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Strawberry Culture *In Vitro*: Applications in Genetic Transformation and Biotechnology

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ABSTRACT

The cultivated strawberry (*Fragaria* × *ananassa* Duch.), a member of the *Rosaceae*, is the most important soft fruit worldwide. *In vitro* techniques are important for clonal multiplication, germplasm improvement and for gene conservation of this flavourful and nutritious berry crop. The *in vitro* propagation of *Fragaria* species using axillary bud proliferation, adventitious shoot regeneration and somatic embryogenesis has been investigated in a number of previous studies. The morphogenesis seems to be highly dependent on plant growth regulators and media used for culture, which is again genotype specific. In strawberry, genetic transformation has been developed using tissue culture systems with varying rates of success. This review presents the progress in-depth of various aspects of strawberry culture *in vitro*, on gelled and in liquid media using bioreactors, for its improvement and for commercial production. It also discusses the issues that still need to be addressed to utilize the full potential of plant tissue culture techniques in mass propagation, *in vitro* selection, somaclonal variation, haploid recovery, somatic hybridization, genetic transformation and in cryopreservation of strawberries. Application of molecular marker techniques should be useful to verify the clonal fidelity of micropropagated strawberries. Strawberry improvement using *in vitro* and molecular techniques will develop improved cultivars suited to the changing needs of growers and consumers.

Keywords: micropropagation, regeneration, somaclonal variation

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** This work is the Atlantic Cool Climate Crop Research Centre contribution no. 193.

INTRODUCTION

The cultivated strawberry (*Fragaria* × *ananassa* Duch.), a hybrid between the Scarlet or Virginia strawberry (*F. virginiana* Duch.) and the pistillate South American *F. chiloensis* (L.) Duch., is a dicotyledonous, perennial low-growing herb grown in most arable regions of the World. There are about 20 recognized species of strawberries in five chromosome groups (x = 7): ten diploids, four tetraploids, one pentaploid, one hexaploid and four octoploids (Staudt 1999; Jiajun *et al*. 2005). The cultivated strawberry is an octoploid (2n = 8x = 56).

Flavourful and nutritious, strawberries are enjoyed by millions of people in all climates, including temperate, Mediterranean, sub-tropical and taiga zones (Hancock *et al*. 1991) and are predominantly used as fresh fruit. Their use in processed forms such as cooked and sweetened preserves, jams or jellies and frozen whole berries or sweetened juice extracts or flavorings, and their use in making a variety of other processed products made them one of the most popular berry crops, more widely distributed than any other fruit (Childers 1980). The berry is valued for its low-calorie carbohydrate and high fiber contents. Strawberries are good sources of natural antioxidants (Wang *et al*. 1996; Heinonen *et al*. 1998) including carotenoids, vitamins, phenols, flavonoids, dietary glutathionine, and endogenous metabolites (Larson 1988) and exhibit a high level of antioxidant capacity against free radical species: superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Wang and Jiao 2000). Meyers *et al*. (2003) showed that phenolics

Received: 5 February, 2007. Accepted: 2 April, 2007.

Special Feature

in strawberries account for a major portion of the total antioxidant activity of strawberries. Strawberry extracts were found to have higher antioxidant activity, as indicated by the oxygen radical absorbance capacity assay, than extracts from plum, orange, red grape, kiwifruit, pink grapefruit, white grape, banana, apple, tomato, pear and honeydew melon (Wang *et al*. 1996). Similarly, Sun *et al*. (2002), using a total antioxidant oxyradical scavenging assay (TOSC), found that strawberry extracts had higher antioxidant activity than extracts from peach, lemon, banana, pear, orange, grapefruit and pineapple. The benefits of these high antioxidant activity fruit include reduction of carcinogens in humans (Chung *et al*. 2002), protection against tumor development (Kresty *et al*. 2001) and reversal of age-related effects on memory (Bickford *et al*. 2000). Strawberry antioxidant activity levels are affected not only by the genotype but also by both growing temperatures (Wang and Zheng 2001) and cultural practices (Wang *et al*. 2002).

In vitro techniques are important tools for modern plant improvement programs to introduce new traits into selected plants, to multiply elite selections and to develop suitable cultivars in the minimum time (Taji *et al*. 2002). Used in conjunction with classical breeding methods, an efficient *in vitro* shoot proliferation and regeneration system could accelerate cultivar development programs. The ability to regenerate plants is crucial to the successful application of *in vitro* methods (Cao and Hammerschlag 2000). A shoot regeneration system can be used to develop transgenic plants following genetic transformation of plant cells and to identify and/or induce somaclonal variants.

