Thidiazuron in Micropropagation of Small Fruits

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Abstract

Strawberry, raspberry, grape, blueberry, and cranberry are major small fruit crops cultivated widely across the world. They are highly appreciated and have long been enjoyed enormous popularity among consumers. Their superior nutritive components play a significant dietary role in maintaining human health that has led to a dramatic increase of their global production. There has been an immense progress in small fruit micropropagation using semisolid gelled and liquid media containing different plant growth regulators (PGRs). Thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (TDZ)] is a PGR and with its cytokinin- and auxin-like effects, has significant role in in vitro propagation of small fruit crops. Bioreactor micropropagation containing liquid media with TDZ has resulted in significant progresses not only in reducing micropropagation cost but also in speeding up the process significantly for these crop species. However, the optimal plant production depends upon a number of factors including genotype, media types, types and concentration of PGR, and culture environment. The chapter deals with the progress in-depth of various aspects of small fruit micropropagation in semisolid and liquid media containing TDZ and use of TDZ in a bioreactor micropropagation for commercial production. Somaclonal variation can be a major concern in small fruit micropropagation using TDZ. Although strategies have been developed to reduce these variations, DNA-based molecular markers are promising tools to monitor clonal fidelity of TDZ-induced micropropagated small fruit plants. The chapter also describes the use of molecular markers for the assessment of genetic fidelity, stability, and true-to-typeness in small fruit tissue culture plants.

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6.1 Introduction

Small fruits, also known as berry crops, are small- to moderate-sized fruits produced on perennial herbs, vines, or shrubs. Brambles (blackberry, raspberry and their hybrids), *Ribes* (currant and gooseberry), strawberries, table and wine grapes (*Vitis* spp.), and *Vaccinium* species (blueberry, cranberry, lingonberry, and others) are among the important small fruit crops worldwide (Debnath 2003a, 2016a). Native American peoples relied heavily on certain small fruits as a staple in their diet and passed on their knowledge of the fruit to the first European colonists. Many Native Americans and First Peoples of Canada combined dried meat with dried small fruits to add flavor (Trehane 2004). The production of blueberries, cranberries, raspberries, and strawberries is a profitable agricultural enterprise that began in the early nineteenth century. Regionally important minor small fruit crops include *Aronia* (*Aronia melanocarpa* [Michx.] Elliott, Rosaceae), arctic raspberry (*Rubus arcticus* L., *R. stellatus* Sm. and their hybrids; Rosaceae), cloudberry (*R. chamaemorus* L., Rosaceae), mora (*R. glaucus* Benth., Rosaceae), Juneberry/saskatoon (*Amelanchier* sp., Rosaceae), alpine strawberry (*Fragaria vesca* L., Rosaceae), edible honeysuckle (*Lonicera caerulea* L., Caprifoliaceae), elderberry (*Sambucus Canadensis* L., Caprifoliaceae), hardy kiwi (*Actinidia arguta* [Siebold & Zucc.] Planch.ex Miq., Actinidiaceae), sea buckthorn (*Hippophae rhamnoides* L., Elaeagnaceae), schisandra (*Schisandra chinensis* [Turcz.] Baill., Schisandraceae), bilberry (*Vaccinium myrtillus* L., Ericaceae), and muscadine grape (*Vitis rotundifolia* Mich., Vitaceae). Chokecherry (*Prunus virginiana* L.), highbush cranberry (*Viburnum trilobum* Marshall), serviceberry [*Amelanchier alnifolia* (Nutt.) Nutt.], and silver buffalo berry [*Shepherdia argentea* (Pursh) Nutt.] are some of the other small fruit crops that are consumed in the traditional diets of North American tribal communities (Galletta and Himelrick 1990; Finn 1999).

Diets high in small fruits have a positive impact on human health, performance, and disease. They are flavorful providing unique contributions to dietary choices of consumers. Small fruits can satisfy diverse consumer choices and tastes with their different levels of sweetness and acidity, and with a variety of flavors and textures. They are consumed in fresh, dried, juice, and processed product forms. Small fruits are a major human dietary source of phytochemicals including flavonoids and other phenolic compounds, cyanogenic glucosides, phytoestrogens (Mazur et al. 2000), and phenols that are potentially health-promoting and are believed to fight against diseases (Macheix et al. 1991). Consumption of small fruits is likely to decrease the risk of cardiovascular diseases, certain forms of cancer, hypertension, type II diabetes, and other age-related and degenerative diseases (Ames et al. 1993; Rissanen et al. 2003). Fruit and leaf extracts from some small fruit species inhibit some

cancers or have strong antioxidant activities as were evident from in vitro and in vivo studies with animal models (Yau et al., 2002). Ellagic acid of small fruits (Häkkinen and Törrönen 2000; Harris et al. 2001; Cordenunsi et al. 2002) affects cell proliferation and apoptosis, suggesting a potential anticancer role. Flavonoid-rich blueberries and cranberries can limit the development and severity of certain cancers and vascular diseases including ischemic stroke, atherosclerosis, and neurodegenerative diseases of aging (Neto 2007). Lingonberry leaves and fruits are rich in antioxidant properties (Vyas et al. 2015) and can be used to treat stomach disorders, rheumatic diseases, and bladder and kidney infections and to lower cholesterol levels (Novelli 2003). Cranberries produce proanthocyanidins (condensed tannins) that help to prevent urinary tract infections through reduced adhesion of uropathogenic *Escherichia coli* (Howell et al. 2005).

