



## Review

## Walnut Genotypes for High Density Orchards

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**Abstract:** The aim of this review is to check the possibilities and circumstances regarding how to create a high-density Persian walnut orchard. Increasing yields, decreasing tree size, limiting juveniles, and lowering total costs are the most important objectives of breeders and horticulturists. Reducing the size of walnut trees can increase yield. Breeding programs in several countries have led to the production of walnut dwarf rootstocks. For example, Daixiang and Daihui in China, Alvand in Iran, and Fernette in France are all novel-bred dwarfing Persian walnut rootstocks. These precocious walnuts are considered to be a rare resource in the study of precociousness as well as juvenile and flowering mechanisms. Moreover, they play a potential role in breeding and modifying cultivars by genetic engineering, through walnut ameliorating programs. The CRISPR (clustered regularly interspaced short palindromic repeat) technique is used to improve walnuts, which will be used in the near future.

**Keywords:** walnut; yield; early maturation; dwarf cultivars; high density; breeding; juvenility; genetic engineering



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## 1. Introduction

The walnut tree is an important nut tree that produces kernel, cooking oil, and timber. Walnut trees are an important source of income for farmers [1]. Rootstock selection is an effective factor in determining the yield, by enhancing water and nutrient uptake, improving scion growth, reducing biological and abiotic stresses, and granting adaptation to environmental conditions [2]. Tree-height reduction should be evaluated in *Juglans* spp. germplasm, for facilitating cultural applications in high-density plantings [3]. For decades, the high-vigor walnut tree (*Juglans regia* L.), suitable for both timber and nut production, has been used as a seed source in many countries [4]. One of the main production problems is tree size (about 27 m high), which makes pruning, spraying, and/or harvesting unpleasant [5]. Genetically, low-vigor and early-mature-walnut genotypes are, often, found in seedling offspring in Iran [6] or Central Asia [7] and may provide suitable genetic resources for varying degrees of tree-size control in the future [5]. Rootstocks for some fruit trees, such as pear, apple, apricot, peach, cherry, and blueberry, have been reported to have size-control effects on the grafted scion [8–11]. Reducing tree size through the use of dwarf rootstocks is an important component of high-density and mechanized orchards [12]. Dwarf trees may, also, offer benefits in agroforestry settings, by decreasing shading of cover crops [13]. Dwarf walnut trees can reduce labor costs and enhance yields per hectare, by increasing the density of plants [14]. Dwarfing is not the most important purpose of walnut-rootstock-breeding programs, but identifying the source of this characteristic is of interest in countries with high diversity, such as those in Central Asia [7]. In Iran, China, Turkey, and Central Asian countries, mechanical harvesting from traditional orchards of giant walnut trees is difficult [6,7,15]. Due to harvest injuries and worker deaths during manual harvesting, there is a growing interest in dwarfing rootstocks [16]. Independent genetic traits, such

as length between nodes, branch angle, branch position (i.e., basitonic, mesotonic, and acrotonic), and growth rate determine the size of the tree [17]. These features are easy to find among forest, fruit and landscape tree species, and finding them is a matter of effort and time [10]. Moreover, walnuts (*Juglans regia* L.) are not considered early-maturing trees [18]. The grafted trees show vigorous growth, but flowering, rarely, begins within 1–3 years after planting. Early blooming is a priority choice in breeding programs, for producing precocious cultivars [19]. Traditional walnut cultivars bloom within 6 years of planting and enter full fruiting 8 years later, requiring significant investment in the early stages of growth [20]. However, early-maturing-walnut cultivars begin to bloom within a year of planting and enter full fruiting five years later [21]. These early-maturing and dwarf trees have attracted the attention of fruit growers around the world, due to their potential for enhanced planting density, increased production, and photosynthetic efficiency, as well as easy harvesting and efficient spraying [22]. Dwarf and semi-dwarf walnuts are, also, characterized by valuable properties, such as rooting ability [23]. Obtaining dwarf or semi-dwarf status, these forms do not exceed 8 m in size, grow quickly, and reach peak fruiting in a shorter period of time. A notable disadvantage of these forms is their shorter maturity, of about 25 years, while vigorous forms actively bear nuts for 130 to 140 years [24], therefore, enhancing yields and minimizing total costs are one of the breeding objectives for breeders and horticulturists [14]. The goal of this review is to discuss planting of high-density walnut trees, using dwarfing rootstock, early-maturing, lateral-bearing, and early-rooting genotypes, with resistance to environmental factors. This new approach is of interest, due to its convenience of harvesting and increasing yield.

