Adv. Hort. Sci., 2018 32(1): 113-135
DOI: 10.13128/ahs-21330

Review paper

AHS
Advances in Horticultural Science

Bio-techniques for improvement of qualitative and quantitative traits in walnut (*Juglans regia*)

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Citation:
SHAH U.N., MIR J.I., AHMED N., ZAID A., JAN S., FAZILI K.M., WANI S.H., 2018 - *Bio-techniques for improvement of qualitative and quantitative traits in walnut (*Juglans regia*).* - Adv. Hort. Sci., 32(1): 113-135

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 26 September 2017
Accepted for publication 27 October 2017

Key words: antioxidants, molecular markers, nutraceuticals, polyphenols, phytochemicals, walnut.

Abstract: Walnut, *Juglans regia* (L.) is an economically significant plant for its immense nutritive and economic value. The breeding character of walnut has lent it a wide diversity in genetic characteristics. The principal vegetative and common traditional agronomic traits together with biochemical characterization i.e., karyotyping and isoenzyme expression have been the early research methods. However, these techniques are time-consuming and susceptible to the environmental variations. Literature is meager in the distribution, applied applications in general and the use of agriculture biotechnology in particular in case of walnut plants. The bio-techniques like molecular markers are adequate in number and there is little or no diversity in the method employed for research on walnuts. Despite basic research method, the organization of information, its retrieval and presentation structures, form elaboration experienced immense advancement via molecular markers such as RFLP, ISSR, RAPD AFLP, SSR and SNP. This appraisal in its first part provides detailed information regarding the present scenario of data on biogeographical distribution, health benefits of walnut worldwide and current applications in the agroforestry management, biochemical evaluations and applied uses of a walnut tree which is relevant for both basic and applied research. The review in its second part sheds light on the application of sophisticated agricultural biotechnology techniques such as use of molecular markers to evaluate, realize the full potential of walnut for increasing its quality, quantity and for its sustainable production which cannot be obtained through usual breeding techniques to meet the demands of a projected world population.

1. Origin and history of walnut (*Juglans regia* L.)

Walnut *Juglans regia* (L.) is commonly recognized as "Jupiter's royal acorn". English walnut is commonly known as "Persian walnut" as it originated from ancient Persia and was adorned as for royalty. *Juglans regia* (L.) species is extensively cultivated in the temperate zones of the world for its best quality nuts and procurement of commercial timber (McGranahan and Leslie, 1991, 2009). Lateral fruit bearing walnut exhibit more genetic variation particularly among familial varieties. In ancient times, the recognition of walnut was spread vigorously through caravans via sea trade. Greek and Romans have lately acquired trend towards walnut cultivation to avert the speckled walnut growing along the coastal areas.

2. Botanical description of walnut

Plant morphology

Juglans regia (L.) is a deciduous perennial tree belonging to the family *Juglandaceae* and grown chiefly for its edible seeds. Walnut tree is predominantly an extensive canopy-dweller decurrent tree with trunk size ranging from 1.5-2 meters in diameter, 25-35 m in height and its survival rate is about 200 years. Walnut bears smooth bark with olive brownish color adapted to moderate light conditions. Leaves are 30-40 cm long, compound, alternately arranged and odd-pinnate. Male flowers are produced in drooping catkins and the female flowers, in clusters of two to five, are terminal, which ripens in the autumn into a fruit with a semi-fleshy husk and a brown nut. The kernel of the nut is protected by a corrugated woody shell.

Flowering, pollination and fruiting

Juglans are monoecious species, having male and female reproductive organs on separate flowers on the same tree. *Juglans regia* is self-fertile, heterogamous and it can be protandrous or protogynous depending on cultivar. The characteristic inflorescence is catkin bearing about 2-3 pistillate flowers born terminally or laterally. Walnut is self-fertile however occasionally it requires different cultivar due to dichogamy. Majority of walnuts are protandrous and anemophilous. Nuts are borne individually or in groups enumerating 2-3 on shoot tips. Walnut is properly shielded by a fleshy covering which cracks at maturity. Walnut shape is variable from ovoid to round attaining a maximum diameter of 2 meters enclosing two kernels divided by extremely delicate covering expanding from inner cover of the shell.

Taxonomical classification

Walnut is a member of *Juglandaceae* family and includes approximately 60 species, among which 21 species are classified under genus *Juglans* (Table 1). Different varieties of walnut and their commercial uses are given in Table 2. All walnut species are edible and English walnut is easily crackable and enormous in size.

Table 1 - Taxonomic classification of walnut

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Fagales
Family	Juglandaceae
Subfamily	Juglandoideae
Tribe	Juglandeae
Subtribe	Juglandinae
Genus	*Juglans*
Species	● *J. regia* Linnaeus
	● *Juglans ailanthifolia* Carr.
	● *Juglans ailantifolia* Carriere
	● *Juglans bixbyi* Rehd.
	● *Juglans boliviana* (C. DC.) Dode
	● *Juglans californica* S. Wats.
	● *Juglans cinerea* Linnaeus
	● *Juglans hindsii* (Jepson) Jepson ex R. E. Sm.

Different sections and species of walnut

Genus *Juglans* is classified into four types: *Juglans sect. Cardiocaryon, Juglans sect. Juglans, Juglans sect. Rhysocaryon,* and *Juglans* sect. *Trachycaryon.*

Juglans sect. Cardiocaryon. This variety originated from the northeast Asia. This walnut variety bears enormous leaves containing 15 leaflets. The wood is commercially soft, and nuts are thick shelled borne in a raceme inflorescence, such as, for example, *J. mandshurica* and *J. ailantifolia*.

Juglans sect. Juglans. Originated from the southeast Europe to central Asia. This variety bears large leaves with 9 broad hairless leaflets having entire margins. The wood is commercially hard, for example, *J. regia* (L.) and *J. sigillata.*

Juglans sect. Rhysocaryon. (black walnuts) Originated from the North America and the South America. This variety bears enormous leaves with 20