Thidiazuron (TDZ, N-phenyl-N′-1,2,3-thiadiazol-5-ylurea), first used as a cotton defoliant (Arndt et al. 1976), has been shown to exhibit strong cytokinin-like activity similar to adenine derivatives (Mok et al. 1982, 1987; Thomas and Katterman 1986). Although TDZ was categorized as a cytokinin with natural cytokinin-type response (Murthy et al. 1998), it has been found to possess both cytokinin- and auxin-like activities in in vitro culture of various plant species (Mok et al. 1982; Visser et al. 1992).While at higher concentrations, TDZ stimulates callus formation, shoot regeneration, and somatic embryo development, it induces axillary proliferation at low concentration (Huetteman and Preece 1993) although structurally TDZ is different from both auxins and purine-based cytokinins (Murthy et al. 1998).

6.2 Propagation In Vitro

Cultures in vitro (Fig. 6.1) contribute significantly to the small fruit crop development programs. In vitro propagation or micropropagation that includes plant formation from existing meristems and somatic cells has been utilized for propagation and as a part of the genetic manipulation in many small fruit crops. Although micropropagation has been successful in some small fruit crops, there are many species where in vitro methods need to be established for elite selections and to develop

Fig. 6.1 In vitro culture of blueberry on an agar-gelrite gelled medium (left) and in a bioreactor containing liquid medium (right)

genotype-independent routine procedures for increasing the propagation rates and to reduce the probability of somaclonal variation (Larkin and Scowcroft 1981).

Being genetically heterozygous, small fruit crops do not reproduce individuals from seed that are similar to the seed parent (Galletta and Himelrick 1990). Most of small fruit crop species are generally propagated vegetatively to maintain the desired genetic characteristics and to achieve rapidly a fruit-bearing condition. Although conventional vegetative propagation is successful in small fruit crops, the process is very time consuming. In vitro propagation is being used in various small fruit crops for year-round mass propagation of specific genotype and maintenance of pathogen-free (indexed) germplasm and used as the initial step in a nuclear stock crop production system. Shoot regeneration in vitro could accelerate cultivar development programs when used in combination with classical breeding. Successful application of plant tissue culture for shoot regeneration is crucial (Cao and Hammerschlag 2000), but the system can be used for genetic transformation followed by production of transgenic plants and to induce somaclonal variants. Complete plant formation using tissue culture techniques can be achieved either through shoot proliferation from pre-existing buds, through adventitious shoot regeneration, or through the formation of somatic embryos with a shoot meristem and a root (Steward et al. 1970).

Haberlandt (1902) explored plant cell culture in the early nineteenth century to study the concept of totipotency and to explore morphogenesis. He was successful to get survivability of in vitro-grown tissue. While Hannig (1904) was the first to observe plant cell division under in vitro condition, regeneration on callus tissue was first reported by Simon (1908). However, commercial micropropagation started with the work of Boxus (1974) and Anderson (1975) in strawberry and rhododendron, respectively. Since then, micropropagation with small fruit crops has been reviewed in literature by various authors (Debnath 2003a, 2006a, 2007a, 2011a, 2013, 2014a; Graham 2005; McCown and Zeldin 2005; Rowland and Hammerschlag 2005; Skirvin et al. 2005; Debnath et al. 2012).

6.3 Thidiazuron-Induced Micropropagation on Semisolid Gelled Media

6.3.1 Axillary Shoot Proliferation

Shoot tips or nodal segments can be surface sterilized and cultured on an agar or agar-gelrite solidified gelled medium containing TDZ for axillary bud production (Debnath 2005a). Plant propagation through axillary shoot proliferation is the most reliable method to produce true-to-type plants as they normally retain the genetic composition of the mother plant. A higher cytokinin concentration alone or with low levels of auxins is generally used to induce axillary budding. Cytokinins are used in culture media to overcome apical dominance and to enhance lateral bud formation from the leaf axis. More extra shoots are produced through further axillary bud growth during subculturing (Debnath 2003a). Different basal media supplemented with cytokinins such as TDZ, zeatin, 6-benzyladenine (BA), zeatin

riboside, or N6-[2 isopentenyl] adenine (2iP) and possibly some auxin can be used for small fruit micropropagation (Debnath 2006a). For axillary shoot proliferation of lingonberries (Debnath 2005a), nodal explants can be cultured on Debnath and McRae's (2001a) shoot proliferation medium containing low concentration of TDZ. A concentration of 0.1–1.0 μM TDZ was found effective for shoot proliferation of lingonberries (Debnath 2005a). Shoot proliferation of strawberry was found effective with 4 μM TDZ in a semisolid culture medium (Debnath 2005b).

