Hazel (*Corylus avellana* L.) as a New Source of Taxol and Taxanes

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Received: 5 Apr. 2011 **Accepted:** 20 Feb. 2012

Abstract

Taxol is used to treatment of variety cancers including ovarian, breast, lung, head and neck carcinomas and the AIDS-related Karposi's carcinoma, and was originally obtained from bark of *taxus brevifolia*. However the amount of extracted taxol from taxus is extremely low and cannot be considered as a suitable commercial source and it has led to studies for finding new sources of Taxol. In 1998, Angela Hoffman and her team reported that hazel could be as a taxol-producing species and it has been confirmed by recent studies. On the other hand, the genes and enzymes involved in taxol biosynthesis pathway in *taxus* sp. have been identified and characterized but because of very slow growth and difficult somatic embryogenesis, manipulation of taxus is restricted. Instead, hazel with widely contribution in the world, easy invitro culture and characterization of some genes involved in the pathways of MVA and DXP (such as HMGR, IPI and GGPP), makes possibility of manipulation of taxol biosynthesis pathway. In this paper we reviewed a summary of genetic and biotechnology studies on hazel.

Keywords: Taxol, Hazel, MVA pathway, DXP or MEP pathway

[DOR: 20.1001.1.2717204.2012.11.41.8.4]

Introduction

Paclitaxel is the general name of taxol, a chemotherapeutic agent with a wide range of activity [1, 2, 3]. Like most of the drugs for clinical uses, taxol is produce by semisynthesis, starting from a natural precursor, 10-deacethylbacatin III that is more readily available from the needles of yew species as a renewable source [4, 5, 6]. Taxus species and entophytic fungi have been considered as the only sources to be exploited for the commercial supplies of paclitaxel and its precursors generally named taxanes [7, 8], instead recent studies have been shown that hazel species possess the metabolic pathway (s) for taxane biosynthesis [9], the biological studies of hazel extract on cell human tumor also have been revealed capability to inhibit metaphase to anaphase transition in cell culture [9]. Taxol is one of the products of biosynthesis terpenoids pathway, terpenoids are a class of compounds derived from the universal precursor isopentenyl diphosphate (IPP) and its allelic isomer dimethylallyldiphosphate (DMAPP), also called isoprene units. Two distinct pathways generate the universal C_5 precursors IPP and DMAPP (Fig. 2). The classic mevalonate (MVA) pathway was discovered in the 1950s and was assumed to be the sole source of the terpenoid precursors IPP and DMAPP. The MVA pathway is active in bacteria, plants, animals and fungi and functions in the cytosol to generally supply the precursors for production of sesquiterpenes and triterpenes. Other pathway, which is named after the first committed precursor, 2-*C*-methyl-d-erythritol-4-phosphate (MEP; the pathway is also sometimes referred to as the DXP pathway), is plastidial in nature and is generally used to supply precursors for the production of monoterpenoids, diterpenoids and tetraterpenoids. Although interaction between these pathways is still largely undiscovered, recent evidence has revealed an exchange of intermediates between the cytosol and the plastid [9, 10, 11]. Recent studies demonstrating cross-talk between the MVA and MEP pathways suggest that engineering of the MVA pathway for IPP formation could be useful in controlling plastidial terpenoid biosynthesis. A common strategy for engineering terpenoid synthesis is to focus on enhancing flux to increase the precursor pools of IPP and DMAPP, by cloning the key genes and understanding regulation of both the MVA and MEP supply pathways [11, 12, 13]. The classic MVA pathway is well defined, and numerous studies have demonstrated the importance of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*). *HMGR* is strictly regulated in plant systems and elicitor-induced *HMGR* genes have been cloned and characterized in numerous plant systems including tobacco, tomato, potato, hazel and euphorbia [14, 15, 16]. Recently two genes of hazel including *IPI* and *GGPP* belong to MEP pathway has been cloned, which are key precursors for diterpenes such as taxol [17, 18]. Because of many studies in the area of biosynthesis pathways of Taxol and its new source, achieving to high commercial production of taxol via manipulation and suspension culture is available. The aim of this study is investigation and feasibility of manipulation of taxol biosynthesis pathway in hazel.

