoccum nigrum MIUG 2.15 in crude extracts and emulsions. Rom. Biotechnol. Lett. 15(3), 59–68 (2008).
35. Murashige, T. & Skoog, F. A inserted medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497, https://doi.org/10.1111/j.1399-3054.1962.tb00523.x (1962).
36. El-Bahrawy, S. A. Effect of gibberellic acid on some microbial growth and spore germination of some fungi. Zentralbl. Mikrobiol. 137(1982), 238–240, https://doi.org/10.1016/S0232-4393(82)80034-6 (1982).
37. Yang, Z., Rogers, L. M., Song, Y., Guo, W. & Kolattukudy, P. E. Homoserine and asparagine are host signals that trigger in planta expression of a pathogenesis gene in Nectria haematococca. Proc. Natl. Acad. Sci. USA 102, 4197–4202, https://doi.org/10.1073/pnas.0500312102 (2005).
38. Wang, C., Wu, J. & Mei, X. Enhancement of taxol production and excretion in Taxus chinensis cell culture by fungal elicitation and medium renewal. Appl. Microbiol. Biotechnol. 55(4), 404–410, https://doi.org/10.1007/s002530000567 (2001).
39. Wickremesinhe, E. R. & Arthera, R. N. Taxus callus cultures: initiation, growth optimization, characterization and taxol production. Plant Cell Tissue Organ Cult. 35(2), 182–193, https://doi.org/10.1021/np003298h (1993).
40. Venugopalan, A. & Srivastava, S. Endophytes as in vitro production platforms of high value plant secondary metabolites. Biotechnol. Adv. 33(6), 873–887, https://doi.org/10.1016/j.biotechadv.2015.07.004 (2015).
41. Rakotominirina, E. F. et al. Endophytic fungi from leaves of Centella asiatica: occurrence and potential interactions within leaves. Antonie Van Leeuwenhoek 93, 27–36, https://doi.org/10.1007/s10482-007-9176-0 (2008).
42. Strobel, G. et al. Taxol from Pestalotiopsis microspora, an endophytic fungus of Taxus wallachiana. Mycobiology 142, 435–440, https://doi.org/10.1009/13500872-142-4-435 (1996).
43. Strobel, G., Daisy, B., Castillo, U. & Harper, J. Natural products from endophytic microorganisms. J. Nat. Prod. 67, 257–268, https://doi.org/10.1021/np030339v (2004).
44. Luo, J., Liu, L. & Wu, C. D. Enhancement of paclitaxel production by abscisic acid in cell suspension cultures of Taxus chinensis. Biotechnol. Lett. 23(16), 345–348, https://doi.org/10.1023/A:1005978207241 (2001).
45. Safaie, N., Alizadeh, A., Saidi, A., Rahimian, H. & Adam, G. Molecular characterization and genetic diversity among Iranian populations of Fusarium graminearum, the causal agent of wheat head blight. Iran. J. Plant Pathol. 41, 171–189 (2015).
46. White, T. J., Bruns, T., Lee, S. & Taylor, J. PCR Protocols: a guide to methods and applications (eds Innis M. A. et al.) Ch. 38, 315–322, https://doi.org/10.1006/B978-0-12-372180-8.50042-1 (Academic Press, 1990).
47. Carbone, I. & Kohn, L. M. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91, 553–556, https://doi.org/10.2307/3761338 (1999).
48. Larkin, M. A. et al. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948, https://doi.org/10.1093/bioinformatics/btm404 (2007).
49. Tamura, K., Dudley, J., Nei, M. & Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599, https://doi.org/10.1093/molbev/msm092 (2007).
50. Salehi, M., Moieni, A. & Safaie, N. A Novel Medium for Enhancing Callus Growth of Hazel (Corylus avellana L.). Sci. Rep. 7(1), 15598, https://doi.org/10.1038/s41598-017-15703-z (2017).

Acknowledgements
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions
A. Moiene and N. Safaie supervised the project, N. Safaie and M. Salehi designed the project. M. Salehi carried out the experiments, data analysis and drafted the manuscript. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-29762-3.

Competing Interests: The authors declare no competing interests.

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