Table 2 - Different varieties of walnut and their commercial uses

No.	Species	Description	Commercial uses
1	English/Persian Walnut (*Juglans regia*)	Native to the region of California, Oregon, Himalayas, Europe and China. Most ubiquitous cultivated in foothills having high water table. Characterized by a relatively thin, gnarled shell enclosing a smooth, large, ivory-colored nut and rich flavor.	As an effective nutraceutical with a high nutritive value. Procurement of high quality timber in the furniture industry for high-end flooring, guitars, furniture, veneers, knobs and handles as well as gunstocks
2	Eastern Black Walnut (*Juglans nigra*)	Native to eastern North America. Characterized by a thick, hard shell with sharp, jagged edges and a darker color. Black nuts are known for their pungent aroma and robust flavor.	Used in timber industry as well as nut production. The hard-black walnut shell is also used commercially in abrasive cleaning, a filtering agent in scrubbers in smoke stacks, cleaning jet engines, cosmetics, and oil well drilling and water filtration
3	White/ Butternut (*Juglans cinerea*)	Native to the eastern United States and southeast Canada. Characterized by slow-growing species, and rarely lives longer than 75 years. Bark is light grey in colour and the whole leaf is downy-pubescent, and a somewhat brighter, yellower green than many other tree leaves. Leaves are alternate and compound and have odd number of leaflets with a terminal leaflet	Used in lumber industry to make furniture, and is a favorite of woodcarvers. It is used to dye fabrics. Used as a medicine owing to its cathartic properties
4	Japanese Walnut/ Heartnut *(J. ailantifolia)*	Native to Japan and Sakhalin. Characterized by light grey bark, pinnate and brighter yellower green leaves, nuts produced in bunches of 4-10 and are of spherical shape	The edible nuts have an oily texture. The husks are also used to make a yellowish dye
5	Manchurian/ Chinese Walnut *(J.mandshurica)*	Native to the eastern Asiatic region (China, Russia and Korea). Characterized by odd, alternate, pinnate, long and broad leaves. Shell is thick and the kernels are edible but small and difficult to extract. Also contain less quantities of allelopathic compounds like Juglone	The timber is in use but is less valuable as compared to English or Black walnut. Cultivated as an ornamental in colder temperate regions
6	Iron Walnut (*Juglans sigillata)*	Native to the Yunnan, Guizhou, Sichuan and Xizang in China. Characterized by oval-shaped nuts with bumps and ridges. Cultivated for its edible nuts	Used as ornamental plant in gardens and parks
7	California Walnut (*Juglans californica)*	Endemic to California and generally found in southern California Coast Ranges, Transverse Ranges, and Peninsular Ranges, and the Central Valley. Characterized by a large shrub or a small tree. It has a small hard nut in a shallowly grooved, thick shell that is difficult to remove. It has a better flavour than *Juglans nigra*	Raw as well as cooked seeds are used in pies, cakes, biscuits, confections etc. An attractive wood, but the frequent branching pattern of the trunk limits the use of this wood commercially. Also has a medicinal value
8	Brazilian/ Argentine walnut (*J. australis*)	Native to Argentina and Bolivia. Characterized by dense, hard and strong wood. Its more frost resistant than *J. regia*	Nutritive value. The immature fruits are pickled whole for human consumption. The mature nuts are also eaten. The concentrated extract of the husk is also used as a vermifuge
9	Northern California walnut (*Juglans hindsii)*	Endemic to Northern California. Characterized by smooth, brown, thick shell, that contains a small edible nutmeat	Commercially important as a rootstock for orchard stock of *Juglans regia*. Also used as an ornamental tree in wildlife gardens, and for habitat gardens, natural landscaping projects. Its wood commonly called claro walnut is used in the lumber industry
10	West Indian walnut (West Indian walnut)	Not native to Jamaica but found in Cuba, the Dominican Republic, Haiti, and Puerto Rico. Characterized by a drupe 2-3 cms long with a black husk and a seed, which is an edible walnut meat, inside. It is listed as an endangered species in the Endangered Species Act of the United States	Used in timber industry. The attractive wood is similar to that of the black walnut
11	Cedro negro *(Juglans olanchana)*	Native to Costa Rica, Guatemala, El Salvador, Honduras, Mexico and Nicaragua. Commonly called cedro negro. Characterized by long branches bearing twigs tipped with 40-50 cm long, glabrous, pinnately compound leaves, darker on the top than on the bottom. The base of the trunk sometimes has buttresses	Used in the timber industry for light construction, cabinet-making, parquet floors, luxurious furniture, turnery, musical instruments, and veneer. The husk is used to dye leather
12	Colombian walnut (*Juglans neotropica)*	Native to Colombia, Ecuador, and Peru. Characterized by slow-growing tree up to 40 mts height with grooved, red-brown bark and an oval-shaped canopy. Leaves are compound with a serrated border	Nuts are used as food. Used in timber industry because the hard, durable wood is highly prized in cabinetry, flooring, veneers, utensils, and other forms of decoration

leaflets, serrate margins. The wood is commercially very hard, such as, *J. californica, J. hindsii, J. australis, J. nigra,* and *J. olanchana.*

Juglans sect. *Trachycaryon.* Originated from the Eastern North America. This variety bears wide leaves containing 20 leaflets having serrate margins. The wood is commercially soft and the fruits are borne in clusters of two to three. The nuts have a thick and rough shell such as *J. cinerea* (L.).

3. World walnut production

Walnut grows abundantly in temperate areas of the world and cultivated commercially in 48 countries on an area of 1.6 million acres. The worldwide production of walnuts has reached maximum particularly in countries of Asia. Global walnut production for the year 2015-16 rose 155,000 tons from the previous year to 2.1 million tons, with China and the United States accounting for over 75 percent of total production (USDA, 2016). China is the world's chief producer of walnut and accounts for 51.49% of world's walnut production followed by the USA (26.86%), European Union (6.02%), Ukraine, Chile, Turkey and India contribute 5.25%, 3.09%, 3.09%, 1.96%, respectively to the world's walnut production (USDA, 2016). Global walnut production for the year 2016-17 is nearly unchanged from the October 2016 record forecast of 2.1 million tons in-shell basis, with China and the United States accounting for nearly 80 percent of total production (USDA, 2016). Figure 1 gives the schematic representation of year wise production of walnuts across the world.

4. Status of walnut in India

Walnuts are commonly called "Akhrot" in India and they are grown in the northwestern Himalayan belt, expanding up to Sikkim and Darjeeling. But the commercial farming of walnut is limited to the states of Arunachal Pradesh, Himachal Pradesh, Uttarakhand and Jammu and Kashmir. The area and production of walnut in India for the year 2014-15 were recorded to be 125,000 ha and 206,000 tons respectively (Government of India, 2015). India has exported 3,291.71 MT of walnuts worth of Rupees. 117.92 crores during the year 2015-16 (APEDA, 2016). Figure 2 gives the schematic representation year wise area versus production of walnut in India.

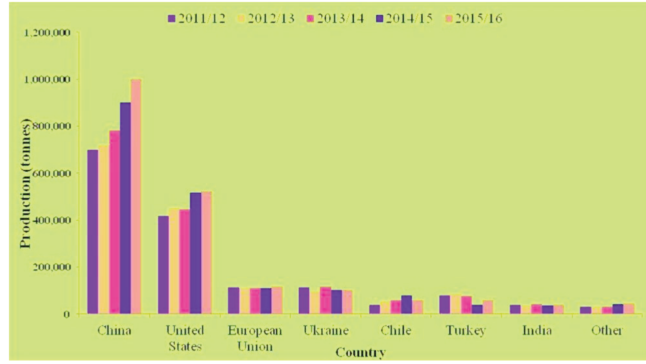

Fig. 1 - Schematic representation of walnut year wise production across the world.

Fig. 2 - Shows the schematic representation of walnut production during three consecutive years in India.

The major walnut importing countries from India are Vietnam Social Republic (15%) followed by Egypt Arab Republic (11%), Netherland (11%), United Kingdom (9%), Spain (8%), United States (7%), Germany (6%), France (4%), Thailand (3%), Australia (3%) and others (23%) (Ministry of Agriculture, 2015) (Fig. 3).

Jammu and Kashmir is the main center for commercial walnut production in India and contributes pretty nearly 98% of the country's output and the

Fig. 3 - Showing principal countries which imports walnuts from India.

state has been declared as the "Agri. Export zone for Walnuts" (Isher *et al.*, 2016). Most walnuts that are produced for export by India are principally produced in this state. According to the United Nations Development Programme Special Unit for South-South Cooperation (UNDP SU/SSC), 63,000 hectares of Jammu and Kashmir state is under walnut cultivation. These plantings produce around 60,000 tones of walnuts worth an estimated 25 million Rupees.