Explant orientation on a TDZ-containing culture medium affects shoot proliferation. Lingonberry explants when placed horizontally on the culture medium responded by callus formation around the cut ends from day 6 to day 8 of culture, while vertical placement induced callus development at the basal end of the explants only. Changing the orientation of explants from vertically upright to horizontal improved the number of shoots per explant but reduced the number of leaves per shoot and shoot height (Debnath 2005a).

Lingonberry explants on cytokinin (TDZ)-free medium produced one unbranched shoot each, suggesting the presence of apical dominance (Debnath 2005a). Apical dominance is a major problem in micropropagation of some plant species (George and Sherrington 1984). Axillary branching in nodal explants occurs only when a cytokinin, e.g., TDZ, is applied exogenously in the culture media (Debnath 2005a). TDZ has an apical dominance release that accelerates shoot proliferation (Huetteman and Preece 1993).

The genotype often profoundly affects explant shoot proliferation performance in a medium containing TDZ (Debnath 2005a). Preece et al. (1991) observed differences in axillary shoot proliferation among woody plant species when cultured on a medium containing TDZ. Lingonberry genotypes belonging to two different subspecies differed in their shoot proliferation potential (Debnath 2005a). This might be due to the fact that the cells within the same plant can have dissimilar endogenous quantities of plant growth regulators (PGRs) and additional difference in receptor affinity or cellular sensitivity to PGRs (Minocha 1987). It is, therefore, expected that in vitro response will vary from genotype to genotype.

Although TDZ promotes callus development and at low concentration promotes shoot proliferation, it inhibits shoot elongation in lowbush blueberry (Kaldmäe et al. 2006) and lingonberry (Debnath 2005a). Since TDZ possesses very high cytokinin activity, it is possible that its inhibitory effect on shoot proliferation is consistent with its high cytokinin activity as shown in cranberry (Marcotrigiano et al. 1996). The inhibition of shoot elongation can take place due to the increase of endogenous cytokinins that hinders the action of cytokinin oxidase (Hare et al. 1994).

6.3.2 Adventitious Shoot Regeneration

Regeneration of adventitious shoots in vitro can be used not only in mass multiplication of difficult-to-propagate crop plant species but also in crop improvement to produce genetically engineered plants and somaclonal variants. In vitro shoot regeneration can be either through the development of unipolar organs (shoots or

roots), known as organogenesis, or of somatic embryos with a root and a shoot meristem (somatic embryogenesis) (Ammirato 1985). Plant regeneration from excised explants through organogenesis includes (i) development of adventitious bud from explants, (ii) elongation of buds to form rootable shoots, and (iii) rooting of the shoots to form plantlets (Qu et al. 2000). Factors like genotype; culture medium; type, concentration, and combination of growth regulators; physical environment; and explant development stage are important for shoot regeneration.

TDZ-induced shoot regeneration in vitro on a semisolid gelled medium has been reported in many small fruit crops including lingonberry (Debnath 2003b, 2005c), strawberry (Debnath 2005b, 2006b; Haddadi et al. 2013), ohelo and bilberry (Shibli and Smith 1996), blackberry (Vujović et al. 2010), and blueberry (Debnath 2009a). While TDZ alone was sufficient to regenerate shoots from strawberry sepal, leaves, and calyx (Debnath 2005b, 2006b), 2,4-dichlorophenoxyacetic acid (2,4-D) (Passey et al. 2003) or 1H-indole-3-butanoic acid (IBA) (Yonghua et al. 2005; Murti et al. 2012) in combination with TDZ was effective for shoot regeneration from strawberry leaves. Marcotrigiano et al. (1996) used TDZ in combination with α-naphthaleneacetic acid (NAA) for shoot regeneration from cranberry leaves but was not very successful as the shoot elongation was limited. Qu et al. (2000) developed a highly efficient shoot regeneration system from cranberry leaves on a basal medium consisting of Anderson's rhododendron salts (Anderson 1975) and Murashige and Skoog's (MS) organics (Murashige and Skoog 1962) with 10.0 μM TDZ and 5.0 μM N6-(g-g-dimethylallylamino) purine (2ip) in five cranberry cultivars. TDZ was found more effective than 6-benzylaminopurine (BAP) for inducing adventitious shoot regeneration from blackberry leaves (Vujović et al. 2010).