Importance of Corylus

Hazel is containing significant amounts of thiamine and vitamin B_6, as well as smaller

[DOR: 20.1001.1.2717204.2012.11.41.8.4]

amounts of other B vitamins. Additionally, 1 cup (237 mL) of hazel flour has 20 g of carbohydrates, 12 g of which are fiber. Hazels are high in protein (19%) and are nutritious, being an excellent source of vitamins E and B_6. Hazels are high in oil content (60%), and are 3 times the oil content of soybean (20% on average). Hazel oil is similar to olive oil, containing 70% monounsaturated fats. The European hazel, a tree, provides most of the world's supply of hazels commercially (Table 1). Taxol is an effective antineoplastic agent, originally extracted from the bark of *Taxus brevifolia* with a low yield. Many attempts have been made to produce taxol by chemical synthesis, semisynthesis and plant tissue cultures. However, to date, the availability of this compound is not sufficient to satisfy the commercial requirements. The aim of the present work was to produce suspension cell cultures from plants not belonging to *Taxus genus* and to verify whether they produced taxol and taxanes [9]. Hoffman and co-workers shown that some american varieties of *Corylus avellana* contaminated by fungal pathogens have, small amounts of taxol and other taxans in the leaves, branches and in the fruits [19]. In other study shells and leaves of hazel plants were analyzed by ELISA and HPLC-MS. Both shell and leaf extracts contained taxanes. Among these, paclitaxel, 10-deacetylbaccatin III, baccatin III, paclitaxel C, and 7-epipaclitaxel were identified and quantified. Hazel extracts also showed biological activity, inhibiting metaphase to anaphase transition in a human tumor cell line. The level of total taxanes in leaves was higher than in shells collected in the same period from the same plants. However, the finding of these compounds in shells, which are considered as a discarded material and are mass produced by many food industries, is of interest for the future availability of paclitaxel and other antineoplastic compounds [20]. The Taxol biosynthetic pathway has been elucidated and many genes encoding certain enzymes, which regulate Taxol biosynthesis pathway, have been cloned and characterized (Table 2).

Table 1- Nutrition* components per 100 g kernel of hazel

Nutrition	Amount	RDA**
Thiamine (Vit. B_1)	0.6 mg	46%
Riboflavin (Vit. B_2)	0.11 mg	7%
Niacin (Vit. B_3)	1.8 mg	12%
Vitamin B_6	0.6 mg	46%
Folate (Vit. B_9)	113 µg	28%
Calcium	114 mg	11%
Iron	4.7 mg	38%
Phosphorus	290 mg	41%
Potassium	680 mg	14%
Energy	2629 J (628 kcal)	
Carbohydrates	17 g	
Dietary fiber	10 g	
Fat	61 g	
Saturated	4 g	
Monounsaturated	46 g	
Polyunsaturated	8 g	
Protein	15 g	

*Nutrition Facts, Bob's Red Mill All-Natural Hazel Meal/Flour (Amazon.com)
**RDA = Recommended Daily Amount

[DOR: 20.1001.1.2717204.2012.11.41.8.4]

Table 2- The cloned genes involved in Taxol biosynthesis pathway in *TaxusI* sp. and *Corylus avellana*