5. Walnut as phytochemical

Walnut is popularly known for its abundant phytochemicals for instance phenolics, fatty acids, melatonin and serotonin (Christopoulos and Tsantili, 2015). In addition to kernels, green shells, walnut husk, seeds, bark and leaves are utilized in the cosmetic and pharmaceutical sector (Negi *et al.*, 2011; Fernández-Agulló *et al.,* 2013; Vinson and Cai, 2012). Table 3 gives a description of walnut and its phytoconstituents in diverse walnut varieties. The level of phenolics in walnut depends on diverse ecological factors, genotype, type of cultivar, geographical site, climatic parameters and developmental stages (Solar *et al.*, 2006; Amaral *et al.*, 2008). Toivonen and Hodges (2011) illustrated tissue and age specific variation in phenolic acids. Walnut polyphenols are potential candidates which work against *in vitro* plasma and low-density lipoprotein LDL oxidation. Regueiro *et al.* (2014) reported several individual phenolic compounds in walnut kernels and characterized approximately thirty-seven compounds. Colaric *et al.* (2005) demonstrated a greater amount

Table 3 - Description of walnut and its phytoconstituents in diverse walnut varieties

No.	Walnut name	Part used	Phytochemical extracted	Reference
1	Persian walnut	Kernels	Phenolic acids (gallic acid, ellagic acid, syringic acid, chlorogenic acid, p-coumaric acid) and flavanols (catechin, epicatechin, gallocatechin, procyanidin B2, epigallocatechin, epicatechin gallate)	Figueroa *et al.,* 2016
2	Persian walnut	Kernels	Ellagic acid	Fukuda *et al.,* 2004; Christopoulos and Tsantili, 2012
3	Heartnut *Juglans ailanthifolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.)	Kernels	Ellagic acid	Li *et al.*, 2006
4	Persian Walnut	Leaves	Phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, p-coumaric, ferulic, sinapic, salycilic, ellagic and trans-cinnamic) flavonoids (catechin, epicatechin, rutin, myricetin and quercetin) and juglone	Nour *et al.*, 2012
5	Persian Walnut	Bark	Gallic acid, chlorogenic acid, catechine hydrate;resorcinol; caffeic acid, rutin trihydrate, syringic acid, vanillicacid,p-coumaric acid,quercitine dihydrate, naphto-resorcinol, trans-cinnamic acid etc	Noumi *et al.*, 2012
6	Persian Walnut	Fruit	Phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, p-coumaric, ferulic, sinapic, salicylic, ellagic and trans-cinnamic acid) flavonoids (catechin, epicatechin, rutin, myricetin and quercetin) and juglone.	Cosmulescu *et al.*, 2014
7	Persian Walnut	Walnut husks	Chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin, and juglone.	Stampar *et al.* 2006
8	Persian Walnut	Kernels and pellicle	Chlorogenic, caffeic, p-coumaric, ferulic, sinapic, ellagic, and syringic acid. Syringaldehyde and juglone	Colaric *et al.*, 2005
9	Persian Walnut	Leaves and husks	Chlorogenic acid, ferulic acid, gallic acid and trans-cinnamic acid	Chrzanowski *et al.*, 2011
10	Persian Walnut	Leaves	Juglone	Thakur, 2011; Babula *et al.*, 2006
11	Persian Walnut	Axillary shoots (microshoots)	Phenolic acids (gallaicacid, chlorogenic acid, p-coumaric acid, syringic acid, vanillin) and flavanoids (quercetin, naphthoquinone) and juglone	Cheniany *et al.*, 2013
12	Persian Walnut	Fruit	Gallic acid, chlorogenic acid, sinapic acid, protocatechuic acid, catechin and juglone	Jakopič *et al.*, 2009

of phenolics in walnut skin than in kernels. Oliveira *et al.* (2008) confirmed protective properties of walnut husk exhibiting higher anti-microbial activity. Walnut kernels are known to be an immense resource of ellagitannins having higher anti-oxidant property (Shimoda *et al.*, 2009). Amaral *et al.* (2008) confirmed that presence of 96% of tannins as major causal agent for the medicinal potential of walnuts along with phenolics and flavanoids. Phenolic compounds are known to confer health benefits**.**

Juglans regia L. leaves are good sources of flavonoids. Many types of research are mainly focused on the extraction/isolation and the antioxidant effect of flavonoids. Walnut flavonoids have not been extensively analyzed, however, English walnut leaf was subjected to vigorous chromatographic evaluation (Pereira *et al.*, 2007). Amaral *et al.* (2008) and Pereira *et al.* (2007) analysed quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and two other partially identified quercetin 3-pentoside and kaempferol 3-pentoside derivatives in walnut leaves along with myricetin as exclusive flavonoid in walnut husk (Lugasi *et al.*, 2003; Stampar *et al.*, 2006). Myricetin exhibit exceptionally high anti-oxidant property along with the potential of imparting beneficial effects on bone heath thereby preventing bone resorption and elevating osteoblastic potential and bone formation (Hsu *et al.*, 2007). Rutin (Quercetin-3-rutinoside) is another significant flavonoid in walnut leaves having elevated levels of anti-oxidant potential that prevent DNA oxidation (Nour *et al.*, 2012).

The previous studies on flavonoids present in *Juglans regia* (L.) leaves provided a conjectural source for an additional study on the organic decoction from *J. regia* leaves. The promising results from the medicinal point of view were procured from flavonoids. Estimation and characterization of phenolic compounds in walnut leaves can be utilized as an important resource. Carvalho *et al.* (2010) reported phenolics and associated anti-oxidant characteristics of methanolic and petroleum ether extracts procured from walnut green shells and leaves. Seeds methanolic extracts exhibited highest phenolic content approximately 116 mg gallic acid equivalent (GAE)/g of extract with EC50 of 0.143 mg/mL using DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity followed by leaf and green husk. Solvent polarity was directly correlated with the distinct disparity in the activity of samples as observed by the solubility of phenolics compounds in respective solvents. Hence, methanol solvent is preferred for phenolics extraction (Do *et al.*, 2014) while petroleum ether is non-polar normally employed for extraction of lesser polar constituents for instance plant pigments, sterols and fatty acids (Carvalho *et al.,* 2010). Carvalho *et al.* (2010) reported maximum phenolics fraction (116.22±3.76 mg of GAE/g of seed extract followed by leaf 94.39±5.63 mg of GAE/g of extract) and green husk (50.18±2.69 mg of GAE/g of extract). Previous research reports have conformed methanolic extracts as most efficient in radical scavenging activity. Al-Okbi *et al.* (2014) reported phenolics content of 47.42 g GAE/100 g in ethanolic extract of walnut seed. Jalili and Sadeghzade (2012) illustrated the average value of 22.16±0.66 (mg/g) of phenolic content in aqueous extracts in diverse walnut cultivars. Ghasemi *et al.* (2011) illustrated the phenolic content of 15.15 to 108.11 mg gallic acid equivalents g^{-1} of extract and flavonoid content ranging from 3.59 to 22.91 mg quercetin equivalents (QE)g^{-1} of extract. Rahimipanah *et al.* (2010) reported total flavonoids and phenolics in walnut green husks via aluminum nitrate and Folin-Ciocalteu colorimetric protocol and their content were 1.44±0.2 mg quercetin and 34.28±1.35mg gallic acid equivalent per gram of dry sample correspondingly. Kale *et al.* (2010) demonstrated flavonoid fraction of walnut bark extracts with maximum quercetin between 3.50 to 32.81 mg/g and phenolics content varied from 20.32 to 44.87 mg/g in the extract. Ethyl acetate extract of walnut showing maximum phenolics and flavonoid sustaining antioxidant potential walnut extracts and recommend their utilization for advanced research to identify secondary metabolites.

6. Phytopharmacology of walnut

All the parts of walnut i.e., bark, leaves, flowers are exclusively used widely in Ayurveda, Unani, homeopathy and allopathic medicine system (Shah *et al.*, 2014). Forino *et al.*, 2016 reported conventional uses of walnut in health maintenance. Diverse activities of walnut utilized are due to enormous phytoconstituents such as saponins, glycosides, alkaloids, and steroids (Muthaiyah *et al.*, 2011). All parts of walnuts particularly leave and husk is extensively used in the pharmaceutical and cosmetic industries (Ribeiro *et al.*, 2015). Table 4 gives the description of walnut as phytochemical and its activity against diverse pathogens.

