Application of Thidiazuron in the Micropropagation of Fagaceae

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Abstract

The Fagaceae family consists of 7 genera and around 1000 species of trees and bushes that are mainly distributed in temperate and warm areas of the northern hemisphere, although few cross the equator in Southeast Asia. In terms of forestry, members of the Fagaceae are of most importance in forests in the temperate regions of the northern hemisphere, a dominance shared with the conifers that replace this family in cold areas and mountain tops. The genera *Quercus* (oaks and holm oaks), *Fagus* (beeches), and *Castanea* (chestnut) are commercially important sources of timber; *Castanea* and *Quercus* (holm oaks) also provide fruits that are used as human food and as animal feed. Many of these trees are also of ornamental value, mainly due to their attractive color of their leaves in autumn.

The majority of these species are difficult to propagate, particularly when the trees reach their adult stage. Biotechnology techniques, such as in vitro tissue culture, would therefore be of great use for their propagation and conservation. These techniques involve the use of growth regulators, especially cytokinins, among which is included thidiazuron (TDZ). This cytokinin has been used to stimulate the development of axillary buds and, mainly, for the induction of adventitious buds and in very few cases in somatic embryogenesis processes. This review presents a summary of the various studies in which TDZ has been used in the micropropagation of diverse species of the family Fagaceae.

Keywords

Castanea · Fagaceae · Fagus · Micropropagation · Quercus · Thidiazuron

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9.1 Fagaceae Family

The Fagaceae are a large family of angiosperms with species that are spread throughout the northern hemisphere, from tropical areas to northern areas, with some species that cross the Equator in Southeast Asia. Beeches (*Fagus*), oaks (*Quercus*), and chestnuts (*Castanea*) are the only genera distributed in Asia, Europe, and North America, where they cover, or used to cover, large forest areas. Evergreen oaks are important members of the forests around the Gulf of Mexico, as well as in southern China and southern Japan. In Southeast Asia, the structure of the mixed mountain forest is largely determined by evergreen members of the family, particularly oaks. In total, therefore, the Fagaceae produce a colossal biomass, possibly exceeded only by the conifers. They also have a wide diversity of uses (biomass, fiber, wood and food products). As well as their economic contribution, they are important specimens in forestry ecosystems and are major drivers of terrestrial biodiversity. In the majority of countries where they are found, they are considered as major patrimonial and cultural resources (Kremer et al. 2012).

The members of this family are deciduous or evergreen trees, and rarely shrubs, with alternate simple, entire to pinnately lobed leaves, and scarious, usually deciduous stipules. The flowers are unisexual (plant monoecious) and usually arranged in catkins or small spikes that may comprise only flowers of one sex, as in oaks, or may have female flowers at the base of otherwise male inflorescences, such as in chestnuts. The perianth is bract-like, with four to seven lobes. The male flowers have as many or twice as many stamens as perianth segments, occasionally up to 40, with the filaments free, with or without a pistillode. The female flowers are in groups of one to three, each group being surrounded by a basal involucre. They appear at maturity. The fruits of the Fagaceae are animal dispersed and have a short viability. The pollen and other features, such as a strongly scented inflorescence, suggest that insect pollination is the ancestral condition in the Fagaceae, and this is retained in most members, except *Fagus* and the temperate oak species (Heywood et al. 2007).

The cupule has a wide variety of forms that surround one or more fruits, which is a unique feature of the family and the origin of which has been controversial (Fey and Endress 1983). Only with the discovery of *Trigonobalanus* in 1961, which is restricted to Sulawesi, northern Borneo, Malaysia, and northern Thailand, has it been possible to suggest firmly that the cupule is derived from a three-lobed extension of the pedicel below each flower, which has been variously fused around single flowers or groups of flowers. It is possible that the cupule provides a link with the pteridosperm ancestors of the flowering plants. The tremendous diversity of scales and spines on the cupules appears to be derived from branches spines (Nixon and Crepet 1989).

The Fagaceae provide some of the most important woods worldwide, especially the oaks (particularly the white oak of North America), beech, and chestnut varieties. Together with clearance for agriculture, this has resulted in the destruction of large areas of forests dominated by these species. *Castanopsis* and *Lithocarpus* have been little exploited, although their wood is also of a high quality. Overall, the woods obtained from the species of this family have a wide range of properties and

uses, from floorboards and furniture to carbon and whisky barrels. The cork is obtained from the bark of the cork oak (*Quercus suber*), and the oak galls were an important source of tannin in Asia Minor and southeastern Europe. Many of the chestnut species, especially *Castanea sativa*, are cultivated for their edible fruits, from which purees, stuffings, and stews can be made, as well as the French delicacy *marrons glacés.* The fruits of the beech are rich in oil (46%) and are used in many regions to feed pig stock, as well as the acorns from the oaks. The acorns produced by *Q. ilex* are used for feeding the so-called Iberian pigs, the meat of which contributes to the development of a high-quality food industry (Cañellas et al. 2007). The form and the colors of many of these species in autumn, particularly the oaks, chestnuts, and beeches, make them used as ornamental in parks and gardens. Only American species of *Castanopsis* (*C. chrysophylla*) and *Lithocarpus* (*L. densiflorus*) may be cultivated in warmer regions (Heywood et al. 2007). Besides their economic importance, the Fagaceae species have a high ecological value, as illustrated by the fact that chestnut blight disease, caused by a pathogenic fungus, caused the worst ecological disaster in the history of the United States (Wheeler and Sedroff 2009).