## 2. Dwarfing Walnut Rootstocks

Induction of dwarfness and precocity will be attractive to walnut growers worldwide, especially in Asia, since most of the trees are harvested by hand, with high costs, and many workers fall down from the trees and are injured or die [18]. For these reasons, walnut producers are, currently, interested in high-density-walnut-planting systems [25]. Dwarf trees have many benefits, including higher yield, high-density, spraying, and photosynthesis efficiency, as well as uniformity in nut size and easy harvesting [10]. High-density-hedge systems have been tested around the world. This type of system is based on the mechanical hedging of walnut genotypes, with lateral bearing cultivars such as Chico, spaced with approx. 7 m between the rows x 4 m in the row [26]. The breeding of dwarf walnut cultivars, by selecting dwarf individuals from a population of second-bearing walnut seedlings, is not only possible but also effective, as a way to develop new cultivars with this effective characteristic [15]. Successful breeding of dwarf walnut cultivars demonstrates the scientific applicability and objectivity of the theory, that walnut-tree height is closely associated with early maturity [18].

The walnut trees are grafted only on walnut rootstocks, thus, the dwarfing rootstocks must be from this species [27]. Early-mature cultivars are selected, primarily, from regions in China characterized by arid climate, low rainfall, and strong evaporation [20].

Chances are low to find another species to be used as rootstock for walnut [27]. Genetically precocious, low-vigor walnut genotypes were found in Persian walnut seedlings (personal observation) [27]. In many walnut nurseries, it is a normal practice to have weak (low vigor) and dwarfing seedlings. The compact and basitonic growth of low-vigor walnut seedlings is reported to originate from Central Asia [28]. In Hungary, there is a walnut genotype with compact vigor, suitable for usage as rootstock, under evaluation (unpublished data). Research has been done on genetically dwarf rootstocks, which revealed that there are differences between these genotypes and the standard genotypes, in terms of vigor, maturity, rooting, etc. [18]. The population of these walnuts not only is very precocious compared to other cultivars but also can produce elite cultivars with genes for dwarfing properties [15]. The shortest seedlings are, commonly, observed in the offspring of families with cluster-bearing and low-vigor [6]. The walnut dwarf genotype has a high proportion of homozygous loci, which cause a decrease in growth vigor, thereby resulting in a high

proportion of dwarf offspring [18]. Dwarf and early-maturing-walnut genotypes are found, genetically, in some seedlings of the Persian walnut genotype [17]. It was reported [6] that the size and early maturity of walnut seedlings vary, depending on the seed source, allowing selection of dwarf and early-maturing seedlings. It was concluded that walnuts of these genotypes retained these traits three years after being replanted in well-spaced orchards [17]. Some seed-source plantations of Iranian walnuts, frequently, have precocious and low-vigor genotypes [29].

Walnut trees of Central Asian origin may represent the ancestors of the trees currently cultivated [28]. Unlike other populations growing in other parts of the world today, they mature precociously [7]. Early-maturity trees are identified by their ability to bloom, within a year of germination. Later, numerous inflorescences from lateral buds result in a large set of nuts, resulting in intense waves of secondary flowering, during the same growing season. Early flowering is responsible for enhancing the branching of early mature trees, which are usually bushier than other cultivars [28]. The early-mature genotype has interesting characteristics for fruit breeders, such as cold hardiness during winter and lateral fruiting, but it, also, exhibits early bud break and high susceptibility to walnut blight [7]. The selection and cultivation of early-maturing genotypes has become the main direction of walnut breeding, in the last two decades [15].