Enzyme	cDNA corresponding to the enzyme			Reference
	GenBank Accession No.	CDS (bp)	Enzyme (kDa)	
Taxadiene synthase	AY364469	2586	98.3	Wildung et al., 1996
GGPPS	AF081514	1182	42.6	Hefner et al., 1998
GGPPS*	EF553534	1122	40.53	Wang et al., 2009
IPI*	EF553533	891		Wang et al., 2010
HMGR*	EF206343	1704	60.83	Wang et al., 2007
TAT	AF190130	1317	49	Walker et al., 2000a
TBT	AF297618	1320	50	Walker et al., 2000b
DBAT	AF193765	1320	49	Walker et al., 2000c
Taxane 10- hydroxylase	AF318211	1494	56.7	Schoendorf et al., 2001
Taxane 13- hydroxylase	AY056019	1458	54.7	Jennewein et al., 2001
BAPT	AY082804	1335	50	Walker et al., 2002a
DBTNBT	AF466397	1323	49	Walker et al., 2002b
Taxane 2- hydroxylase	AY518383	1488	55	Chau et al., 2004a
Taxane 7- hydroxylase	AY307951	1503	56.3	Chau et al., 2004b
Taxane 5- hydroxylase	AY289209	1509	56.8	Jennewein et al., 2004
PAM	AY582743	2094	76.5	Walker et al., 2004

* Genes from *Corylus avellana* L

Incompaitability

Pollination of hazel is by wind, and only takes place between different trees (a tree cannot pollinate itself). Hazel lives for 50-70 years, but the ancient technique of coppicing can dramatically extend the life-span [21]. It is known that there is self and cross incompatibility in hazel. Pollen–stigma incompatibility in this plant is an important consideration in planning crosses in a breeding program and pollinizers for orchard planting. Therefore, it is necessary to know about the incompatibility between the main and the pollinizer cultivars in the orchards [22, 23]. Incompatibility in hazel is of sporophytic type and under the control of a single locus with multiple alleles [24]. The stigmatic surface is the site of the incompatibility reaction and pollen tube growth is arrested in the stigmatic surface [25, 26]. In other hand Callan and Thompson's research (1985) have shown that exogenous sugar overcame incompatibility in hazel [27].

Origin

The geographical distribution of the European hazel extends from the Mediterranean coast of North Africa northward to the British island and the Scandinavian Peninsula, and eastward to the Ural Mountain of the Russia, the Caucasus mountains, Iran, and Lebanon [28]. 75 accessions from Spain, Italy, Turkey, and Iran were analyzed using 13 chloroplast microsatellite to investigate the origin and diffusion of hazel cultivars. Four loci were polymorphic and identified a total of four different chlorotypes. Their distribution was not uniform in each geographical group. The most frequent chlorotype A was present in all groups. An increase in chlorotype number and diversity from Spain eastward to Italy, Turkey, and Iran was observed. Results suggest that some spread of cultivars occurred from East to West and that hazel cultivation was not introduced from the eastern Mediterranean basin into Spain and Southern Italy by Greeks

[DOR: 20.1001.1.2717204.2012.11.41.8.4]

or Arabs. Moreover, the results suggest considerable exchange of germplasm between Italy and Spain, probably by the Romans. Hazel appears to have been domesticated independently in three areas, the Mediterranean, Turkey, and Iran [29].

Cytology

The chromosome base number of *corylus* (15 species) has long been considered to be x=14 based on the early report of Woodworth [30], but in the later publications, different chromosome numbers have been reported in most species of the genus. On the basis of review of the earlier publication and additional observation in three species of the *corylus* (*C. Americana, C. heterophyla* var. *thunbergii, C. sieboldiana*), demonstrates that the generic chromosome base number is not x=14 [31], but x=11 (Table 3). Molecular phylogeny of betulaceae suggests that x=11 is an autapomorphy of *Corylus* derived from x= 8 common to three other genera of Coryloideae [31].

Biotechnology

Tissue culture and micropropagation

Hazel micropropagation has started from three decades ago and continued till present. Like other woody plants micropropagation success has high dependency on the type of genotype, kind and age of explants. The best explants have obtained from upper branches. To prevent browning of explants, which is caused by phenolic components, were applied solution of ascorbic acid and systein. Also The root induction have been done by many researchers that results reviled effect of different factors such as type and amount of hormones, the type of medium and bacteria [36, 37, 38, 39].