Table 4 - Description of walnut as phytochemical and its activity against diverse pathogens

Walnut	Part used	Target cell/ pathogen	Activity	Reference
Juglans regia	Bark	*Staphylococcus aureus, E. coli*	Antibacterial	Farooqui *et al.*, 2015
Juglans regia	Bark	*Candida albicans, Candida glabrata*, and *Candida parapsilosis* strains	Antifungal	Noumi *et al.*, 2010
Juglans regia	Kernels	MCF-7 (estrogen receptor positive breast adenocarcinoma), KB (oral and mouth), HepG-2 (liver), Caco2 (colon), and WRL-68 (liver)	Anti-proliferative	Negi *et al.*, 2011
Juglans regia	Leaves	Tobacco mosaic virus	Antiviral	Zhai *et al.*, 2007
Juglans regia	Leaves	*Sindbis virus herpessimplex* (HSV), *Sindbis* (SINV), and *Poliovirus*	Antiviral	Mouhajir *et al.*, 2001
Juglans regia	Leaves	*769-P renal* and *Caco-2 colon* cancer cells	Anti-proliferative	Carvalho *et al*., 2010
Juglans regia	Leaves	A375 human melanoma cell	Anti-proliferative	Shah *et al.*, 2015
Juglans regia	Kernels	COLO-205	Anti-proliferative	Anjum *et al.*, 2016
Juglans regia	Walnut oil	*Herpes simplex* (HSV), *Parainfluenza* (PI-3) *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Enterococcus faecalis,* Fungi*: Candida albicans and Candida parapsilosis*	Antiviral, Antibacterial, Antifungal	Orhan *et al*., 2011
Juglans regia	Leaves	*Bacillus cereus*, *Bacillus subtili*s, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, Fungi: *Candida albicans, Cryptococcus neoformans*	Antibacterial and antifungal	Pereira *et al.*, 2007
Juglans regia	Husks	*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, Fungi: *Candida albicans*, *Cryptococcus neoformans*	Antibacterial and antifungal	Oliveira *et al*., 2008

Antimicrobial activity

Antimicrobial potential of walnut especially bark (Farooqui *et al.*, 2015) leaves (Pereira *et al.*, 2007) and fruits (Pereira *et al.*, 2008) have been extensively utilized against various infections.

Studies have also demonstrated the antimicrobial activity of walnut products, particularly of bark, leaves, fruits and green husk of *Juglan regia* (L.) was subjected to hot and cold extraction and resulting solution exhibited high antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli*, *Klebsiella pneumoniae, Staphylococcus epidermidis, Micrococcus luteus, Salmonella typhimurium, Enterococcus faecalis, Bacillus thuringiensis, Protomonas extroquens,* and Proteus sp., as evaluated through agar streak and disc diffusion assays (Biyik, 2010; Deshpande *et al.*, 2011).

Antifungal activity

Aqueous extracts of leaves and bark display antifungal activity against an extensive spectrum of fungi using disc diffusion, agar dilution, agar streak dilution and reddish assays. Pereira *et al.* (2008) demonstrated the enormous anti-fungal potential of walnut extracts refluxed with petroleum ether (b.p. 40-60°C) against *Candida albicans* and *Cryptococcus neoformans.* Noumi *et al.* (2010) demonstrated antifungal properties of *Juglans regia* (L.) alongside oral *Candida* strains.

Antiviral activity

Zhai *et al.* (2007) confirmed inhibition of tobacco mosaic virus (TMV) by 95% ethanol and ethyl acetate leaves extract of *J. regia*. At a minimum concentration of 1.5 µg/ml of methanolic extract of *J. regia,* there was distinct inhibition Sindhis (Mouhajir *et al.*, 2001).

Antioxidant activity

Several research reports demonstrated antioxidant activity of ethyl acetate, butanol, ether and aqueous methanol extract of walnut kernels, husks and leaves as evaluated by DPPH radical scavenging assay and lipid oxidation inhibition by β-carotene linoleate (Oliveira *et al.*, 2008; Pereira *et al.*, 2008; Carvalho *et al.*, 2010; Rahimipanah *et al.*, 2010;

Qamar and Sultana, 2011).

Consumption of walnuts and walnut skin diet by C57BL/6 mice showed decline oxidative stress as observed by a change in the enzymatic and non-enzymatic system (Bulló *et al.*, 2010). Furthermore, research report demonstrated ameliorating effect of walnut consumption on LDL oxidizing potential owing to their elevated levels of omega-6 PUFAs.

Antidiabetic activity

In Mediterranean and Asiatic countries, air-dried leaves of *Juglans regia* have been employed to treat diabetic symptoms (Hosseini *et al.*, 2014), and the potential of walnut leaves to ameliorate hyperglycemia in humans has been evaluated by both *in vitro* and *in vivo*. Pitschmann *et al.* (2014) reported inhibition of protein-tyrosine phosphatase 1B and no effect on peroxisome proliferator-activated receptors-gamma (PPARγ) in myocytes fed with methanolic extracts from walnut leaves. This study confirmed walnut leaf extracts drastically decline levels of serum fasting HbA1c, blood glucose and insulin (Hosseini *et al.*, 2014). Hence the study concluded hypoglycemic potential of walnut leaves wielding lowering effect on sugar levels in the liver as well as kidney. The mechanisms of action underlying the antihyperglycaemic effect of the walnut leaf extracts were investigated by *in vitro* studies. Inhibition of significant enzymes involved in sugar metabolisms like α-amylase (Rahimzadeh *et al.*, 2014), PTP1B (Ahmad *et al.*, 2012) and associated processes like glycation and oxidation reactions have been reported with walnut leaves (Pitschmann *et al.*, 2014).

Antihelmintic activity

A wide range of anti-worm activities was also reported from bark extracts of walnut. Kale *et al.* (2011) reported antihelminthic activity against *Eiciniafeotida* using reference Albendazole. Methanolic, ethanolic and benzene extracts of *Juglans regia* (L.) exhibited inhibitory activity against annelids like *Pheretimaposthuma* using standard drug Piperazine citrate (Upadhyay *et al.*, 2010).

Anti-inflammatory activity

Anti-inflammatory potential of walnut is comparatively inadequate. Papoutsi *et al.* (2008) demonstrated anti-inflammatory activity because of ellagic acid.

Hepatoprotective activity

Ameliorative effect of walnut polyphenols from kernel pellicle against a decline in glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) was reported in mice model after a single oral administration (200 g/kg) (Shimoda *et al.*, 2008). These results confirmed higher values of a hepatoprotective effect than curcumin.