Of the seven genera that make up the Fagaceae family (*Fagus*, *Chrysolepis*, *Castanea*, *Castanopsis*, *Lithocarpus*, *Quercus*, and *Trigonobalanus*), three of them are specially highlighted for their economic value: *Castanea*, *Fagus*, and *Quercus*. These three genera are the most widely micropropagated, and on which we are going to focus this review.

9.2 Thidiazuron

The cytokinin activity of TDZ was first reported in 1982 and, since then, has been gaining acceptance due to its efficient role in the cultivation of plant cells and tissue. TDZ has been successfully used for the induction of adventitious buds, to promote the proliferation of axillary buds, and somatic embryogenesis being especially effective in the case of recalcitrant woody species (Huetteman and Preece 1993; Lu 1993; Murthy et al. 1998; Guo et al. 2011).

There are several derivatives of urea with cytokinin capacity, with the most active compounds being N-phenyl-N′-1,2,3-thidiazol-5-ylurea or thidiazuron (TDZ), N-N′-diphenylurea (DPU), and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU). TDZ is one of the most potent diphenylureas evaluated in plant tissue culture (Mok et al. 1982). It was initially developed by the Schering AG group as a cotton defoliant (Arndt et al. 1976) and has a high activity at concentrations as low as 10 pM. The exposure of plant tissue to TDZ for a short period of time is sufficient to stimulate regeneration (Preece et al. 1991; Visser et al. 1992; Li et al. 2000; Matand and Prakash 2007).

The structure of TDZ is nothing like the natural cytokinins, and it appears that it is directly responsible for the morphogenic responses induced by this regulator. Any change in the molecules that affects its functional groups (phenyl or thiadiazole) reduces its activity (Mok et al. 1982; Mok and Mok 1985).

Different physiological and biochemical processes are induced or increased in the cells due to the action of TDZ, but its exact mode of action is still not known (Guo et al. 2011). Some examples of the diversity of the physiological effects mediated by TDZ include efficient seed germination, expedited bud break, induction and stimulation of sprouting, cotyledonary growth and development, formation of trichomes and stomata appearance on floral parts, and berry weight of grapes. More recently, the morpho-regulatory potential of this compound has led to its application in the cultivation of plant cells, tissues, and organs, with the aim of improving regeneration protocols (Guo et al. 2011). Several studies cite the use of TDZ as a unique growth regulator to induce regeneration in different species, meeting both the auxin and cytokinin requirements (Visser et al. 1992; Murthy et al. 1996; Murthy and Saxena 1998). However, TDZ is currently considered as a cytokinin due to induction of natural cytokinin-like responses. In some cultivation systems, it has replaced the habitual cytokinins, provoking similar responses. This substitution is confirmed with the suppression of the effect of the TDZ provoked by the inhibitors of the metabolism of cytokinins with purine ring, which suggests that there is a common site of action for both types of regulators (Nagata et al. 1993; Hutchinson and Saxena 1996). Its role in morphogenesis seems be closely related to the metabolism of the endogenous growth regulators, especially cytokinins, auxins, abscisic acid, and ethylene (Yip and Yang 1986; Murthy et al. 1995, 1998).

Besides its cytokinin activity, TDZ is capable of inducing somatic embryogenesis in some species, which leads to thinking that it might have some auxin activity or it is associated with auxin metabolism (Saxena et al. 1992; Lu 1993). Murthy et al. (1995) established that TDZ can stimulate auxin synthesis. Other authors indicate the possibility that TDZ could affect the action of certain enzyme systems, such as those associated with cell walls and membranes, or the cytokinin oxidase system, in which the activity seems to be inhibited with TDZ (Wang et al. 1991; Hare et al. 1994).

Other theory is that TDZ may produce a stress situation in plant tissue, for which they respond by developing a survival mechanism in order to perpetuate the plant by means of the increase in morphogenic response (Murch et al. 1997).

TDZ has an effect on a large number of species, even in those that show little or no response to conventional cytokinins. This makes it especially effective in woody species of difficult regenerative capacity in which organogenesis is only achieved with very high concentrations of adenine-type cytokinin (Fellman et al. 1987; Preece et al. 1991; Baker and Bhatia 1993). The reason for this may be found in its greater stability, since it is more resistant to enzymatic action than the endogenous cytokinins and is degraded less during the culture medium autoclaving process (Huetteman and Preece 1993).

TDZ has been employed for the multiplication of various woody species, owing to its capacity to induce the proliferation of axillary shoots (Wojtania et al. 2011; Sedlák and Paprštein 2015; Castillo et al. 2015). In the induction of caulogenesis, it has been shown to promote the differentiation of meristemoids at concentrations much lower than other cytokinins, and the regeneration also occurs with a comparable, or even greater, efficiency (San José et al. 2014; Liu et al. 2016). Its greater

organogenic capacity is also shown by producing a higher number of shoots per explant than benzyladenine (BA), kinetin (KIN), or zeatin.

TDZ also promotes callus formation, in many cases more intensely than other regulators, particularly at concentrations greater than0.02 mg/l. This callus is, in many cases, an essential step for the regeneration of adventitious buds (Huetteman and Preece 1993; Murthy et al. 1998; Traore et al. 2003). More recently, TDZ alone, or in combination with other regulators, has been used as a somatic embryogenesis stimulant in tissue culture media (Nhut et al. 2006; Jones et al. 2007; Kahia et al. 2016; Rugini and Silvestri 2016).