Debnath (2009a) developed a two-step procedure for adventitious shoot regeneration on an agar-gelrite gelled semisolid nutrient medium containing TDZ. Wild lowbush blueberry leaf segments were cultured on modified cranberry medium of Debnath and McRae (2001a) that contained three-quarter macro-salts and micro-salts of Debnath and McRae's (2001b) shoot proliferation medium D. The cultures were incubated in the dark at 20 ± 2 °C for 14 days and then exposed to light and maintained at 20 ± 2 °C with a 16-h photoperiod (PPF density at culture level was 30 $\mu molm^{-2}\ s^{-1}$). The TDZ concentration affected the frequency and growth of calli, buds, and shoots on leaf explants. A range of 2.3–4.5 μM of TDZ concentration on a semisolid gelled medium was found the most suitable range for shoot regeneration of wild lowbush blueberry clones (Debnath 2009a). In strawberry, TDZ at 2–4 μM induced adventitious meristem, bud, and shoot regeneration, but the formation of buds and shoots was completely stopped in a semisolid gelled medium with 8 μM TDZ (Debnath 2005b, 2006b).

Shoot regeneration on a TDZ-containing medium is influenced by a number of factors including genotype, TDZ concentration, and the polarity and orientation of the explants on the culture medium. The concentration of TDZ affects callus size and regeneration percentage, shoot number, and the vigor of regenerated shoots. In lowbush blueberry, the leaf explants produced less shoots but more callus on a nutrient medium with 4.5 μM than those treated with 2.3 μM of TDZ. Shoot vigor declined with the increase of TDZ concentration on the culture medium (Debnath

2009a). Vujović et al. (2010) reported the highest shoot regeneration rate from blackberry leaves on a medium containing 4.5 μM TDZ. Swartz et al. (1990) obtained shoot regeneration from *Rubus* leaves on a MS medium containing 10 μM thidiazuron.

Polarity of shoot regeneration can vary from genotype to genotype and can be upturned by PGR treatments (George 1993). In lowbush blueberries, TDZ was found to induce shoot formation on the whole leaf surface. However, more regeneration was observed on basal and medial segments of leaves than on apical segments (Debnath 2009a). This could be due to the fact that the distal portion of the leaf has less meristematic cells than those at the proximal portions. The effect of polarity on regeneration on a TDZ-containing medium was evident by more callus growth and higher number of buds and shoots formed from the apical than in the central and basal segments of lingonberry hypocotyl segments from seedlings (Debnath 2003b). Regenerative capacity increased substantially from the base toward the tip of the hypocotyl (Debnath 2003b). In strawberry, bud and shoot regeneration occurred on both sides of the sepals on a TDZ-containing medium (Debnath 2005b) as were observed in lingonberry (Debnath 2005c) and cranberry leaf cultures on a semisolid medium with TDZ (Marcotrigiano et al. 1996). However, shoot regeneration was on adaxial side of cranberry leaves on a medium with TDZ (Qu et al. 2000). Regeneration of lingonberry shoots from leaves was better when the adaxial side was in contact with the TDZ-containing medium (Debnath 2005c). Shoot regeneration was best when young expanding basal leaf segments of lowbush blueberry were placed with the adaxial side in contact with the culture medium supplemented with 2.3–4.5 μM TDZ and kept for 14 days in darkness (Debnath 2009a). TDZ induces shoot regeneration in various small fruit crops (Debnath 2003b, 2005b, c, 2007a, 2009a).

TDZ concentration required for the regeneration of adventitious shoots depends on genotype. A high concentration of TDZ (37.8–40.5 μM) in combination with 2.5–0.5 μM IBA was effective for strawberry shoot regeneration by Murti et al. (2012). However, excessive PGR concentration in culture media may cause somaclonal variation in micropropagated plants (Larkin and Scowcroft 1981).

6.3.3 Somatic Embryogenesis

Induction of somatic embryogenesis in blueberries has been reported recently by Ghosh et al. (2017) where callus formed from leaf segments after 4 weeks of culture on a semisolid gelled medium containing TDZ. Highest percentage (98%) of callus formation was observed in a hybrid blueberry obtained through crossing between highbush blueberry cvs. Chippewa and Patriot, at 4.5 μM of TDZ. Reports on plant regeneration via somatic embryogenesis are not available in *Vaccinium* species on gelled media, but it has been observed in the diploid (*Fragaria vesca* subspecies *vesca* "Hawaii 4") (Zhang et al. 2014) and octoploid strawberries (Donnoli et al. 2001; Biswas et al. 2007; Husaini and Abdin 2007; Husaini et al. 2008; Kordestani and Karami 2008). Strawberry shoot regeneration from leaf culture was noticed via

somatic embryogenesis or direct shoot regeneration based on the concentration of TDZ (Husaini and Abdin 2007). Strawberry leaf discs were cultured on a nutrient medium containing 4.0 mg l^{-1} TDZ and maintained at 10 ± 1 °C under darkness for 1 week followed by 3 weeks under 16-h photoperiod to get somatic embryos (Husaini et al. 2008). Initiation of strawberry somatic embryos was successful with dark (Donnoli et al. 2001; Husaini et al. 2011) and cold treatments (Husaini et al. 2011) of the culture. Nakajima and Matsuda (2003) reported somatic embryogenesis from filaments of eight grape cultivars using a combination of 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM TDZ or 10 μM 2,4-D and 10 μM TDZ. TDZ has been used to induce somatic embryo formation from filaments in grapevines (Nakajima and Matsuta, 2003; Oláh et al. 2003). Bouamama et al. (2007) used 11.35 μM of thidiazuron and 9 μM of 2,4-D for the induction as well as the development of somatic embryos in several grapevine cultivars, using anther culture.