7. Walnut and cancer

Due to the increasing inclination towards plant based drugs, more vigorous initiatives have been taken to action to develop more advanced plant based drug formulations. Among the diverse phytochemicals procured from plants, polyphenols are known to be most active candidates against cancer (Kim *et al.*, 2016). Singh and Sukhla (2015) demonstrated polyphenols as potent cancer inhibiting phytoconstituents as revealed by inhibitory effects of walnut on cancer promotion and progression. Polyphenols inhibit cancer progression by hampering nuclear factorkappa B - (NF-kB) activation. NF-kB is a nuclear transcription factor exhibiting significant role in gene regulation implicated in inflammation and carcinogenesis. Upregulation of NF-kB is implicated in cell-cycle progression. Polyphenol suppresses NF-kB and activator protein-1 (AP-1), its inhibition is considered as a potential step in chemoprevention. Polyphenols further induce inhibition in mitogen activated protein kinases (MAPK), protein kinases (PK), of the growth-factor receptor (GFR) leading to cell cycle arrest, the onset of apoptosis and decline in angiogenesis (Bonfili *et al.*, 2008). Mertens-Talcott *et al.* (2005) illustrated on the synergistic, additive, or antagonistic interface of polyphenolic compounds to counteract cancer growth. Shah *et al.* (2015) reported numerous chemical constituents possessing anticancer activity due to omega-3 fatty acids (Gerber, 2012), vitamin E (mainly the c-tocopherol form) (Albanes *et al.*, 2014), phytosterols (Ramprasath and Awad, 2015), ellagic acid (Eskandari *et al.*, 2016), gallic acid (Ho *et al.*, 2013) and flavonoids namely quercetin (Firdous *et al.*, 2014), carotenoids (Virtamo *et al.*, 2014), and melatonin (Travis *et al.*, 2014). Hardman (2014) reported 80% inhibition of breast cancer in mice following walnut consumption. Due to the presence of omega-3 fatty acids along with phytosterols in walnut breast cancer was declined remarkably. Phytosterols bind with estrogen receptors and subsequently decline breast cancer. Consumption of walnut diet declined a breast tumour by about 60% in transgenic mouse. Hardman (2014) confirmed presence of numerous complex constituents in walnut exhibiting additive or synergistic effect on cancer suppression. Further presence of γ-tocopherol in walnut exhibit anti-cancer activities against prostate, colon, renal and lungs as demonstrated by anti-proliferative and anti-angiogenic mechanisms (Rafieian-Kopaie and Nasri, 2015). Walnut inhibits inflammation by inhibiting endothelin (Ma *et al.*, 2010). Research on prostate cancer established higher levels of endothelin in men indicating the need for further research to decipher relation between walnut and prostate cancer. Reiter *et al.* (2013) investigated prostate cancer and a determined decline in tumor and LNCaP (Lymph Node Carcinoma of the Prostate) xenograft growth. Negi *et al.* (2011) determined anti-proliferative activity of chloroform and ethyl acetate fractions of walnut in human cancer cell lines such as MCF-7 (Michigan Cancer Foundation-7) (estrogen receptor positive breast adenocarcinoma), KB (oral and mouth), HepG-2 (liver), Caco2 (colon), and WRL-68 (liver) cancer cell lines. All the cell lines exhibited inhibition against kidney and colon cancer however for 769-P renal and Caco-2 colon cancer cells, walnut leaf revealed the elevated rate of anti-proliferative competence than walnut seeds and husk. The observations procured confirmed lack of relationship among total phenols and anti-proliferative activities signifying some class of compounds implicated in cancer inhibition (Carvalho *et al.*, 2010). This study was further validated by Tsoukas *et al.* (2015) who demonstrated a converse association between walnut intake and colon cancer incidence. Paur *et al.* (2010) reported dose dependent inhibition in lipopolysaccharide(LPS)-induced NF-κB activity in the monocytic cell line (U937-κB) by blending clove, oregano, thyme, walnut and coffee. This study confirmed the comparative efficacy of the combination of different extracts than individual plant constituent. Hence, walnut can be utilized as a natural source of anti-oxidants as well as chemo preventive candidate. Thakur (2011) demonstrated inhibition of carcinogenesis in rats by azoxymethane and can be utilized as chemo preventive agent against neoplasia. During recent years, juglone is established as effective cytotoxic agent and induce cell apoptosis via mitochondria dependent pathway via human lung cancer (A549) cells (Cenas *et al.*, 2006), human leukemia (HL-60) cells (Xu *et al.*, 2012 a), and human cervical carcinoma (HeLa) cells (Zhang *et al.*, 2012). Ji *et al.* (2008) reported inhibition of growth and induction of apoptosis of sarcoma 180 cells *in vivo.* Juglone, being an inhibitor of Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) is an impending remedial target for anticancer research. Role of Pin1 in relation to tamoxifen-resistant breast cancer has been an important determinant factor in drug resistance. These observations demonstrate Pin1 augment E2F-4 and Egr-1-driven expression of LC-3, an autophagosome marker is over expressed in cancer cells than non-cancerous. Namgoong *et al.* (2010) further established juglone as potent inhibitor having a distinct declining effect on the TPA and induced overexpression of E2F-4 and Egr-1 transcription factors controlling LC-3 gene expression.

8. Walnut as a nutraceutical

The term “nutraceutical” is used to illustrate food or food nutrient supplement that suggests a medical or health benefit by providing simple nutrition (Wong *et al.*, 2015). Functional foods are known to be a remedy for chronic diseases (Luciano, 2014). Walnut has been used as functional food. They are important components of the Mediterranean diet (Ley *et al.*, 2014). Nutritional profile of walnut is tabulated in Table 5. Not only the kernel of walnut but also green shells and bark have also been used in the cosmetic and pharmaceutical industries (Ribeiro *et al.*, 2015; Adeel *et al.*, 2017). Walnuts are chiefly consumed for their organoleptic and nutritional aspects since they are resources of important anti-oxidants and preserve food attributes (Martinez and Maestri, 2016). Walnut seeds are now established as major nutraceutical owing to their effects on coronary heart disease (Nasri *et al.*, 2015; Schwingshackl *et al.*, 2017). Walnuts possess numerous nutritional aspects including a maximum fraction of polyphenolics due to which they are incorporated into recommended dietary supply (Sanchez-Gonzalez *et al.*, 2017). Christopoulos and Tsantili (2015) reported numerous phytochemicals in walnut for example phenolics, fatty acids, melatonin and serotonin. In addition to phytochemicals, various hydrolyzable and condensed tannins have been reported in walnut (Figueroa *et al.*, 2016). High oil and protein content of kernel of *Juglans regia* (L.) (Juglandaceae) validates this fruit requisite for human nutrition. FAO has categorized walnut as a deliberate candidate for human nutrition and phytomedicine (Gandev, 2007). Most of the energy in walnuts generates from fats which account for 65% which makes them energy-rich and high-calorie food (Tapsell *et al.*, 2009). Martinez and Maestri (2008) reported triacylglycerols, in which monounsaturated fatty acids (MUFAs) mainly oleic acid (18:1 n-9) and polyunsaturated FAs (PUFAs;

Table 5 - Nutritional profile of Walnut

Nutrients	Total amount /100 gm walnut
Calories	654
Water	4%
Protein	15.2 g
Carbs	13.7 g
Sugar	2.6 g
Sucrose	2.4 g
Glucose	0.1 g
Fructose	0.1 g
Fiber	6.7 g
Fats	65.2 g
Saturated	6.13 g
16:00	4404 mg
18:00	1659 mg
Monounsaturated fatty acids	8.933 g
18:01	8799 mg
20:01	134 mg
Polyunsaturated fatty acids	47.174 g
Omega-3	9.08 g
Omega-6	38.09 g
Trans fat	0
Vitamins	
Vitamin A	1 µg
Vitamin C	1.3 mg
Vitamin D	0 µg
Vitamin E	0.7 mg
Vitamin K	2.7 µg
Vitamin B1 (Thiamine)	0.34 mg
Vitamin B2 (Riboflavin)	0.15 mg
Vitamin B3 (Niacin)	1.13 mg
Vitamin B5 (Panthothenic acid)	0.57 mg
Vitamin B6 (Pyridoxine)	0.54 mg
Vitamin B12	0 µg
Folate	98 µg
Choline	39.2 mg
Minerals	
Calcium	98 mg
Iron	2.91 mg
Magnesium	158 mg
Phosphorus	346 mg
Potassium	441 mg
Sodium	2 mg
Zinc	3.09 mg
Copper	1.59 mg
Manganese	3.41 mg
Selenium	4.9 µg
Cholesterol	0 mg