Prolonged exposure to TDZ can lead to problems like hyperhydricity, leaves with abnormal morphology, short and compact shoots, as well as difficulty for the elongation and rooting of the regenerated shoots (Huetteman and Preece 1993; Lu 1993; Ahmad and Anis 2007).

9.3 Biotechnological Approaches in the Propagation of Fagaceae Using TDZ

The majority of members of the Fagaceae are included in the group of long-rotation hardwoods for which no long-term improvement programs have been carried out. However, these species play a vital role in the conservation of the soil and water and make a significant contribution to the sustainability and viability of the ecosystems. The low natural regeneration using seeds, their irregular fructification, the consumption of the seeds by animals, and the loss of viability during storage exacerbates the regeneration problems suffered by most of the species of this family. Furthermore, there are great difficulties in their vegetative propagation when adult material is used.

Several reviews have described the possibilities offered by forest biotechnology as an emerging opportunity in relation to tree improvement (Vieitez and Merkle 2005; Vieitez et al. 2012; Nelson et al. 2014; Ballester et al. 2016; Corredoira et al. 2016; Monteuuis 2016). In vitro culture techniques have been applied to different woody species since they provide the appropriate tools for the rapid production of genotypes with desired traits and to capture all the genetic superiority without involving any gene segregation. These techniques may alleviate, at least in theory, the lack of seed production, as well as the difficulties in the production of offspring with desired traits, storage of the seeds, and the low rooting capacity of the cuttings, helping to rapidly increase the number of individuals in a species with reproduction problems and/or in extremely reduced populations (Vieitez et al. 2012). The plant material obtained can be of great value for research, living collections, to reduce pressure on natural populations and, where possible, for reintroduction programs (González-Benito and Martín 2011).

9.4 Genus *Castanea*

Castanea is a small genus native to the temperate zones of Asia, Europe, and Eastern United States (Camus 1929). The precise number of species is uncertain due to the use of synonyms and the lack of accurate characterization for some species of chinkapins. The most representative species and of greater economic importance within this genus are *C. crenata* Sieb. and Zucc. (Japanese chestnut), *C. dentate* (Marshall) Borkh. (American chestnut), *C. mollissima* Blume (Chinese chestnut), and *C. sativa* Mill. (European chestnut). The other species are small trees or bushes of importance only for breeding, as rootstocks, or for special uses. The chestnuts are long-living trees and can reach 500–1000 years. However, like the majority of large seeded hardwoods, they are very difficult to propagate vegetatively. These trees are also threatened by pollution, economic and social changes, and two major diseases: ink disease caused by *Phytophthora cambivora* and *P. cinnamomi* and chestnut blight caused by *Cryphonectria parasitica*, which have destroyed a large number of trees in Europe and North America (Nelson et al. 2014).

9.5 TDZ and Proliferation of Axillary Buds

Micropropagation by means of axillary bud proliferation is the preferred method for the commercial propagation of woody species, since it is usually easier to do and is also considered more appropriate in order to maintain genetic stability of the regenerated plants (Bonga and von Aderkas 1992; Monteuuis et al. 2008; Gomes and Canhoto 2009).

Although TDZ has been selected for the propagation of numerous woody species due to its enormous capacity to stimulate the proliferation of the shoots (Huetteman and Preece 1993; Guo et al. 2011; Panda et al. 2016; Singh and Agarwal 2016), however, in Fagaceae positive results with TDZ are limited, and most of the cultures of these species are maintained in media supplemented with BA or zeatin (Table 9.1).

Among the works carried out, we should mention those of Wilhem and Rodkachane (1992), who evaluated the capacity of TDZ (4–11 mg/l) with and without 0.5 mg/l BA in the proliferation of juvenile and adult material of *Castanea sativa*. The results indicate that, after induction, a subculture with a high concentration of TDZ combined with BA improved the micropropagation. However, if TDZ is maintained in the medium, it promotes massive callus growth and inhibits elongation of the shoots. Fernández-Lorenzo et al. (2001) tested the effect of 0.1 mg/l BA or 0.01 mg/l TDZ in the multiplication of cultivars of adult origin of this same species, observing that TDZ produced a significant decline in the multiplication rate and the production of vitrified deformed shoots with excessive callus. In material of juvenile origin (1 year and 2–3 months) of two chestnut cultivars, Maraval 74 (*C. sativa* x *C. crenata*) and Marigoule 15 (*C. crenata* x *C. sativa*), Soylu and Ertük (1999) tested the effect of BA and in some cases 0.1 mg/l TDZ in the proliferation of buds. They pointed out that, although this concentration of TDZ in combination with BA had been mentioned previously by Waidinger and Rodkachane (1993) as

Table 9.1 Summary of studies on micropropagation of different species of *Castanea*