The very early onset of fruiting of substantial nuts disrupted the balance between vegetative growth and reproduction of the tree [30]. This can lead to poor uniformity, susceptibility to premature aging, pests, and diseases, and reduced plant growth potential [19]. These factors have hindered sustainable high yields and prevented the sustainable expansion of the walnut industry [21]. Tree-height measurements are sufficient to identify dwarf genotypes because a correlation was observed between seedling height, seedling diameter, number of nodes, and internode length [31,32]. The Xinjiang region program, also, evaluates the benefits of the second-bearing habit found in many precocious dwarf walnuts [15]. Endogenous hormones and histological differences have been reported between vigorous and dwarf walnut seedlings [23]. Dwarf walnut trees are, reportedly, short-lived. Therefore, breeders are trying to graft slow-growing scions on the vigorous rootstocks [2].

For the walnut trees grafted on dwarfing rootstocks, spacing of 5 m in the row  $\times$  5 m between the rows is selected and increases to 10 m  $\times$  10 m as they mature (high planting density) [33]. In Europe, spacing of 3.5 m in the row and 7 m between the rows is being tested in some locations [34].

### 3. Dwarfing by Different Treatments

Plant-growth inhibitors, such as acylcyclohexanedione and prohexadione-calcium, are, often, used in the agricultural industry [35]. Many species are, regularly, treated with chemicals to control their height [36]. Most of these growth regulators act as inhibitors of gibberellin biosynthesis. Prohexadione-calcium has been considered not only for its special inhibitory effect on seedling size and stem length, without any problems remaining in the plant and soil, but also to enhance the yield, fruit quality, and fruit set of some species [37]. Prohexadione-calcium shortened the annual shoots of the walnut cultivar. The concentration of prohexadione-calcium ( $250 \text{ mg} \cdot \text{L}^{-1}$ ) can reduce the summer growth of the shoots on walnut trees [38].

Stimulated mutations may be obtained chemically or through physical mutagens. One of the first mutation research trials was performed on walnut seeds in Ukraine, where walnut seeds were exposed to gamma rays from 1 Gy to 150 Gy. Fast-growing seedlings of walnut were observed from gamma doses below 200 Gy, but slow-growing seedlings were obtained at certain higher doses [39].

### 4. Dwarfism Mechanism

Dwarf rootstocks have smaller root systems (i.e., root volume, root diameter, number of root tips and twigs), thus, the soil absorbs less water and nutrients. A smaller volume of water is transferred to the scion, due to lower hydraulic conductivity and reduced

vessel size, which affect the net rate of photosynthesis and plant growth. Indole 3-acetic acid (IAA) was less absorbed by the dwarf rootstock/scion combination, which affects the production of cytokinin (CK) and gibberellin (GA) by the root system and IAA is, ultimately, transported to the aerial part, reducing plant growth (Figure 1) [40,41].