linoleic (18:2 n-6) and α-linolenic acids (18:3 n-3) in walnut oil are present in high amounts in all genotypes. Walnuts are a chief resource for omega-6 fatty acid called linoleic acid. They also contain a relatively high percentage of omega-3 fat called alpha-linolenic acid (ALA) contributing up to 8-14% of the total fat content. Deckelbaum and Torrejon (2012) reported walnut as the sole source of ALA which declines inflammation and augments blood profile. Muradoglu *et al.* (2010) and Martinez *et al.* (2010) reported average protein estimation of about 18.1% comprising of 70% of total seed protein along with lesser amounts of globulins (18%), albumins (7%) and prolamins (5%). Walnut proteins possess all essential amino acids requisite for humans. Amino acids exist in proper ratio in walnuts e.g., lysine/arginine ratio in walnut proteins is comparatively lower than in daily vegetable proteins. The lower lysine/arginine ratio declines atherosclerosis development (Martinez *et al.*, 2010). In addition to macronutrients, walnuts are richest sources of vitamins and minerals, Potassium (K), phosphorus (P), magnesium (Mg) and iron (Fe) (Cosmulescu *et al.*, 2009). Calcium (Ca), sodium (Na), zinc (Zn) and Copper (Cu) are present in moderate fractions (Siahnouri *et al.*, 2013). Macro elements are necessary for proper development and growth of organisms (Jeszka-Skowron *et al.*, 2016). Most of macroelements assist in enzymatic reaction involved in the general metabolism of the organism. Trace elements promote heart health, maintains bone, nerve and immune system functions (Abdel-Aziz *et al.*, 2016). Vitamins are indispensable organic substances and in addition to their role in important metabolic functions, they are also required for proper functioning of hormone regulation. Anjum *et al.* (2016) confirmed presence of ellagic acid, gallic acid, tocopherol (vitamin E), ellagitannins (tannins) and phytochemicals with potential antioxidant activity, for instance, melatonin. Walnut also comprises of elevated levels of α- tocopherol, a vitamin E, which prevents lipid oxidation process and decline risk of cancer and coronary heart disease (Jiang, 2014).

Health effects of walnut (Table 6)

Cardiovascular benefits. Nijike *et al.* (2015) reported daily consumption of walnut lowers risk of heart diseases. Higher anti-oxidant and elevated levels of the fat fraction in walnut are important for heart health.

Brain health. Brain function is immensely advanced by eating nuts. Walnuts are also associated with alleviation of depression and age-linked diseases (Grosso and Estruch, 2016). Arab and Ang (2015) reported daily consumption of walnuts enhance memory retention.

Walnuts help reduce problems in metabolic syndrome. Metabolic syndrome is complex disorder encompassing increased triglycerides, high blood pressure, insufficient high-density lipoproteins, cholesterol, and obesity. Saneei *et al.* (2013) reported daily consumption of one ounce of walnuts for 2-3 months can lower risk of MetS- associated disorders.

Benefits in treatment of type 2 diabetes. Type 2 diabetes is a growing problem in developed and industrialized countries. A better understanding of dietary changes is needed to improve this disorder. The significant diet regime for type 2 diabetes patients for lowering cardiovascular issues has been reported by consumption of walnuts on daily basis (Farr *et al.*, 2017).

Anti-cancerous effects. Walnuts contain a wide variety of antioxidant and anti-inflammatory bioactive components that may have anti-cancerous properties (Byerley *et al.*, 2017). Presence of polyphenolic compounds, phytosterols, gamma-tocopherol, omega-3 fatty acids and ellagic acid decline threat of chronic oxidative stress and ameliorate inflammatory properties to counteract intimidation of cancer advancement (Perugu and Vemula, 2014). Choi *et al.* (2016) and Al-Mahmood *et al.* (2016) reported a lower incidence of colon, breast and prostate cancer associated with walnut consumption.

Other health benefits. Walnut nutrients exhibit significant function in the maintenance of bone structure (Kajarabille *et al.*, 2013). It has been recently confirmed that walnut consumption decline blood levels of N-terminal telopeptideNTx of type 1 collagen. More collagen composition signifies higher bone stability as well as lower mineral loss from bones (Griel *et al.*, 2007). Katz *et al.* (2012) confirmed the potential significance of walnut consumption in body weight management.

9. Application of molecular markers in horticultural crops

Horticultural crops are important for dietary purpose and source of revenue for farmers in the emerging countries (Behera and France, 2016). Genetic

Table 6 - Health effects of walnut

Cardiovascular aspect	Walnut benefit
Blood quality	Lowering LDL, the “bad” cholesterol; decreased total cholesterol; increased gamma-tocopherol; increased omega-3 fatty acids in red blood cells (alpha-linolenic acid)
Vasomotor tone	Decreased aortic endothelin; improved endothelial cell function
Risk of excessive clotting	Decreased maximum platelet aggregation rate; decreased platelet activation
Risk of excessive inflammation	Decreased C reactive protein (CRP); decreased tumor necrosis factor alpha (TNF-a)
Blood vessels	Improves the function of blood vessels by cutting the risk of plaque buildup in the arteries
Brain tonic	Relieves depression and age-related decline in brain function
Decline in metabolic syndrome	Reduction of various MetS-related problems
Treatment of type 2 diabetes	Regulates blood sugar and insulin metabolism
Development of body structure	Decrease blood levels of N-telopeptides of type 1 collagen (NTx); weight maintenance and prevention of obesity
Anti-cancer activities	Anti-inflammatory properties help lower risk of chronic inflammation

improvement in horticultural crops is not yet as noteworthy as it has been achieved in case of cereals and grasses. The advancement and accessibility of genomic resources in these crops can be utilized resourcefully to conserve and investigate existing genetic diversity and to understand the association between genotype-phenotype and to accelerate breeding (Chaturvedi and Sahijram, 2015). Current progress in mechanization and high-throughput sequencing can be utilized to decipher ambiguous and intricate genomes. Gene pyramiding and polygenic resistance evaluation in diverse genotypes with extensive resistance have been made possible through the advent of molecular markers (Ansari, 2015). Walnut is valued for its wood and nut, phytochemicals but so far as its transcriptomics and genomic data is concerned, it is very limited (Hu *et al.*, 2016).

From the past few decades, a significant progress has been achieved by the use of molecular markers in plant biotechnology and unraveling their genomic intricacies for detecting and exploiting DNA polymorphism in the plants. There are different markers, i.e. markers based on the morphology of plants, on biochemical functions and DNA-based molecular markers (Parveen *et al.*, 2016). These molecular markers are classified as hybridization-based or non-PCR-based markers. By using, these DNA based molecular markers, one can get complete information about a particular plant from the level of the nucleotide to a segment on DNA and frequencies of alleles, population structure and distribution of genetic diversity. Molecular markers explicitly reveal the polymorphism at DNA level (Deoxyribonucleic acid). They are routinely used in various crop improvement programs, ecological, physiological, genetic studies of plants and to progress the effectiveness and accuracy of traditional plant breeding through marker assisted selection (Allwright and Taylor, 2016). Important information for genetic diversity can be evaluated for different horticultural crops by using RAPD markers, for example, eggplant and watermelon (Trivedi *et al.*, 2016), *Moringa oleifera* (L.) (Kumar *et al.*, 2017). AFLP markers have effectively been utilized for evaluating genetic diversity in horticultural crops like *Celosia argentea* (Olawuyi *et al.*, 2016), *Rosa platyacantha* (Yang *et al.*, 2016) and olive (Mnasri *et al.*, 2017). Owing to their hyper variability and competence in distinguishing of polymorphisms, SSR have become ideal markers for assessment of genetic diversity in horticultural crops like potato (Ghebreslassie *et al.*, 2016), grapes (Rao, 2017), high density genetic map construction (Xue *et al.*, 2008), population and conservation genetic studies, clonal identification (Liu *et al.*, 2016 a, b), controlled crosses certification, species and hybrid identification, paternity determination (De La Rosa *et al.*, 2013), marker assisted selection (Ashraf and Foolad, 2013) and genotyping (Billot *et al.*, 2013). ISSR is extensively utilized in population genetics of horticultural crops i.e., *Elaeis guineensis* (Chagas *et al.*, 2015) and *Rhododendron triflorum* (Xu *et al.*, 2017) and determining the genetic diversity of horticultural crops such as bael (*Aegle marmelos* Corr.) (Mujeeb *et al.*, 2017). This section of the chapter provides a detailed account of the exploitation of different molecular markers used in case of walnut study. Use of some like random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), single Nucleotide Polymorphism (SNP) are briefly discussed in improving, increasing the quality and quantity of walnut is summarized.