Species	Treatment	Observations	References
		Axillary shoots	
C. sativa	GD + TDZ (4–11 mg/l) + BA	A subculture improves micropropagation	Wilhem and Rodkachane (1992)
	P24 + TDZ (0.01 mg/l) + BA	In combination with BA, it favors bud expansion	Waindinger and Rodkachane (1993)
	GD, WPM + TDZ 0.01 mg/l	Decreases the multiplication rate; vitrified shoots; excessive callus	Fernández Lorenzo et al. (2001)
	MS (1/2NO_3) + TDZ (0.025–0.1 mg/l)	BA more effective than TDZ	Roussos et al. (2016)
		Adventitious shoots	
	MS + 0.1 mg/l TDZ	Preconditioning for genetic transformation	Corredoira et al. (2005)
	MS + 0.2 mg/l TDZ + IBA	More effective than BA	Tafazoli et al. (2013)
		Somatic embryogenesis	
	MS, DKW + 0.1 mg/l TDZ + BA/KIN+ IBA		Sezgin and Dumanoglu (2014)
		Axillary shoots	
C. sativa x C. crenata	MS (1/2NO_3) + TDZ 0.1 mg/l	Does not stimulate growth	Soylu and Ertük (1999)
C. crenata x C. sativa			
		Adventitious shoots	
C. sativa x C. crenata	MS + 0.1–2 mg/l TDZ + NAA	Cotyledon nodes; preconditioning with BA	San José et al. (2001)
		Axillary shoots	
C. dentata	WPM + TDZ 0.1–0.5 mg/l	Bud proliferation increases (reddish color)	Yang et al. (2009)
	− + TDZ 0.01 mg/l	Positive results	Herman (1995)
		Somatic embryogenesis	
	WPM +0.1 mg/l TDZ ± 2,4-D	TDZ did not give an embryogenic response	Carraway and Merkle (1997)
		Axillary shoots	
C. crenata	BW + TDZ 1 mg/l	Less effective than zeatin	Tetsumura and Yamashita (2004)

GD Gresshoff and Doy's medium (1972), *TDZ* thidiazuron, *BA* benzyladenine, *P24*'s medium (Waidinger and Rodkachane 1993), *WPM* Lloyd and McCown's medium (1980), *MS (1/2 NO_3)* Murashige and Skoog's medium (1962) with half strength nitrates, *IBA* indole-3-butyric acid, *DKW* Driver and Kuniyuki's medium (1972), *KIN* kinetin, *NAA* naphthalene acetic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *BW* Sato's medium (1991)

bud growth stimulator in adult chestnut material, it was not effective in these two varieties. In a recent work, Roussos et al. (2016) evaluated the efficacy of several cytokinins (BA, KIN, isopentenyladenine (2iP), forchlorfenuron (FCF), and TDZ) and growth retardants on the multiplication and rooting in in vitro cultures of the European chestnut from material of juvenile origin. BA was the most effective

cytokinin, followed by KIN and 2iP. The two phenylureas used (FCF and TDZ) were not as effective as the adenine-type cytokinins.

Different culture media (Sato's medium (BW, 1991), Lloyd and McCown (WPM, 1980), and Driver and Kuniyuki (DKW 1972)) were used in the micropropagation of *C. crenata* from plantlets of 2 months (Tetsumura and Yamashita 2004). These media were supplemented with BA, zeatin, or TDZ (1 mg/l). The best results in the establishment phase were obtained with BW medium supplemented with zeatin and thus were subsequently used for shoot multiplication.

According to the study by Herman (1995), the use of TDZ (0.01 mg/l) has given positive results in American chestnut (*C. dentata*). In this same species, Yang et al. (2009) investigated the role of different growth regulators in increasing shoot proliferation and callus formation, although no details were given on the type of explant or the age of the material used. TDZ and CPPU cytokinins at low concentrations (0.1 and 0.5 mg/l) produced the best multiplication rates compared with KIN or zeatin. However, the shoots obtained from explants cultivated in medium with CPPU or TDZ medium showed a reddish coloration, while with KIN or zeatin, they had a more normal morphological appearance.

9.6 TDZ and Differentiation Adventitious Buds

Although the formation of adventitious buds is not desirable for clonal propagation, it does offer an excellent opportunity for the regeneration of genetically manipulated plants using biotechnology. The in vitro regeneration via de novo differentiation of buds may be used for the production of more resistant plants and/or more productive genotypes after the insertion of new genes into the cells and could accelerate tree breeding programs. TDZ is currently considered to be one of the most effective triggers of morphogenesis in differentiated cells of woody species, having been successfully used in several species (Pavingerova 2009; Lenz et al. 2016; Zaytseva et al. 2016), although, as with the proliferation of axillary buds, there are few works on Fagaceae (Table 9.1).

The works found in the literature only mention the European chestnut or its hybrids. Thus, San José et al. (2001) studied TDZ on the adventitious bud formation capacity in preconditioned explants of *C. sativa* x *C. crenata*. In order to do this, they germinated the embryonic axes isolated in Murashige and Skoog medium (1962) supplemented with 0.1 mg/l TDZ or 1 mg/l BA. Segments of the hypocotyl, epicotyl, and cotyledon nodes were isolated from these embryos preconditioned and cultivated in induction medium with 0.01 mg/l of naphthalene acetic acid (NAA) and different concentrations of TDZ (0.1–2 mg/l). The best results were obtained with cotyledon nodes preconditioned in BA for 12–14 days and subsequently treated with 1–2 mg/l TDZ for 4 weeks (Fig. 9.1a, b). These shoots were elongated in medium with 0.1–0.05 mg/l BA. Cotyledon nodes pre-cultivated with 0.1 mg/l of TDZ have subsequently been used in genetic transformation works with *Agrobacterium tumefaciens* in *C. sativa* (Corredoira et al. 2005). Tafazoli et al. (2013) also established a protocol for the regeneration of adventitious buds from