Callus induction

Calli has been induced from different parts of plant such as leaves, petiole, stems and seeds under the influence various combination of growth regulators. The best hormonal combination to callus induction was combination of BA (benzylaminopurine) and 2, 4-D (2, 4-dichlorophenoxy acetic acid) [40], (Fig. 1) Also previous researches indicatet the important role of BA and 2, 4-D on callus induction in hazel [9].

Table 3- A summary of chromosome data in *corylus* sp.

Taxon	Woodworth (1929)	Other authors[1]		Kazuo et al. (1998)
C. americana Mill	n= 14	n= 6	2n= 22	2n= 22
C. avellana L.	n= 14	n= 10, 11, 12	2n= 22	
C. colurna L.	n= 14	n= 7-10	2n= 18, 22, 28	
C. cornuta Marsh	n= 14	n= 6	2n= 22	
C. ferox Wallich			2n= 22	
C.heterophylla Fisch.	n= 14		2n= 22	2n= 22
C. hostrata Ait.		n= 11	2n= 22	
C. lolurnoides Schneider			2n= 22	
C. maxima Mill.	n= 14	n= 11	2n= 22	
C. pontia C. Koch	n= 14			
C. sieboldiana Blume	n= 14			2n= 22
C. spinescens Rehd	n= 14			
C. tibetica Betalin	n= 14		2n= 22	
C. vilmorinii Rehd.	n= 14			

[1)] Data from Fedorov [32], and "index to plant chromosome numbers" edited by Moore [33], Goldblatt [34] and Goldblatt and Johnson [35]

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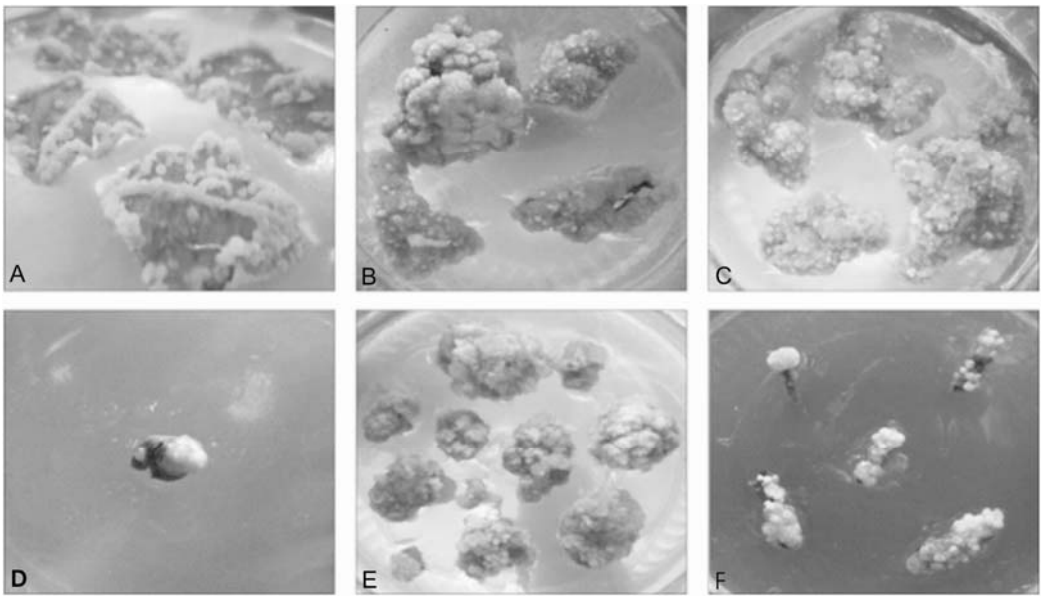