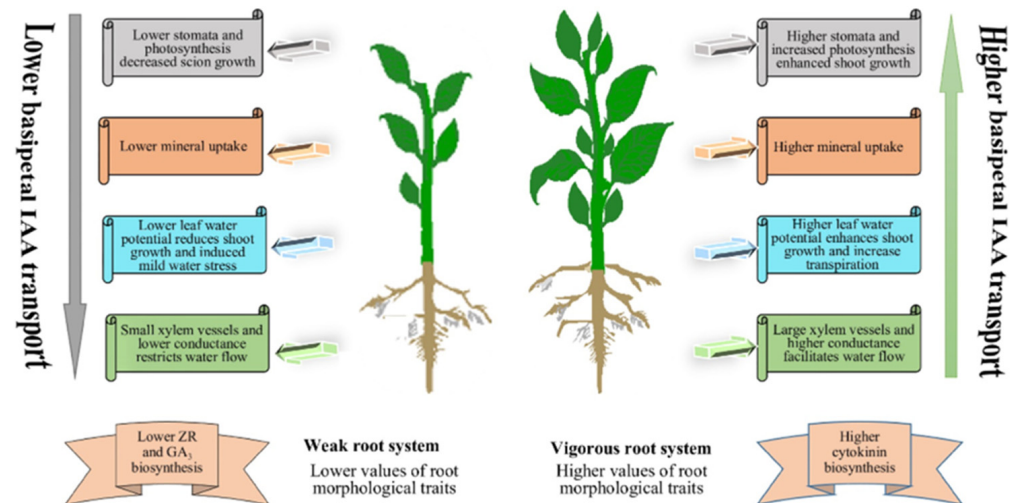


Figure 1. Schematic diagram of rootstock-control scion vigor [41].

## 5. Type of Dwarf Rootstock

Iran's walnut breeding program was initiated in 1983 and led to the release of six commercial cultivars, including Jamal, Damavand, Persia, Caspian, Chaldoran, and Alvand. Alvand was the shortest of all these cultivars. Persia and Caspian were on par with Chandler, in terms of growth power [42].

China's walnut breeding program led to the release of dwarf and compact cultivars: Daixiang (1992), Luguo 7 (1997), and Daihui (2003) [1].

The French walnut breeding program, in 1995, led to the release of Fernette and Fernor, both lateral-bearing cultivars, from the cross between Franquette and Lara. Another cultivar, Ferjean, a lateral carrier cultivar, became available, in 1999, from a cross between Grosvert and Lara [31].

## 6. Tree Architecture

Plant height plays a significant role in the productivity of fruit crops [43]. The development of dwarf and semi-dwarf fruit trees enables high-density planting, increasing productivity as well as efficient use of water and nutrients [44]. Hypotheses intending to explain rootstock dwarfing should be associated with vigor control [45]. Phytohormones play a significant role in plant growth and structure [46]. Gibberellin is known to increase plant height in many crops [43]. Dysfunction of the genes involved in gibberellin biosynthesis or oxidation and signaling can cause dwarf plants [47].

The auxin/cytokinins hypothesis states that dwarf rootstocks reduce Indole-3-acetic acid basipetal transport within the phloem and cambial cells of the rootstock stem to the root, which restricts root growth, and, subsequently, cytokinins biosynthesis and transport to the scion, which restricts the growth of shoots [48]. The second hypothesis states that dwarfism occurs, due to decreased water and solute transport, throughout the graft [49]. The third hypothesis states that phenol accumulation in the graft decreases tissue viability, reducing the rate of auxin degradation and subsequent root cytokinins response [48], but no causal relationship has been shown between growth and phenol accumulation [50].

## 7. Grafting and In Vitro Propagation

It was shown that the best grafting success and survival rate of the walnut tree were obtained by using dwarf rootstocks [17]. Grafting success on dwarf rootstocks is higher

than on standard rootstocks, which may be due to the higher quality of the callus on the grafted union. The use of dwarf walnut seedlings as rootstock can limit the growth of scions [17]. The scion of the dwarf rootstock produced fewer leaves, probably due to limited shoot growth, than standard rootstocks. The use of clustered walnut seedlings as a rootstock reduced the average scion growth, compared to the standard rootstock. Clustered cultivars can be used as a dwarf rootstock, to control the vigor of walnut scion; in addition, the number of nodes is considered a significant factor in assessing the vigor of grafted trees. The use of dwarf rootstocks on Persian walnuts can enhance node formation in the scion and limit internode growth [17]. However, grafting is one of the most significant strategies for ameliorating fruit quality and yield, leading to the development of new homoploid species [49].