6.3.4 Rooting and Acclimatization

Thidiazuron-induced small fruit microshoots can be rooted either under in vitro or ex vitro conditions (Qu et al. 2000; Debnath 2005a, b, 2009a). For rooting on a gelled medium, microshoots are excised and cultured onto an auxin-free medium (Qu et al. 2000). Ex vitro rooting of micropropagated shoots can be done in shredded sphagnum moss (Qu et al. 2000) or in a peat-perlite medium (Debnath 2003b, 2005a, c, 2009a). *Vaccinium* species can be rooted under ex vitro condition, while rooting in vitro is very common for strawberries and *Rubus* species (Debnath 2005b, 2006b, 2007b, 2010). Rootings ex vitro are rapid and less expensive, but in vitro rooting reduces disease contamination and environmental stress during rooting period (Pedroso et al. 1992).

Ex vitro rooting of *Vaccinium* microshoots can be done by treating the excised shoots by 39.4 mM IBA powder and planting them in a peat-perlite medium (Debnath 2009a). In vitro-derived strawberry shoots can be planted in a potting medium and maintained in a humidity chamber with a vaporizer at a temperature of 20 ± 2 °C, humidity 95%, PPF = 55 μmol m^{-2} s^{-1}, and 16-h photoperiod. Acclimatization of the plantlets can be done by gradually dropping the humidity over 2–3 weeks. Hardened-off plants can be transferred in a greenhouse and grown at 20 ± 2 °C, humidity 85%, maximum PPF = 90 μmol m^{-2} s^{-1}, and a 16-h photoperiod (Debnath 2005c).

Debnath (2006b) observed that TDZ, in a semisolid culture medium, strongly inhibited root formation of adventitious strawberry microshoot. Media with TDZ promoted more callus formation but suppressed shoot elongation and rooting of shoots. TDZ-induced strawberry shoots when proliferated in a medium containing 1 or 2 μM zeatin rooted well (Debnath 2006b). Lower cytokinin concentration may be required to form roots as the formation of roots is generally inhibited when the

cytokinin is adequately concentrated for the initiation of shoot proliferation (Gaspar and Coumans 1987). Endogenous cytokinins play a role in the formation of adventitious root (Bollmark et al. 1988). It is probable that TDZ is more efficient than zeatin to enhance endogenous cytokinin production which, in turn, might have prevented rooting (Bollmark et al. 1988). Induction of rooting for strawberry microshoots is possible without exogenous auxin. This might be due to the effect of exogenous auxins that can inhibit root growth (Scott 1972).

6.4 Bioreactor Micropropagation in Liquid Culture with Thidiazuron

Haberlandt (1902) was the first to use a liquid medium to culture isolated cells from bracts of *Lamium purpureum* in Knop's solution supplemented with sucrose (Preil 2005) although the cells did not divide. Later, Kohlenbach (1959) observed that differentiated mesophyll cells of *Macleaya* develop into cell clusters and calli forming organs and somatic embryos. Use of a liquid culture medium for in vitro propagation offers much more uniform culturing conditions than a semisolid medium (Debnath 2011a). Use of a gelled medium for propagating plants is difficult to automate and costly for large-scale production. Automated bioreactors that use liquid media are important for large-scale production of small fruit crops.

Bioreactors are designed for intensive culture and control over microenvironmental conditions (aeration, agitation, dissolved oxygen, etc.) (Paek et al. 2005). Liquid culture in a bioreactor system can cut down cost and labor in terms of agar, medium volume, and subculture periods (Sandal et al. 2001). Micropropagation can be done in bioreactors in agitated and non-agitated vessels (Ziv 2005). However, under submersed condition, a bioreactor system can limit the gas exchange to the culture that may lead to suffocation, hyperhydricity, and abnormal plantlet formation (Detrez et al. 1994) with chlorophyll-deficient glossy hyperhydrous leaves, cell hyperhydricity, hypolignification, reduced deposition of epicuticular waxes, and changes in enzyme activity and protein synthesis (Ziv 1991a, b). Temporary immersion bioreactors (TIBs) and use of growth retardants in culture media can be used to overcome some of these problems (Ziv et al. 2003). Cultures are alternately exposed to air and dipped into a liquid medium in a TIB system. Some of the other alternative procedures include putting a liquid medium on top of an established culture on agar and mist bioreactors and use of supports over stationary liquid media such as cellulose blocks, rafts, sponges, or paper bridges (Etienne and Berthouly 2002).

Bioreactor micropropagation in small fruit crop has not been used with many species but reviewed in literature (Debnath 2011a; Debnath et al. 2016). Some results that used TDZ-containing liquid media in *Fragaria*, *Rubus*, and *Vaccinium* species are presented below.