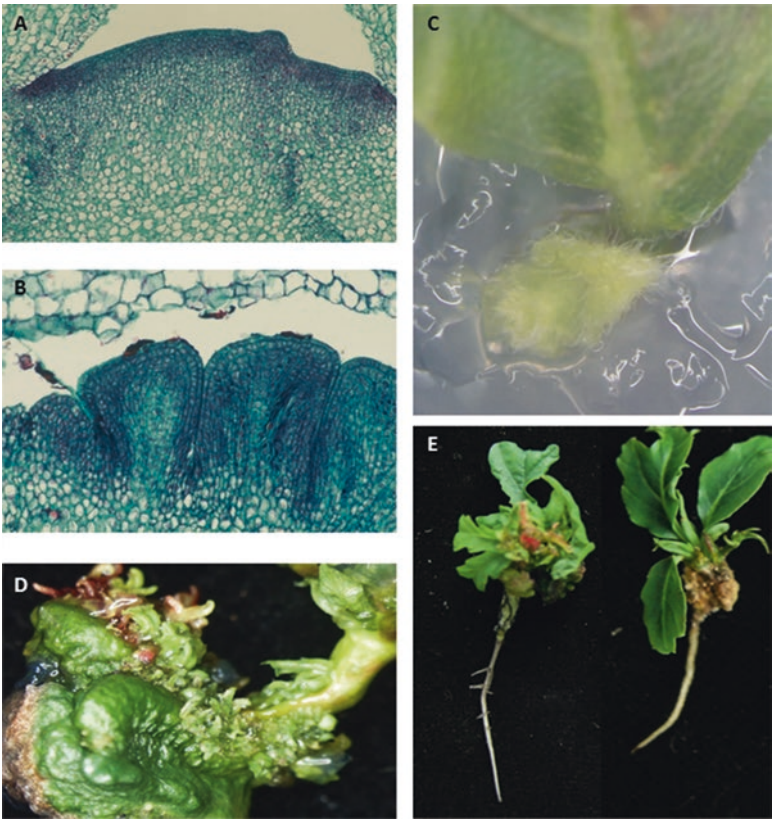

Fig. 9.1 Micropropagation of Fagaceae using TDZ. (**a**) Longitudinal section of axillary bud meristem of chestnut formed at the cotyledonary node region cultured in preconditioning medium with 0.1 mg/l TDZ (×66.9); (**b**) multiple shoot formation in a BA-preconditioned explant of chestnut cultured on 1 mg/l TDZ induction medium (×124.5); (**c**) adventitious shoot differentiation in *Fagus sylvatica* leaf explant treated with 0.5 mg/l IAA and 0.5 mg/l TDZ; (**d**) adventitious shoot regeneration in the cotyledon of a somatic embryo of *Quercus robur* after 1 week culture in 0.1 mg/l TDZ and 7 weeks in germination medium; (**e**) germinated embryos of *Quercus robur* following 1 week exposure to TDZ and 7 weeks in germination medium

roots, nodes, and internodes of 3-month-old plantlets of *C. sativa*. The results showed the superiority of TDZ compared to BA as a shoot – inducing cytokinin in the in vitro induction of adventitious shoots from nodal segments of chestnut. The highest regeneration rates were achieved with the concentration of 0.2 mg/l.

9.7 TDZ and Somatic Embryogenesis

Somatic embryogenesis is an alternative regeneration process that offers numerous advantages as regards the differentiation of adventitious buds, is currently considered as one of the main biotechnology techniques for the mass propagation of

plants, and is potentially of enormous use in genetic improvement programs (Lelu-Walter et al. 2013). In woody species, somatic embryogenesis is the most promising propagation method, enabling the implementation of multi-varietal forestry (Park et al. 2016) and biotechnological approaches such as the large-scale propagation of selected material, genetic transformation, and cryopreservation of elite genotypes (Corredoira et al. 2006; Bonga 2016; Guan et al. 2016).

TDZ was used for the induction of somatic embryogenesis in the genus *Castanea* by Sezgin and Dumanoglu in 2014 (Table 9.1). These authors established a protocol for the induction of somatic embryogenesis and regeneration of plants from immature cotyledons in two cultivars of *C. sativa* using different combinations of growth regulators (BA, TDZ, KIN, indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and NAA). For both cultivars (Osmanoglu and Sariaslama), the best results were obtained with the MS and DKW formulas and the combination of different cytokinins and auxins (BA+KIN+IBA, BA+TDZ+IBA, KIN+TDZ+IBA). According to these authors, TDZ at 0.1 mg/l has been used in binary combinations with other auxins (2,4-D, indole-3-acetic acid (IAA), or NAA) in the somatic embryogenesis of different chestnut species and hybrids. Carraway and Merkle (1997), using *C. dentata*, tested the effect of the combination of three auxins (2,4-D, IAA, and NAA) with BA or TDZ (0.1 mg/l) in the induction of somatic embryos in ovules and immature zygotic embryos, obtaining the best results with 3 mg/l of 2,4-D. NAA or TDZ gave no embryogenic response.

9.8 Genus *Fagus*

The genus *Fagus* consists of ten species of monoecious trees, natives of the Northern hemisphere temperate zones of Eurasia and Eastern North America. *Fagus sylvatica* L. (European beech) is the most important species of the genus and one of the economically most important of central Europe, which together with the oaks define the climax vegetation of this region. *F. orientalis* Lipski (oriental beech) is a native of the temperate zones of Eastern Europe, the Balkan Peninsula, the Caucasus, and Asia Minor. *F. grandiflora* Ehrh. (American beech), of great ornamental value and with a high price as timber, is a native of Eastern North America. *F. japonica* Max. and *F. sieboldi* Engl. are Japanese species similar to the European beech (Chalupa 1996). Beech trees are important from an economic, as well as an ecosystem point of view. Its fine grain wood is used for flooring, furniture, and veneers, as well as an excellent wood for burning. The ornamental varieties are also of great economic importance (Vieitez et al. 2003).