Grafting allopolyploid species ( $2n = 32$ ) in hybrid and precocious walnuts may improve plant growth traits [50]. This solves the problem of premature aging of early-mature walnuts and yields high-quality hybrid walnut wood [21,51].

Rootstocks can affect crop quality, through their effects on water and nutrient uptake as well as photosynthetic rate [2]. The self-rooted Chandler tree had better kernel quality (higher proportion of kernel) than the Chandler tree grafted onto the cloned Paradox [52].

It is more difficult to propagate walnuts vegetatively than other fruit species [53]. It has been confirmed that the rooting ability varies in walnut populations. The rooting ability of dwarf seedlings, by stool layering, is superior to that of high-vigor seedlings [5]. A number of seedlings showed that rooting ability with dwarf seedlings was about two times greater than high-vigor seedlings. The ability of high-vigor seedlings to take root may be impacted, due to higher levels of gibberellin, and as a result of wood density, greater lignification, and sclerenchyma-ring stiffness. Thus, the rooting ability of low-vigorous seedlings was improved, and the fruit yield was increased in dense orchard systems by dwarf rootstock-vegetative propagation [18]. The success of rooting of dwarf walnuts is better than vigorous walnut seedlings, in response to stool layering [5].

The number of adventitious shoots generated from micro-cutting was highest in the dwarf and semi-dwarf cultivars, compared to high-vigor in vitro conditions. In reaction to the modified stool layering in low-vigor seedlings, an average number of roots ameliorated, compared to high-vigor seedlings [16]. The number of axillary branches emerging from micro-branches was the highest on the dwarf genotypes, compared to the high-vigorous ones [54].

The effects of rootstock on scion structure include changes in the number of lateral branches and the angle to the trunk [55]. Clustered-walnut rootstocks reduced scion height and internode length of the scion branch as well as increased lateral shoot formation, compared to standard rootstocks [18]. Cluster-bearing rootstocks increased precocity, in comparison to standard rootstocks [18]. However, researchers reported that scion growth rate, 45 days after grafting, was affected by tree vigor [5]. In addition, the growth rate (height) of scions was decreased, at the end of the growing season, with low-vigor seedlings [5]. This may be ascribed to their smaller root system and slower growth percentage. Thus, the effect of seedling rootstocks, obtained from the clustered-walnut trees, on controlling walnut-tree size by grafting in field conditions, is confirmed [18].

Size decrease may be achieved through the selection of smaller-size trees or through the use of dwarfing rootstocks. *Juglans ailantifolia* interstocks have been illustrated to decrease *Juglans regia* scion growth [13].

Plant dwarfing is done genetically and by various genes involved in this phenomenon [14]. The GAI gene is a candidate gene in walnut dwarfism [14]. A single genetic system can, also, be proportional to dwarf walnut trees, as the most dwarf offspring were obtained from parents, which were dwarfed and cluster-bearing [18]. It is feasible not only to develop dwarf walnut cultivars, by selecting dwarf individuals from the seedling population of second-bearing walnuts, but also to develop new cultivars with this useful characteristic [15]. It, also, seems that the early-bearing characteristic has a high heritability [18].



It has been reported that genetically engineered Persian walnuts, with the flavodoxin (*fld*) gene, showed better growth under both osmotic and salinity stress [56]. In the near future, the use of CRISPR/Cas9-mediated techniques to improve walnuts, as previously observed in tomatoes [57], will be envisaged.

## 8. Early Maturation, Yield, Nut Quality

Early maturity of walnut seedlings is the primary goal for walnut growers and breeders [58], but some early-mature-walnut cultivars are susceptible to walnut-blight disease and late-spring frosts [7].

Breeding for precocity is performed in all countries where walnut production occurs [24]. In France, within the breeding program, walnuts that bear nuts in the first year of growth were obtained [7], while in the second year of growth, similar results were obtained with the selection of walnuts in the Krasnodar region [17].