Figure 1- Callus induction from different explants of *corylus avellana*. (a), (b), (c), and (e) calli from laves, (d) callus from cotyledons, and (f) callus from petioles

Somatic embryogenesis

Generally for explants derived from embryogenic and juvenile phase two approaches have been used, direct and indirect induction. Two different basal salt concentration used to induced somatic embryogenesis, modified MS (Murashige and Skoog) medium or T medium (Tuleck and McGranahan), supplemented with: 0.6 mM m-inositol, 0.04 mM nicotinic acid, 0.03 mM pyridoxine hydrochloride, 0.04 mM glycine, and 0.01mM thiamine hydrochloride. Two different growth regulators treatment could have been successfully used Kinetin (K) (9 µM) plus BA (5 µM) plus IBA (0.05 µM) for 40 to 60 days, or IBA(5 µM) plus BA (0.5 µM) for 25 days, followed by IBA(0.5 µM) plus BA (5 µM) during other 25 days. Indirect embryogenesis obtained by use same basal media supplemented with 2,4-D (1.0-4.5 µM) in combination with K (0.1-1.0 µM) and/or BA (0.1 µM). Variable result also obtained in presence of several concentrations of BA plus K or BA plus IBA after longer periods of culture [41].

Hazel as a new source of taxol

During years 1950s, a joint research between the National Cancer Institute (NCI) and the United States Department of Agriculture (USDA) led to recognition of taxol and comptothecin [42]. Taxol (generic name paclitaxel) was first isolated from the bark of the pacific yew tree, *T. brevifolia*. Its complex, the highly oxygenated diterpenoid structure was determined in 1971 [43]. Taxol is one of the most effective drugs to treatment of variety cancers (including ovarian, breast, lung, head and neck carcinomas and the AIDS-related Karposi's carcinoma) [44]. Taxol is belonging to terpenoid components that over 40,000 different terpenoids have been isolated from plant, animal and microbial species [45, 46]. Terpenoids are a class of compounds derived from the universal precursor isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyldiphosphate (DMAPP), also called isoprene units (Fig. 2). Two distinct pathways generate the universal C_5 precursors

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IPP and DMAPP The classic mevalonate (MVA) pathway was discovered in the 1950s and was assumed to be the sole source of the terpenoid precursors IPP and DMAPP. The MVA pathway is active in bacteria, plants, animals and fungi and functions in the cytosol to generally supply the precursors for production of sesquiterpenes and triterpenes (Fig. 3).

Because the yew is one of too slowly growing trees, commercial produce of taxol has not been possible yet. Therefore finding a new source of taxol is so important. Hoffman [19] reported paclitaxel from hazel for the first time. Although the amount of taxol in every gram of branches and leaves of hazel is about 5 micrograms, hazel has faster growing and wide distribution [47]. Recent researches have been indicated that taxanes recovered from hazel culture are produced by hazel metabolism not by endophytic fungus contamination. In other hand, the amount of taxanes has been increased through elicitation with methyljasmonate or methyljasmonate plus chitosan. The HPLC analysis of medium after 2, 4 and 6 days after elicitation, has shown that the production of total taxan, consisting of taxol,10-deacethylbacatin III, bacatin III, 10-deacethyltaxol, 7-xylosyltaxol, increased after elicitation [9]. In other research [20] analysis by ELISA and HPLC-MS has been indicated that both shell and leaf extracts contained taxanes such as taxol, 10-deacethylbacatin III, bacatin III, paclitaxel C, 7-epipaclitaxel. Thus, hazel has a high potential to be new commercial source of paclitaxel.