6.5 *Fragaria* (Strawberry)

The strawberry is enjoyed by millions of people across the world (Hancock et al. 1991) and is used fresh or in processed forms including jams, jellies, and frozen whole berries or sweetened juice extracts or flavorings. It is one of the most popular small fruit crops more extensively distributed than any other fruit crops (Childers 1980). The cultivated strawberry (*Fragaria* × *ananassa* Duch.) is a hybrid between *F. virginiana* Duch. and *F. chiloensis* (L.) Duch. Strawberries are a low-growing, dicotyledonous, perennial herb. They are grown in most arable regions of the world. Strawberries are a high source of vitamin C and fiber (Galletta and Bringhurst 1990).

Although strawberries can be propagated vegetatively by runner cuttings, a limited number of propagules are produced though this process and the product are not free of fungal diseases (Dijkstra 1993). Virus-free plants can be produced through micropropagation, and they can be saved under refrigeration (Mullin and Schlegel 1976). This makes it a reliable technique for germplasm storage.

Strawberry liquid culture with cell suspensions was started by Keßler et al. (1997) in bioreactors with different stirrer types. Adventitious shoot regeneration was successful in strawberry cultivars using a TIB bioreactor (RITA®) in a liquid MS medium supplemented with 9 μM TDZ and 2.5 μM IBA although regeneration frequency was not as good as on semisolid medium (Hanhineva et al. 2005). A combination of semisolid gelled medium and a liquid medium has been used by Debnath (2008a) where shoots were regenerated from leaf, sepal, or petiole explants of strawberries on a semisolid culture medium containing 2–4 μM TDZ (Debnath 2005b, 2006b), followed by culturing in the same liquid medium in a TIB bioreactor system with a 15-min immersion of explants every 4 h. Shoots can be proliferated and rooted in the bioreactor system with the same medium with 0.5–1 μM zeatin. In vitro-derived rooted shoots can be transferred planted on ProMix BX (Premier Horticulture Limited, Riviere-du-Loup, QC) potting medium and acclimatized following Debnath (2008a).

6.5.1 *Rubus* Species

The members of the genus *Rubus* (Tourn.) L. are called brambles that include raspberries, blackberries, and dewberries. They are distributed in both hemispheres except desert regions (Daubeny 1996). Ideobatus (raspberries) is the most important domesticated subgenera of genus *Rubus* containing around 200 species (Debnath 2011a, 2016a). The cloudberry (*R. chamaemorus* L., family Rosaceae) is a less known small fruit crop in *Rubus* species. This boreal circumpolar fruit species is a perennial, rhizomatous, and dioecious herb common to bogs. Cloudberries are rich in vitamin C and tannins and are used in traditional medicine to treat scurvy and diarrhea (Thiem 2003).

Bioreactor micropropagation using a liquid medium in *Rubus* species was first described by Debnath (2007b). Three cloudberry wild clones were cultured in an airlift bioreactor containing liquid medium with 0.45–2.3 lμ TDZ. A concentration

of 1.1–2.3 μM TDZ was needed for shoot proliferation. Liquid culture system enhanced the micropropagation efficiency of cloudberry clones; shoot number was almost double those on semisolid gelled medium for two cloudberry wild clones. A concentration of 1.1 μM TDZ was found to produce five to seven 4-cm-high shoots per nodal explant in a bioreactor containing a liquid medium in wild cloudberries (Debnath 2007b). However, TDZ induces hyperhydricity in liquid culture; 20–30% of the cloudberry shoots were hyperhydric after 8–10 weeks of culture in liquid medium (Debnath 2007b). Generally, hyperhydricity takes place in liquid media because of high water potential of leaves (Paek and Han 1989).This happens when a culture medium is rich in cytokinin (Gaspar 1991). Liquid culture-derived hyperhydric microshoots cannot root properly and give rise to malformed plants with poor survivability. In the reversible process, it was found that the quality of the cloudberry shoots could be improved by transferring them onto a gelled medium with 8.9 μM BAP and 5.8 μM gibberellic acid (GA_3) (Debnath 2007b).

Bioreactor micropropagation in a TDZ-containing liquid medium was also reported in red raspberry (*R. idaeus* L.) (Debnath 2010, 2014b). Shoot regeneration can be achieved from raspberry leaves in a liquid medium-containing bioreactor system combined with a semisolid gelled medium with 2.3–9.0 μM TDZ (Debnath 2014b). The polarity and orientation of red raspberry leaves and TDZ concentration played a significant role for callus and bud formation and for bud and shoot number per regenerating explants. Although regeneration was observed on the whole surface of the leaf, it increased markedly from the tip toward the base of the leaf. Regeneration was more in explants from basal segments (proximal ends) than the apical segments (distal regions) (Debnath 2010). As was in semisolid gelled media with small fruit crops, TDZ also inhibits shoot elongation in liquid media. In red raspberry, shoot inhibition can be improved by culturing in a BA-added medium. BA-induced elongated shoots can be rooted in the same liquid medium that contains no plant growth regulator (Debnath 2010, 2014b).