Single Nucleotide Polymorphism (SNPs) have been widely used in plant germplasm management and breeding of fruit tree crops (van Nocker and Gardiner, 2014). These benefits have resulted in SNPs gradually becoming the markers of choice for exact genotype identification and diversity analysis in horticultural crops such as cacao (*Theobroma cacao*) (Fang *et al.*, 2014 a), grapevine (*Vitis vinifera*) (Cabezas *et al.*, 2011), pummelo (*Citrus maxima*) (Wu *et al.*, 2014), strawberry (*Fragaria* spp.) (Longhi *et al.*, 2014) and tea (*Camellia sinensis*) (Fang *et al.*, 2014 b).

10. Molecular characterization in walnut

Molecular characterization of walnut germplasm is mandatory requirement for establishing breeding purposes and establishment of proprietary rights. Conventional approaches for germplasm characterization are evaluated based on comparative morphological analysis. Due to the ecological effects on phenotypic expression together with juvenile phase of walnut, there are ample hindrances in proper classification of walnut. To surmount these restrictions, molecular markers are employed for discrimination and identification of walnut accessions. These DNA based markers are not influenced by environment and can be identified in all development stages of plant tissues. Characterization of genetic variability in walnut was done using isozymes (Malvolti *et al.*, 1994), to identify interspecific hybrids (Arulsekar *et* *al.*, 1985), in species and cultivars differentiation (Vyas *et al.*, 2003) and to evaluate mating aspects (Rink *et al.*, 1994). RFLP markers has been employed for evaluating parentage and ascertaining phylogenetic associations and discriminate among species and cultivars in the genus *Juglans* (Aly *et al.*, 1992; Fjellstrom *et al.*, 1994; Fjellstrom and Parfitt, 1995). Description of various molecular markers used in molecular characterization of various walnut genotypes is given in Table 7.

Randomly amplified polymorphic DNA (RAPD)

RAPD is immensely significant in deciphering closely associated cultivars in walnut and results obtained are harmonized with preexisting data procured from RFLPs owing to more vigorous polymorphism exhibited by RAPD (Nicese *et al.*, 1998). Furthermore, RAPD markers were also employed to assess polymorphism intensity at interspecific level among Persian walnut (*J. regia*) and in the Northern California black walnut [*J. hindsii* (Jeps.)] (Woeste *et al.*, 1996 a) and to recognize a marker associated with hypersensitivity to the cherry leaf-roll virus (Woeste *et al.*, 1996 b). Nicese *et al.* (1998) evaluated eighteen RAPD primers among nineteen walnut genotypes that comprised of strongly associated cultivars and parents in breeding programs. Following cluster analysis of closely associated genotypes, genotypes can be segregated into two classes having association with their closely related alleles. RAPD markers can investigate polymorphism to decipher variation among walnut genotypes particularly strongly associated genotypes and genetic similarity subsisting on RAPDs revealing can detect enough polymorphism to differentiate among walnut genotypes, even among closely identified (Hayward *et al.*, 2015). RAPD markers are highly useful for evaluating genetic variability in genus *Juglans* through DNA fingerprinting to differentiate between valuable genotypes for selection (Pop *et al.*, 2010; Ahmed *et al.*, 2012). RAPD was employed to evaluate association among eight walnut genotypes thriving in Turkey and discrimination among indigenous and exotic genotypes together with their early bearing progenies (Erturk and Dalkilic, 2011). Xu *et al.* (2012 b) illustrat-

Table 7 - Description of various molecular markers used in molecular characterisation of various walnut genotypes

Walnut species/variety	Molecular marker used	Traits associated	References
Juglans regia	RFLP	Inheritance and linkage	Fjellstrom *et al.*, 1994
Juglans regia	RAPD and SSR	Phenotypic	Pop *et al.*, 2013
Juglans regia	SSR	Morphological	Mahmoodi *et al.*, 2013
Juglans regia	SSR	Morphological	Karimi *et al.*, 2014
Butternut (*Juglans cinerea* L.), Japanese Walnut (*Juglans ailantifolia*) and Buartnut *(Juglans cinerea* × *Juglans ailantifolia*)	SSR	Nut phenotype	Chen *et al.*, 2014
Juglans regia	RAPD	Butternut canker disease resistance	Zhao *et al.*, 2014
Juglans nigra, *Juglans regia*, and hybrid (*Juglans x intermedia* (Carr)	RAPD	Association between native/foreign genotypes with their early-bearing natural hybrids	Erturk and Dalkilic, 2011
Juglans regia	SSR	Identification of interspecific hybrid	Pollegioni *et al.*, 2014
Juglans regia	SSR	Morphological	Ebrahimi *et al.*, 2011
Juglans regia	ISSR	Morphological and biochemical traits	Malvolti *et al.*, 2010
Juglans regia	ISSR	Distinction between native and international cultivated genotypes	Christopoulos *et al.*, 2010
Juglans regia	SLAF	Disease resistance to anthracnose	Zhu *et al.*, 2015.
Juglans regia	AFLP	Geographical proximity	Bayazit *et al.*, 2007
Juglans regia	SNP	Fingerprinting of 30 walnut genotypes	Ciarmiello *et al.*, 2013
Juglans regia	SNP	Genome sequencing	Liao *et al.*, 2014

ed RAPD and AFLP for deciphering genetic diversity of walnuts in western Sichuan plateau and Qinba mountainous regions. The walnut genotypes were procured from 8 different regions and 32 RAPD primers and 28 AFLP primers in combination were recognized with polymorphic bands in entire walnut. In this study, high allelic number and elevated genetic diversity identified with RAPD and AFLP signify western China has significant genetic diversity resource and profuse genetic variance among walnuts. Zhao *et al.* (2014) employed RAPD as chief molecular implement for characterizing genome of butternut (*Juglans cinerea*), Japanese walnut (*Juglans ailantifolia*), black walnut (*Juglans nigra*), Persian walnut (*Juglans regia*), Manchurian walnut (*Juglans mandshurica*), and an interspecific hybrid (*J. ailantifolia cinerea*) for species-specific markers.

The study revealed 38 amplicons were exclusive to Japanese walnut and buartnut hybrids lacking in butternut. RAPD markers were relatively insufficient to differentiate intraspecific variability inside Japanese walnut, butternut, or their hybrids. RAPD can also decipher hybrid lineage as in the case for discriminating whether Japanese walnut or Manchurian walnut was the progenitor of hybrid line or not. The results demonstrated that some of the hybrids can be distinguished through morphological and genetic resemblances through RAPD as it demonstrates pedigree data. The resulting RAPD markers provide their utility in the conservation of butternut, an endangered North American species.