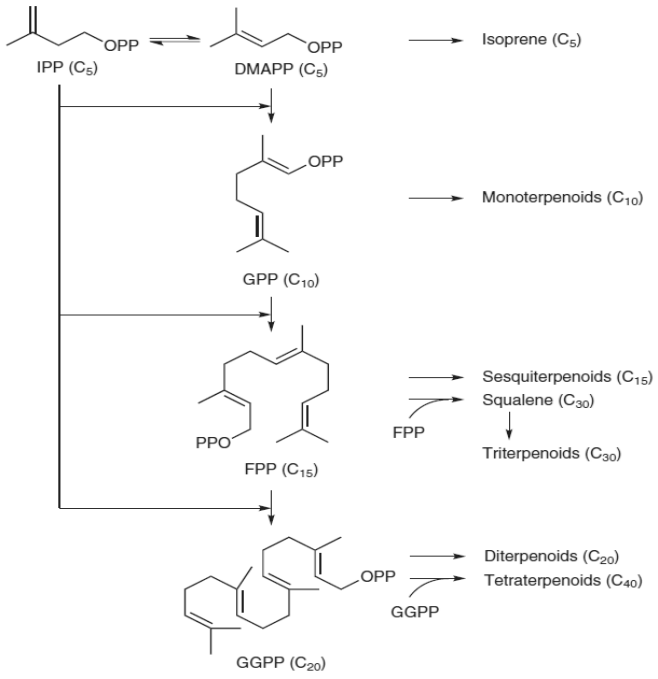

Figure 2- Overview of terpenoid biosynthesis and the generation of terpenoid starting blocks. Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) condense to form higher order terpenoid precursors, including the monoterpenoid precursor geranyl diphosphate (GPP), the sesquiterpenoid precursor farnesyl diphosphate (FPP) and the diterpenoidprecursor geranylgeranyl diphosphate (GGPP). Two molecules of FPPcondense to form the triterpenoid precursor squalene, and two molecules of GGPP condense to form higher order tetraterpenoids [10]

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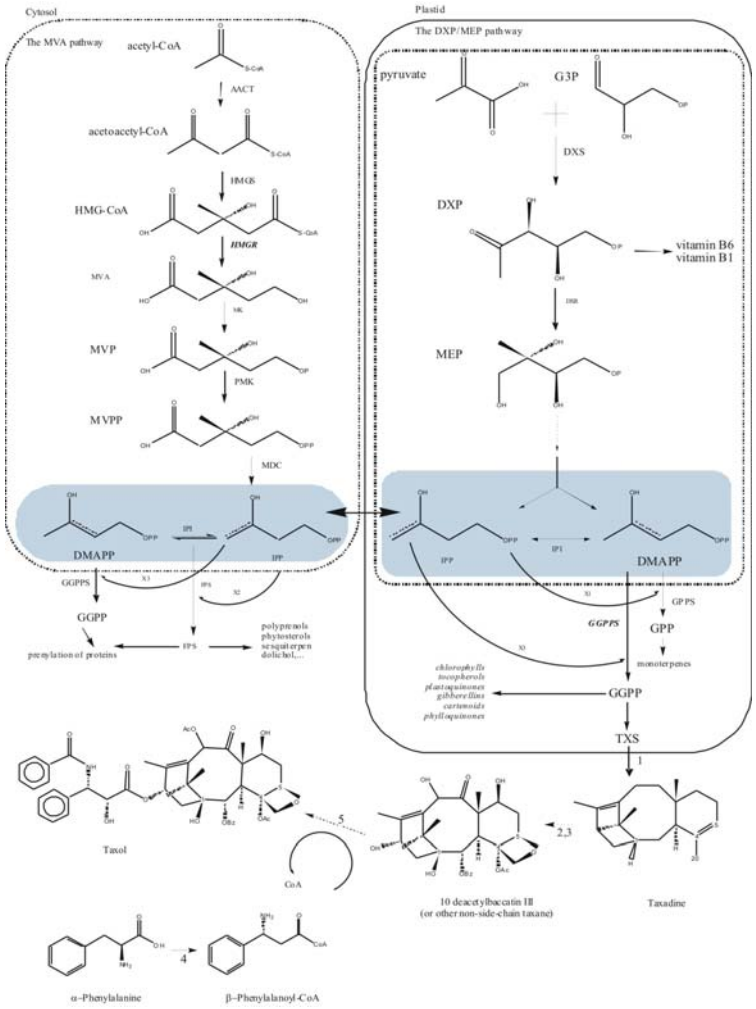