The beeches are slow-growing trees with a life-span of 150–200 years, but some examples have been reported that have reached 300 years. Some ornamental beeches are propagated by bench grafting, with conventional vegetative propagation of beech generally being difficult (Meier and Reuther 1994; Chalupa 1996; Vieitez et al. 2003). They are extremely difficult to propagate if the cuttings are of adult origin, particularly if they are taken from branches of the crown (Ahuja 1984). The beeches are mainly propagated by seeds, which can be stored at 5 °C with a

humidity of 20–25% in order to maintain their viability. However, good seed harvests are only obtained every 4–6 years, and storage over long periods has its problems. Furthermore, the use of seeds in tree improvement can take 30–50 years for trees to attain maturity; a similar period of time is needed in the propagation via cuttings taken from seedlings. Hybridization is very limited; thus the genetic basis for the selection of elite trees is also restricted. New methods for the rapid clonal propagation of the beech is thus necessary due to the lack of techniques for the production of large quantities of selected plant material using vegetative propagation (Vieitez et al. 2003).This need is especially acute in the case of *F. grandiflora* affected by the so-called beech bark disease, an introduced insect-fungus disease complex incited by an initial infestation by the scale insect, *Cryptococcus fasifuga* Lind., followed by an infection with one of the *Nectria* fungi, primarily *Nectria coccinea* var. faginata Lohman et al. (Ramírez et al. 2007). Thus, attention has been focused on the development of in vitro culture techniques, viable and rapid propagation methods that will enable new individuals to be obtained from the few tolerant/resistant trees that can be identified in the zones affected.

9.9 Proliferation of Axillary Buds and TDZ

The first attempts at propagating beeches employing in vitro axillary bud proliferation were carried out by Chalupa (1979, 1985) using apices and nodal segments of plantlets of the *F. sylvatica* species (Table 9.2).The explants were cultivated in different media (MS, WPM, and DKW) supplemented with various cytokinins: N-(phenylmethyl)-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine (PBA), BA,

Table 9.2 Summary of studies on micropropagation of different species of *Fagus*

Species	Treatment	Observations	References
		Axillary shoots	
F. sylvatica	WPM+ TDZ 0.001–0.005 mg/l	PBA and BA more effective than TDZ	Chalupa (1979, 1985)
		Adventitious shoots	
	WPM + 0.5 mg/l TDZ + IAA	Leaves. TDZ 3 w more effective than BA	Vieitez and San José (1996)
	WPM + 1 mg/l TDZ + IAA	Internodes. More effective than BA	Cuenca et al. (2000)
		Adventitious shoots	
F. orientalis	WPM + 0.5–1 mg/l TDZ + IAA	Leaves, originating from the callus	Cuenca and Vieitez (1999)
	WPM + 1 mg/l TDZ + IAA	Internodes. More effective than BA	Cuenca et al. (2000)
	WPM + 1 mg/l TDZ + IAA	Leaves, internodes. Glucose 3–4%	Cuenca and Vieitez (2000)

WPM Lloyd and McCown's medium (1980), *TDZ* thidiazuron, *PBA* N-(phenylmethyl)-9-(tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine, *BA* benzyladenine, *IAA* indole-3-acetic acid

and TDZ. The best results were obtained with PBA and BA in combination with IBA. Low concentrations of TDZ (0.001–0.005 mg/l) stimulated the growth of axillary buds, but used in higher concentrations, it promoted callus formation and inhibited shoot elongation (Chalupa 1985). When the explants came from selected adult trees, the in vitro development was more difficult, and the majority of the works have used media supplemented with BA (Vieitez et al. 2003).

9.10 Differentiation of Adventitious Buds and TDZ

Although Chalupa (1996) mentions a preliminary study in which he obtained adventitious and axillary buds in embryonic axes, the first protocol for organogenesis induction in *F. sylvatica* was developed by Vieitez et al. (1993). These authors observed the induction of adventitious buds in hypocotyls of plantlets resulting from the germination of zygotic embryos cultivated in the presence of BA. The capacity to differentiate adventitious buds was also observed in cotyledon and hypocotyl segments isolated from plantlets of 3 weeks and cultivated in media with BA and NAA. These observations led to subsequent studies for the development of adventitious buds in leaves and internodes of juvenile cultures maintained in vitro.

Vieitez and San José (1996) describe the induction of adventitious buds in foliar explants of shoots cultivated in vitro originally established from 2-month-old and 3-year-old plants of *F. sylvatica*. The explants were cultivated in WPM medium supplemented with 0.5 mg/l IAA and different concentrations of BA (1, 2, or 4 mg/l) or TDZ (0.05, 0.5, 1, or 2 mg/l) (Table 9.2). The efficiency of the regeneration depended on the type of explant (proximal or distal half of the leaf), the cytokinin added to the culture medium, and the genotype. TDZ at 0.5 mg/l was more effective than BA (Fig. 9.1c). The concentration of TDZ and the time of exposure were critical for optimizing the results. The continued exposure to TDZ produced stunted and compact bud clusters, thus more difficult to elongate than those induced by BA. The problem can be overcome by reducing exposure time to 1–3 weeks, in accordance to the two-stage procedure proposed by Huetteman and Preece (1993) (culture in TDZ medium followed by culture in a second medium without TDZ or with other growth regulators). The histology study confirmed the direct or indirect origin of the adventitious buds.