In the nursery, early-maturing walnuts begin to bloom 1 to 3 years after planting, [59]. The flowering capacity of clustered walnut trees provides a resource for breeding projects, aimed at enhancing nut production and shortening the juvenile time of the tree.

Subsequently, multiple inflorescences resulting from lateral buds lead to the formation of nuts, and secondary-flowering waves occur during the same growing season. Precocious trees are responsible for increasing the branching of early-maturing trees, which are usually smaller than other genotypes [28]. To establish a high-density walnut orchard, lateral-bearing cultivars should be used, since these genotypes/cultivars have profitable yield on their side branches, even if their terminal buds are pruned off. With removal of the terminal buds, walnut trees cannot produce any yield, so their growing will not be evaluated on an economic level. The ratio of female flowers on the lateral buds is the key factor for high-density production; with more female flowers on the lateral buds, more nuts can be produced on them. The lateral-bearing characteristics depend on the origin of genotypes and the location of orchard. Genotypes derived from the Carpathian race have a low lateral-bearing capacity, that is, up to 25–30% [60]. The genotypes from the Californian race can produce 30–90% of their nuts on the lateral buds [61]. This rate is much higher in those genotypes, which are from the Iranian race; especially, the novel-bred cultivars, which have up to 100% lateral-bearing capacity [62–64]. Genotypes bred from the French race have nuts on lateral buds at a low rate, such as 5% to 15% [61,65]. Going from the safe walnut-producing zones in the Northern Hemisphere to the Southern Hemisphere, a cultivar can produce more female flowers on the lateral buds; e.g., a US-bred cultivar can have up to 60–70% in Hungary [66], while up to 90% in Greece [67,68] and Turkey [69]. However, it can be stated that the cultivars with a lateral-bearing characteristic are more sensitive to low temperature, especially during dormant periods, necessitating that fruit-site conditions must be checked before planting [70–73], as those cultivars need safe locations, to grow successfully. Szügyi-Bartha et al. [74] did not confirm these results, examining the Hungarian-bred walnut assortment; based on their data, the novel-bred Hungarian lateral-bearing-walnut cultivars did not have significant lower frost tolerance, compared to the terminal-bearing cultivars. Another alternative method, to increase the yield, is to plant cultivars with a cluster-bearing characteristic. Cluster-bearing cultivars bred in Serbia reach 3.3 g to 29 g dried-nut weight, 26.2% to 64.3% kernel rate [75], and 10.5 g/nut in Romania [76].

Breeders and growers are seeking walnut cultivars with a premium nut size, meaning 32 mm in diameter. The most preferred nut characteristics are: smooth shell surface, light shell and kernel color, at least 40% in breaking yield, and low susceptibility to walnut bacterial blight caused by *Xanthomonas arboricola* pv. *juglandis* [62,75,77–88].

## 9. Conclusions

Rootstock selection, by increasing water and nutrient uptake, improving scion growth, and reducing biological and abiotic stresses, as well as adaptation to environmental conditions are effective factors in determining yield. The decrease in plant height should

be assessed in the walnut germplasm, to promote cultural application in high-density planting. Breeding programs in some countries have led to the production of dwarf walnut rootstocks. China's walnut breeding program led to the release of dwarf and compact cultivars: Daixiang and Daihui. The Iranian walnut breeding program led to the production of the Alvand dwarf rootstock. The French walnut breeding program led to the release of Fernette. Moreover, slow-growing walnut trees were obtained at higher doses of 200 Gy gamma rays. Dwarf rootstocks have smaller root systems; thus, the soil absorbs less water and fewer nutrients. Plant dwarfing is done genetically, by different genes involved in this phenomenon. The GAI gene is a candidate gene in walnut dwarfism. In the near future, use of CRISPR/Cas9 techniques to improve walnuts is expected.

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