Figure 3- Biosynthesis of paclitaxel and related taxanes [10]

Molecular studies

DNA typing can be a convenient method for accurately identifying hazel cultivars. Microsatellite or Simple Sequence Repeat (SSR) molecular markers have been routinely isolated from plants and have been very useful because they are locus-specific, codominant, highly polymorphic and highly reproducible [48]. Ixchel et al [49] studied phylogeny and biogeography of *Corylus* (Betulaceae) by the use of Inferences from ITS Sequences. The ITS phylogeny supports the recognition of two divisions within the genus: division of *Acanthochlamys* comprising the basal extant lineage *Corylus ferox*, and division of *Corylus* consisting of the remainder of the genus. Within division *Corylus*, recognition of three subdivision is supported, subdivision *Corylus* (species with leafy, more or less bell-shaped involucres), subdivision *Colurnae* (tree species with deeply dissected involucres), and subdivision *Siphonochlamys* (species with tubular involucres). The ITS sequence divergence among species within *Corylus* is low, corresponding with that of most other North Temperate disjunct taxa. A few

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morphological characters were added to the analysis to boost support for branches. Migration between Asia and North America via the Bering land bridge is suggested by subdivision *Siphonochlamys* while long distance dispersal from europe to north america during the late tertiary is suggested by the close relationship and lack of sequence divergence among members of subdivision *Corylus.* A close biogeographic relationship among areas of eastern Asia, the Himalayas, and the Mediterranean region is suggested by the subdivision *Colurnae* clade [51]. In other phylogenetic study by ITS and chloroplast *matK* gen distinguished 12 species of the genus *Corylus* (Betulaceae) also results appeared two clusters on the consensus tree, one with Asian and European species, and the other with North American species. However, this classification based on *matK* sequences was not in agreement with currently accepted taxonomic classification, and the paucity of informative characters precludes more definitive inferences from the *matK* data [50]. Recently a linkage map for European hazel (*Corylus avellana* L.) constructed using random amplified polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) markers and the 2-way pseudotestcross approach [51].

Conclusion

Taxol is widely used as an anticancer drug, but the challenging supply and demand balance has driven an extensive search for new sources to replace the yew tree *T. brevifolia.* Phytochemical analysis has shown that hazel species possess the metabolic pathway (s) for taxane biosynthesis because hazel can produce taxol when it is in aseptic conditions, likely in the absence of microorganisms such as endophytic fungi. In other hand many of the taxol bio- synthesis pathway's enzymes are now known, and the corresponding genes have been cloned and characterized. Three key rate-limiting enzyme genes (*HMGR*, *IPI* and *GGPP*) from hazel involved in terpenoids biosynthesis have been cloned and characterized. Therefore taxol synthesis can be regulated by over expression the key genes of Taxol biosynthesis pathway. In parallel studies, positive effect of elicitors such as methyljasmonate or methyljasmonate plus chitosan on increasing of taxol production has been revealed. Producing taxanes through hazel cell culture is better than yew because hazel is widely available, grows at a much faster rate invivo, and is easier to cultivation invitro than yew. In addition the finding of these compounds in shells, which are considered as a discarded material and are mass produced by many food industries, is of interest for the future availability of paclitaxel and other antineoplastic compounds.

Acknowledgment

We are grateful from Dr. Hasanali Naghdi Badi to his assistants. The authors would like to acknowledge Dr. Shawn A. Mehlenbacher for his useful articles. This work was supported by Institute of Medicinal Plants (IMP), Academic Center for Education Culture and Research (ACECR), in Karaj, Iran.

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