Cuenca and Vieitez (1999) also achieved the induction of adventitious buds in foliar explants of shoots cultivated in vitro established from plants of 2 months and 4 years of *F. orientalis.* These authors used WPM supplemented with 0.5 mg/l IAA and 0.5–1 mg/l TDZ. Histologically, it could be shown that, although some buds developed directly from the epidermis or sub-epidermis, the majority of them originated from cell file proliferation produced by the periclinal division of cells subjacent to the epidermis.

Cuenca et al. (2000) evaluated the formation of adventitious buds in internodes of shoots cultivated in vitro from European and Oriental beech (*F. sylvatica* and *F. orientalis*, respectively). Induction medium consisted of WPM supplemented with

0.5 mg/l IAA and 0.1, 0.5, 1, 2, and 4 mg/l TDZ or BA. As in the case of foliar explants (Vieitez and San José 1996), TDZ was much more effective than BA for the induction of adventitious buds in this type of explant, with 1 mg/l being the optimum dose for TDZ. An increase in the concentration of TDZ leads to the formation of clusters of tiny buds that subsequently do not develop. The frequency of explants forming buds and the number of adventitious buds was significantly affected by the genotype and the concentration of TDZ. These results are in agreement with those of Murthy et al. (1998), who confirm that TDZ is more effective for the induction of adventitious buds than other purine-type cytokinins. The combination of TDZ with IAA or IBA at 0.5 mg/l increases the capacity in forming buds in the internodes.

In a study carried out by Cuenca and Vieitez (2000) to see the effect of the carbon source on adventitious bud differentiation in leaves and internodes of *F. orientalis* with 0.5 mg/l IAA and 1 mg/l TDZ, these authors showed that both the percentage and number of adventitious buds per explant were significantly affected by the type and the concentration of sugar used, with 3–4% glucose giving the best results.

The elongation of the adventitious buds was promoted by storing them under a dim light at 3–4 °C for 2 months (Vieitez et al. 2003). The importance of a cold storage period for elongation of shoots induced with TDZ has been described previously by Huetteman and Preece (1993).

9.11 Genus *Quercus*

The genus *Quercus* consists of about 400 species distributed throughout the temperate regions of the world (Johnson et al. 2002). These trees can reach a height of 30–40 m and live for 800 years or more. Of the 24 species of oak and their hybrids that inhabit Europe, *Q. robur* L. and *Q. petraea* L. have a higher number of specimens and are the most important from an economic and ecological point of view. Together with these species, *Q. suber* L. and *Q. ilex* L. should also be mentioned as species characteristic of Mediterranean ecosystems. In America, *Q. rubra* L. and *Q. alba* L. are the most important and most widely represented (Vieitez et al. 2012). There are also more than 35 species of *Quercus* reported in the Himalayan region (Singh et al. 2011).

As in the majority of the Fagaceae, the regeneration of *Quercus* spp. is reported to be gradually deteriorating due to extensive harvesting, irregular fructification, unavailability of seed every year, and predation, as well as the difficulties encountered with their propagation using conventional methods, particularly when they are adult specimens (Vengadesan and Pijut 2009). There has also been the loss of a large number of specimens in the last few years, among other causes, due to that known as oak decline syndrome. This is a gradual and episodic phenomenon characterized by a loss of vigor that seems to be caused by an interaction between several biotic and abiotic factors (Vieitez et al. 2012). The need for plantations with high value genetic material has favored the application of micropropagation techniques in this genus.

9.12 Proliferation of Axillary Buds and TDZ

Benzyladenine, in different concentrations, is the most widely used cytokinin for the proliferation of axillary buds in the genus *Quercus*. However, Chalupa (1988) evaluated the effect of TDZ in cultures of juvenile (3–6 months) and adult (30–50 years) origin from *Q. robur*. TDZ significantly affected the elongation and morphology of the shoots. At very low concentrations (0.001–0.004 mg/l), it promoted proliferation, but at higher concentrations (0.01–0.02 mg/l), it stimulated the formation of a large amount of callus, and the shoots developed were short and fewer in number (Table 9.3).

In *Q. euboica* Pap., an endemic species of Greece, Kartsonas and Papafotiou (2007) studied the effect of different cytokinins (KIN, zeatin, 2iP, BA, and TDZ) in the multiplication of shoots established from material of adult and juvenile origin.

Table 9.3 Summary of studies on micropropagation of different species of *Quercus*

Species	Treatment	Observations	References
		Axillary shoots	
Q. robur	WPM o BTM + 0.001–0.004 mg/l TDZ	Promotes shoot proliferation	Chalupa (1988)
		Adventitious shoots	
	WPM o BTM + 0.01–0.02 mg/l TDZ	Also combinations of TDZ with BA or PBA	Chalupa (1988)
	MS + 0.1–1 mg/l TDZ	Cotyledons of somatic embryos	Martínez et al. (2008)
		Somatic embryogenesis	
	MS + 0.01–0.02 mg/l TDZ	Favors the conversion of the somatic embryos	Martínez et al. (2008)
		Axillary shoots	
Q. euboica	WPM + 0.02 mg/l TDZ	Inhibits bud development	Kartsonas and Papafotiou (2007)
	WPM + 0.1 mg/l TDZ	Totally ineffective	Kartsonas and Papafotiou (2009)
		Axillary shoots	
Q. serrata	WPM + 1.5 mg/l TDZ	Very short shoots and small leaves	Pandey and Tamta (2014)
		Axillary shoots	
Q. rubra	MS + 0.1 mg/l TDZ+ BA	Elongation of the shoots in medium with BA and GA_3	Vengadesan and Pijut (2009)
		Somatic embryogenesis	
Q. ilex	MS + 0.2 mg/l TDZ+ picloram	TDZ did not produce somatic embryos. Better treatments: NAA or IAA with BA	Martínez et al. (2017)