Simple sequence repeat (SSR)

SSR is enumerated among most precise molecular marker owing to their high polymorphism, co-dominant transmission, higher reproducibility, exhibiting higher resolution and more convenient PCR detection in deciphering genetic relationship (Miah *et al.*. 2013). Wang *et al.* (2015), Ali *et al.* (2016), Ebrahimi *et al.* (2016), and Pang *et al.* (2017) illustrated research reports where in genetic characterization of walnut was evaluated using SSR. Han *et al.* (2016) described genetic diversity and population structure study of *J. regia* germplasm using ten primers developed from expressed sequence tags (EST-SSR) and sequence polymorphisms among the phenylalanine ammonia lyase (PAL) gene. The result showed high level of population differentiation. In addition, SSR markers informative in *Juglans regia* may also be polymorphic in other *Juglans* (L.) species (Aldrich *et al.*, 2003) and have utility for breeding hybrid rootstocks. SSR are more consistent and trustworthy system for molecular characterization. SSR has wide numerous relevance in case of walnut comprising of cultivar characterization, cultivar identification, pedigree substantiation for cultivar and in deciphering evaluation of superior rootstock for breeding and paternity analysis (Dangl *et al.*, 2005). Ruiz-Garcia *et al.* (2011) employed 32 SSR primer pairs to characterize 57 walnut cultivars originated from Spain and USA. In this research reports, 32 primer pairs flanking simple sequence repeats, were established in *Juglans nigra* to screen elite variety with high rate of polymorphism. Further selection of 19 selected microsatellite markers provided differentiation of evaluated cultivars exhibiting 97 alleles and on an average 5 alleles per locus establishing SSR as primary choice for characterization of walnut germplasm. The data generated from SSR characterization further confirmed molecular parameters of Spanish walnut to be different from Californian genotypes. Ahmed *et al.* (2012) illustrated genetic association of 82 walnut genotypes using 13 SSR and 20 RAPD primers exhibit higher level of genetic diversity in these walnut cultivars thriving rigorously in climate of Jammu and Kashmir. These results demonstrate applications of SSR markers in walnut breeding and conservation. Pollegioni *et al.* (2014) illustrated genetic diversity and spatial genetic structure of 39 autochthonous Persian walnut populations analyzed transversely along Asian range via 14 neutral microsatellite markers. Shah *et al.* (2016) reported genetic characterization of 96 walnut genotypes growing in North western Himalaya as investigated by 19 SSR markers and the study displayed high polymorphism rate of 89.6%. Chen *et al.* (2014) demonstrated potential of SSR markers in the cultivar characterization together with intellectual property rights. Topcu *et al.* (2015) developed genomic libraries augmented with CA, GA, AAC, and AAG repeats via genomic DNA from *J. regia* cv. Maraş-18 to develop SSR markers for walnut. This study confirmed GA-enriched library as preferred alternative in terms of allelic number, polymorphism, productivity, and information content.

Inter simple sequence repeat (ISSR) markers

ISSR-PCR is a most convenient approach that surmounts most of these restrictions encountered by other molecular markers (Karimi *et al.*, 2014). ISSR is most popular among plant improvement. Potter *et al.* (2002) has demonstrated the application of inter-simple sequence repeat (ISSR) markers in genetic characterization of English or Persian walnut (*Juglans regia* L.). In this study eight ISSR primers presented exclusive fingerprint for 48 cultivars investigated. The dendrogram developed from data presented classes pertaining to known pedigrees which does not provide this data anticipating the fact that there is restriction in context to utility of ISSR in determining genetic association among species. Christopoulos *et al.* (2010) reported immense application of ISSR markers in genetic characterization for deciphering genetic diversity among Greek natives of walnut (*Juglans regia* L.). In this study, similarity coefficient values signified presence of a higher extent of genetic variability. Majority of international cultivars were classified together whilst majority of Greek native populations cannot be classified in separate class. International cultivars exhibit lesser diversity than Greek native population genotypes. The pairwise regional PhiPT values signified that most geographically isolated regions are the most genetically differentiated. The intense variability in Greek germplasm along with their desired traits anticipated that native germplasm can be utilized for breeding purposes and preservation of walnut germplasm. Li *et al.* (2011) employed ISSR markers to evaluate the genetic variation and genetic structure to provide a theoretical basis and technical support for appropriate conservation and application of existing genetic resources of walnut. Analysis of ANOVA (Analysis of variance) also showed genetic variance among populations was larger than within a population. Mantel test and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram based Nei's genetic distance displayed that genetic distance between populations had more significant correlation with geographic distance. Ji *et al.* (2014) developed ISSR primers to assess the degree and design of genetic diversity among eight populations of North China Mountain Walnut (NCMW). This diversity was further confirmed through ANOVA analysis. Structure of NCMW and UPGMA cluster analysis demonstrated restricted gene flow, habitat devastation and geographical isolation might be determining factors for population structure. UPGMA cluster further signify that eight populations of walnut can be classified into three discreet groups as per similarity coefficient and geographic origin but exhibited significant association with morphological traits especially nuts. Hu *et al.* (2016) in their classical experiments use transcriptomic information from RNA-Seq to understand development of polymorphic simple sequence repeats (SSRs, microsatellites) for understanding the population genetics of walnut. They studied more than 47.7 million clean reads, 99,869 unigenes having length of 747 bp. They further identified 39,708 (42.32%) genes, 63 new transcriptome-derived microsatellite markers. The identification and characterization of microsatellite markers in their study could help to explore the diversity, genetic structure, population genetics for improved walnut future breeding practices, besides providing a useful genetic resource information for studying and understanding the genomic and transcriptome aspect of walnut. Doğan *et al.* (2014) evaluated genetic association of 59 walnuts (*Juglans regia* L.) genotypes, international and Turkish by using three different types of molecular markers i.e. RAPD, ISSR and SSR primers. These results exhibited that SSR markers offer elevated rank of polymorphism as compared to RAPD and ISSR.

Single nucleotide polymorphism (SNP) markers

SNP is the most abundant type of DNA variation in most species. The introduction of next generation sequencing (NGS), together with high throughput genotyping technology, makes it relatively easy to identify and use SNPs (Elshire *et al.*, 2011). The new generation of molecular markers based on single nucleotide polymorphisms (SNP) represent a promising and effective tool for fast and accurate species identification. Ciarmiello *et al.* (2013) developed a simple amplification refractory mutation technique, based on SNP markers of rDNA and cox2 intron I sequences, to fingerprint 30 walnut genotypes. rDNA sequences revealed the presence of 402 variations and Cox2 intron I sequences showed 769 variable positions. The findings revealed that the cox2 intron I region, either alone or in conjunction with rDNA, could be used effectively in identifying these walnut genotypes. Liao *et al.* (2014) performed genome sequencing of walnut and then all the sequence reads were mapped against genome assembly of walnut. In total, 49,202 nucleotide variations were detected including 48,165 single nucleotide polymorphisms (SNP5) and 1037 insertions/deletions (InDels).

11. Conclusions

The nutritional factors of walnut are improved due to the presence of numerous micro elements. The notable nutritive feature of walnuts relates to a rich range of polyphenols. The breeding character of walnut has bestowed it an inclusive diversity in genetic features. These results have been attained after an extended progression of evolution and by going through intricate environmental abnormalities. However, these techniques are susceptible to the environmental variations. The markers are adequate in number and there is little or no diversity in the method employed for research on walnuts. Despite basic research method, the organization of information, its retrieval and presentation structures, form elaboration experienced immense advancement via molecular markers such as RFLP, ISSR, RAPD AFLP, SSR and SNP. This assessment offers data regarding health benefits of walnut at the global level and existing applications in the horticulture. The present work comprehensively describes the utilization of molecular markers in walnut plants which could help to improve/enhance the traits linked to its optimum/yield growth and sustainable production of innumerable essential metabolites having the ability to find applications in horticulture and allied science (Fig. 4).

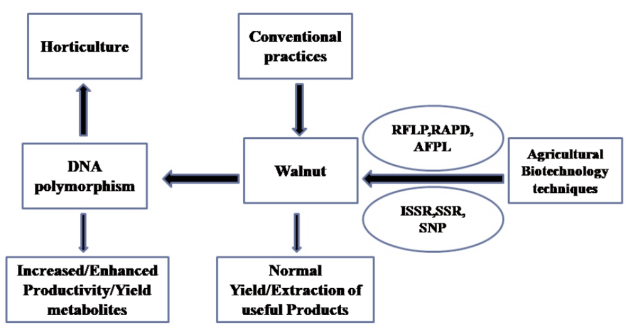

Fig. 4 - Schematic representation of utilization of molecular tools to improve walnut traits.

Acknowledgements

The authors express their gratitude to Central Institute of Temperate Horticulture (Indian Council of Agricultural Research, (ICAR), Aligarh Muslim University and University Grants Commission New Delhi, India for financial support of this work.

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