6.5.2 *Vaccinium* Species

The genus *Vaccinium* L., with about 400–500 species, is native to all continents except Antarctica and Australia (Vander Kloet 1988; Vander Kloet and Dickinson 2009). Genetically they are dicot and heterozygous angiosperms with small- to medium-sized fleshy edible fruits on woody perennial shrubs or vines. Although *Vaccinium* species includes blueberry, cranberry, lingonberry, bilberry, huckleberry, and whortleberry, the first three are commercially cultivated *Vaccinium* fruit crops.

Not many reports are available where bioreactor micropropagation has been used in *Vaccinium* species in a TDZ-supplemented liquid medium. A bioreactor system containing a liquid medium with TDZ combined with a semisolid gelled medium was used by Debnath (2011b) to propagate wild lowbush blueberries. Leaf segments were cultured on a semisolid gelled medium with 2.3 μM TDZ for 4 weeks followed by in liquid medium containing 1.2–2.3 μM TDZ for another 4 weeks. Leaf polarity and TDZ concentration had significant influence in callus formation

and shoot regeneration. Regeneration percentage was highest in the basal leaf segment followed by medial and apical leaf segments (Debnath 2011b) that collaborates the previous findings on a semisolid gelled medium (Debnath 2009a). Shoot regeneration took place on both sides of the leaves but was better when leaf segments were cultured with their adaxial surface in contact with the culture medium (Debnath 2011b).

6.6 Characteristics of Micropropagated Plants

Enhanced vegetative growth including increased branching and/or rhizome production is often observed in micropropagated small fruit crop plants (Debnath et al. 2012; Fig. 6.2). The effect of propagation methods on the morphological and biochemical properties of *Vaccinium* species was reported by various authors (Gustavsson and Stanys 2000; Debnath 2005d, 2006c, 2007c, 2008b; Foley and Debnath 2007; Debnath et al. 2012; Vyas et al. 2013; Goyali et al. 2015). In strawberry, Debnath (2009b) compared TDZ-induced regenerated strawberry tissue culture (TC) shoots that were elongated by treating with zeatin with those propagated by conventional runner cutting (RC) plants. TC plants produced more vegetative growth with more berries than those of RC plants. Berries produced by TC plants had also more anthocyanin contents and antioxidant activities than did RC plants (Debnath 2009b). This might be because the in vitro hormonal treatment (TDZ, zeatin) could have effects to increase crown, runner, leave, and berry number per plant (Debnath 2009b). However, increased vegetative growth and berry yield of TC plants over RC plants are genotype dependent, and all genotypes did not produce enhanced growth and berry yield in raspberries (Debnath 2014b). TC plants had higher berry yield and more and longer canes and more berries than root cutting plants in cultivar "Festival" but not in "Latham" indicating genotype-dependent juvenile branching characteristics of "Festival" TC plants but not in "Latham" TC plants (Debnath 2014b). Similar results with micropropagated strawberries were also reported by Dalman and Malata (1997) for overwintering. Increased resistance to frost damage was observed in micropropagated strawberries than the runner plants (Rancillac and Nourrisseau (1989).

Fig. 6.2 Greenhouse-grown root cutting (left) and tissue culture (right) raspberry plants

6.7 Clonal Fidelity and Molecular Analysis in Micropropagules

True-to-type propagules and clonal fidelity are prerequisites for commercial micropropagation. The use of in vitro propagation has concerns about genetic changes resulting from the process (Dale et al. 2008). Although production of true-to-type micropropagules is the main objective for mass propagation or conservation of a specific genotype, in vitro culture is also a tool to create new variation. In vitro culture-derived variation or somaclonal variation (Larkin and Scowcroft 1981) can broaden the genetic variation in small fruit crop plants resulting in a range of genetically stable variations useful in crop improvement (Jain 2001). Somaclonal variation can be genetic (heritable) and epigenetic (nonheritable). Somaclones were found to be regenerated from leaf culture (Popescu et al. 1997), from somatic embryogenesis (Donnoli et al. 2001), and from leaf and petiole cultures irradiated with gamma rays (Kaushal et al. 2004). Debnath (2017) reported somaclonal variations in strawberries for fruit yield under field condition. Two TC plants had higher berry yield than those of other tissue culture plants and the runner cutting mother plant. However it was not identified whether these variations were genetic or epigenetic (Debnath 2017). Somaclonal variation can be due to changes in the structure and number of chromosomes, sister chromatid exchanges, transposable element activation, DNA methylation pattern alteration and activation of hypervariable DNA regions, and point mutations including deletion, addition, or substitution of nucleotides and rearrangements in the nuclear and cytoplasmic genomes (Kaeppler et al. 1998). Factors like genotype, ploidy level, degree of departure from organized meristematic growth, explant type, donor plant age, types and concentrations of growth regulators used, auxin-cytokinin balance, duration of culture period, and number of subcultures are the possible causes for the origin of somaclonal variation (Henry et al. 1998).