WPM Lloyd and McCown's medium (1980), *BTM* broad-leaved tree medium (Chalupa 1981), *TDZ* thidiazuron, *BA* benzyladenine, *PBA* N-(phenylmethyl)-9-tetrahydro-2H-pyran-2-yl)-9H-purin-6-amine, *MS* Murashige and Skoog's medium (1962), *GA_3* gibberellic acid, *NAA* naphthalene acetic acid, *IAA* indole-3-acetic acid

The percentage of explants that formed shoots was affected by the cytokinin type and concentration. The cultures in medium with BA and zeatin gave the best results; with TDZ (0.02 mg/l) in the medium, the percentage of shoot formation was zero. This result was corroborated in a subsequent work carried out on this same species (Kartsonas and Papafotiou 2009).

With material of juvenile origin from *Q. serrata* Thunb., a native species of east Asia, Pandey and Tamta (2014) studied the effect of different cytokinins (BA, 2iP, KIN, and TDZ) (0.5, 1, 1.5 mg/l) in the proliferation of shoots. Although TDZ (1.5 mg/l) enabled the maximum number of explants to be obtained, these were short and with swollen bases and small leaves without expanding. The medium supplemented with BA (1 mg/l) produced a relatively lower number of shoots per explant, but these reached the greatest lengths, and their leaves appeared healthy and well expanded.

The in vitro propagation of *Q. rubra* was achieved from plantlets of 8 weeks by Vengadesan and Pijut (2009) using MS medium supplemented with 1 mg/l BA and 0.1 mg/l TDZ. TDZ together with BA stimulates the development of a large number of shoots, but this combination did not allow the subsequent growth and development of the shoots. According to these authors, TDZ produced cell division, but not elongation as occurred in *Acer grandidentatum* (Bowen-O'Connor et al. 2007). The addition of BA (1 mg/l) and gibberellic acid (0.05 mg/l) to the medium was necessary in order to stimulate the elongation of the shoots.

9.13 Differentiation of Adventitious Buds and TDZ

Adventitious buds were differentiated in the callus developed on the basal part of oak shoots (*Q. robur*) cultivated in medium with TDZ (0.01–0.02 mg/l). A higher concentration of TDZ (0.05–0.1 mg/l) promoted the formation a large quantity of callus and inhibited the elongation of the buds. The combinations of PBA or BA with TDZ also promoted callus formation and the differentiation of adventitious buds (Chalupa 1988) (Table 9.3).

Martínez et al. (2008) observed the differentiation of adventitious buds in cotyledons of somatic embryos of *Q. robur* treated with0.1–1 mg/l TDZ (Fig. 9.1d).

9.14 Somatic Embryogenesis and TDZ

Martínez et al. (2017) studied the effect of different growth regulators (BA, IAA, NAA, IBA, picloram, and TDZ) in order to induce the formation of somatic embryos in leaves and apices from in vitro shoots cultures of *Q. ilex*. The best results were obtained with the apical explants and the combination of NAA or IAA (4 mg/l) with BA 0.5 mg/l. Thidiazuron was not effective in the induction of somatic embryogenesis in this species (Table 9.3). Martínez et al. (2008) determined the ability of TDZ to improve the conversion capacity of somatic embryos induced in adult material of

Q. robur, incorporating it into the germination medium (MS) for short periods of time. According to these authors, the addition of TDZ to the germination medium induces the formation of multiple buds in the epicotyl area. The exposure of somatic embryos to 0.01–0.02 mg/l TDZ for 7 days increases the frequency of somatic embryos with elongated shoots (Fig. 9.1e). TDZ at 0.02 mg/l increases the conversion percentages by up to 64% in one of the genotypes studied (Sainza).

9.15 Concluding Remarks and Future Prospects

In forestry terms, the members of the Fagaceae family are considered to be of great economic and ecological importance, occupying a wide ecological niche in the northern hemisphere. However, the vegetative propagation of the majority of the species of this family presents considerable difficulties, especially when the selected material is of adult origin. From a few decades ago, biotechnology offers new methods for the propagation, improvement, and conservation of plant material and is particularly suitable in the case of recalcitrant species.

In the in vitro culture of plant material, we are able to consider (1) the proliferation of axillary buds, considered as the simplest and efficient method for maintaining the genetic stability of the regenerated plants; (2) the differentiation of adventitious buds (although it is not desirable for clonal propagation, it does offer an excellent opportunity to capture somaclonal variations, obtain chimeral modifications, and apply selection and mutagenic pressures due to the adventitious nature of regeneration); and (3) the somatic embryogenesis, which is considered as an ideal regeneration system for genetic transformation studies and for the mass propagation of plants.

The application of micropropagation requires the use of culture media supplemented with various growth regulators, mainly auxins and cytokinins. Among these latter, TDZ has been used successfully in the micropropagation of numerous species and is considered as one of the most powerful diphenylureas evaluated in plant tissue culture. Despite this, it has not been widely used in the micropropagation of Fagaceae, being replaced by other purine-type cytokinins, such as benzyladenine or zeatin. Further studies will lead to the development and use of better and more powerful growth regulators that will make it easier for the in vitro propagation of material collect from selected adult trees or the differentiation of adventitious buds and somatic embryos that will allow carrying out works on genetic improvement and conservation of these species.

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