There are many ways to monitor variation in micropropagated plants including evaluation at morphological, biochemical, physiological, and genetic levels. Vujović et al. (2010) used cytological, flow cytometry, and isozyme analyses to monitor somaclonal variation in blackberry regenerants. Chromosome counting in root tip meristems and flow cytometry analysis indicated identical ploidy level in all TC plants although the peroxidase patterns showed differences between some in vivo and micropropagated plants (Vujović et al. 2010).

DNA markers are independent of environmental influences (Weising et al. 1995) and can be a powerful tool for assessing clonal fidelity in micropropagated small fruit crops. Markers that are available for genetic analysis of tissue culture-raised plants include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), restriction fragment length polymorphism (RFLP), arbitrary primed polymerase chain reaction (AP-PCR), sequence characterized amplified region (SCAR), DNA amplification fingerprinting (DAF), simple sequence repeat (SSR), short tandem repeat (STR), sequence-tagged sites (STSs), expressed sequence tag-polymerase chain reaction (EST-PCR), and cleaved amplified polymorphic sequences (CAPS) derived from

EST-PCR markers (Debnath 2011a). While ISSR markers have been used to confirm trueness-to-type of bioreactor-derived micropropagated strawberries (Debnath 2009b), EST-PCR markers showed similar monomorphic amplification profiles in lowbush blueberry micropropagules (Debnath 2011b). SSR markers have been used for monitoring clonal fidelity in raspberry micropropagules (Debnath 2014b). Somaclonal variation is likely to be associated with regeneration of plants through unorganized callus formation (Piola et al. 1999). However, axillary buds can also produce variant plants (Soneji et al. 2002).

6.8 Conclusions

Small fruit crops are being propagated increasingly using tissue culture methods to multiply massive amounts of disease-free, genetically uniform plants. Axillary shoot proliferation is a very simple and reliable method to produce true-to-type micropropagules, and it is more preferred over adventitious shoot regeneration and somatic embryogenesis in small fruit crops. The latter two, however, are also powerful tools for rapid propagation of small fruit crops, provided clonal fidelity of the micropropagated plants is maintained. TDZ possesses both cytokinin- and auxin-like effects in in vitro culture (Mok et al. 1982; Visser et al. 1992) and can provide significant role in small fruit micropropagation. Compared to other cytokinins, TDZ at lower concentration induces proliferation of axillary shoots, while at higher concentration it promotes both axillary and adventitious shoot formation in small fruit crops (Debnath 2005a, b, c, 2008a). In vitro organogenesis was found to produce genetically uniform and true-to-type micropropagules in strawberry (Debnath 2009b), blueberry (Debnath 2011b), and raspberry (Debnath 2014b). Although bioreactor micropropagation is more cost-effective and ideal for automation, hyperhydricity including morphological and physiological disorders is common in liquid culture-derived micropropagules (Debnath 2011a). Optimization of TDZ concentration and culture conditions is needed for TDZ-induced bioreactor micropropagation in various small fruit crops.

Clonal fidelity is a major concern in small fruit micropropagation and can be monitored by DNA-based markers. Occurrence of variation during in vitro culture depends on factors like explant donor genotype, explant type, explant polarity and orientation on a culture medium, presence of chimeral tissue, media type, types and concentrations of plant growth regulators, culture duration, and cultural environment (temperature and light) (Debnath 2011a; Graham 2005). Micropropagated small fruit crops exhibit enhanced vegetative growth and can be used for rapid establishment and early fruit production. In vitro and molecular techniques are powerful tools, and combined with classical breeding, they can be used in small fruit improvement program (Debnath 2011a, 2016b; Fig. 6.3).

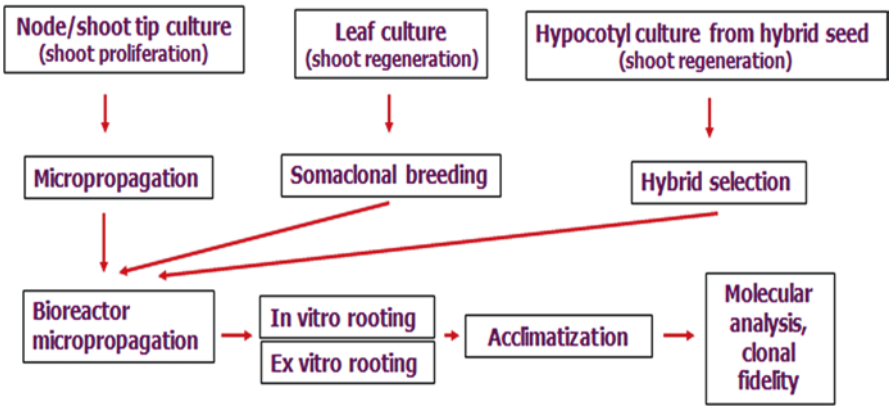

Fig. 6.3 Small fruit improvement program using in vitro and molecular techniques

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