MICROPROPAGATION

The totipotency exhibited by the apical meristem and the adjacent shoot tip region is the cornerstone for commercial micropropagation. Micropropagation of strawberry plants was introduced about thirty years ago (Boxus 1974). Immediately, the most important European nurseries producing several millions plants per year, were interested in this technique as it gave a definitive answer to the problems of soil fungi, causing a lot of damage to the strawberry fields and by another way, tissue culture plants seemed to produce more runners per mother plant in a short time (Mohan *et al*. 2005). Micropropagation has also been widely used in the USA (Zimmerman 1981) in commercial propagation of strawberries and in breeding programs to produce many plants rapidly. Conventionally, strawberries are vegetatively propagated by runners arising from axillary leaf buds on the plant crown. Plant propagation through runner produces a limited number of propagules. Although production of propagules through runner has been reported to contribute 90% of total Dutch strawberry production, the product in 'Elsanta' cultivar was found to be susceptible to several fungal diseases (Dijkstra 1993). Micropropagated strawberry plants can be stored under refrigeration (Mullin and Schlegel 1976), making it a valuable technique for storage of germplasm. Complete new plants can be derived from tissue either from pre-existing buds through shoot proliferation, following shoot morphogenesis through adventitious shoot regeneration or through the formation of somatic embryos. Micropropagation differs from all other conventional propagation methods in that aseptic conditions are essential to achieve success.

Shoot proliferation and virus elimination

Plants produced by axillary branching normally retain the genetic composition of the mother plant and this method has proven to be the most applied and reliable method for true-to-type *in vitro* propagation in general. Successful shoot proliferation has been obtained in strawberry from single meristems (Boxus 1974), meristem callus (Nishi and Oosawa 1973) and from node culture (Bhatt and Dhar 2000).

Morel (1960) was the pioneer for meristem culture. Meristem-tip culture alone or in combination with heat treatment (Yoshino and Hashimoto 1975) is widely used to obtain virus and fungus-free strawberry plants (Molot *et al*. 1972). Posnette (1953) originally developed the technique of hot air therapy to eliminate viruses, and Belkengren and Miller (1962) began the practice of excising heat-treated meristems for placement on tissue culture media. Potted plants are placed in a growth chamber at ambient temperatures and then this temperature is raised a few degrees a day up to 38°C and grown for 6 weeks (Lines *et al*. 2006). Shoot tips are removed after treatment and the meristem, a dome of actively dividing cells, about 0.1 mm in diameter and 0.25 mm long, with two or three leaf primordia are removed and cultured on a nutrient medium. Most commonly used explant for strawberry micropropagation is the meristem from the tip of runners (Sowik *et al*. 2001). The explant is placed on a medium containing no or low levels of auxins and higher levels of cytokinins to promote axillary budding while preventing callus formation. The cytokinins are used to overcome apical dominance and enhance the branching of lateral buds from the leaf axis. Additional shoots are produced through further axillary bud growth (Debnath 2003). Mullin *et al*. (1974) grew strawberries with strawberry mild yellow edge (SMYE) viruses for 6 weeks in a 36°C growth chamber before excising 0.3 to 0.8 mm meristematic tips with leaf primordia. The result was that 33% to 75% of the resulting plants were SMYE-free according to leaf insert graft indexing to indicator strawberry plants. In fact, Mullin *et al*. (1974) maintained strawberries that had received heat therapy and were propagated using apical meristematic regions, free from detectable graft transmissible diseases for seven years in a greenhouse and a screenhouse.

Cultures can be initiated and maintained on Boxus (Boxus 1974) medium containing Knop's (Knop 1965) macronutrients and Murashige and Skoog (1962, MS) micronutrients and organic components, or MS medium supplemented with 2.2-4.4 µM 6-benzyladenine (BA), 0.5-2.5 µM indole-3-butyric acid (IBA) and 0.3 µM gibberellic acid (GA_3) (Cerović and Ružić 1989) at 23-25°C during the light period, and 17°C in the dark; the quantum irradiance is 46 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod (Sowik *et al*. 2001). Runners can be initiated from *in vitro* culture on media containing $AgNO_3$ (10-20 mg/l). GA_3 (28.9-57.7 µM) increased the efficiency of $AgNO_3$ significantly (Zatykó *et al*. 1989). Borkowska (2001) initiated strawberry cultures according to Boxus (1974, 1992), but modified medium by lowering the concentration of cytokinin (BA, 2.2 µM) and auxin (IBA, 0.5 µM). Although agar (0.6-0.8%, w/v) is the most commonly used gelling agent for *in vitro* strawberry culture on semi-solid medium, Lucyszyn *et al*. (2006) reported that the agar/galactomannan (gour, Indian Gum Industries, Jodhpur, India) mixture in the proportion of 0.3/0.3 (w/v) in MS medium showed better performance and enhanced shoot proliferation compared to medium containing agar (0.6%, w/v) only. Cultures were maintained at 23°C under a photosynthetic photon flux density (PPFD) of 30 µmol $m^{-2}s^{-1}$ from'warm-white' fluorescent lamps and 16 h photoperiod. The use of light-emitting diodes, or LEDs, in particular 70% red and 30% blue at 60 µmol $m^{-2}s^{-1}$ resulted in greatest shoot proliferation, plantlet and total fresh weight when three-leaved explants of cv. 'Akihime' derived from conventional mixotrophic cultures were placed in a photoautotrophic culture vessel, the "Culture Pack", using rockwool as a substrate, 0.2. mg/l BA in MS basal medium and with CO_2-enrichment (3000 ppm) (Nhut *et al*. 2000, 2003). Plantlets from the same treatment also resulted in 100% acclimatization. Similar results could be obtained in a much more simplified system using conventional 3.6 l polyethylene vessels, in which one culture vessel was estimated to cost less than 1 $US, and in which up to 150-200 strawberry plantlets could be developed and rooted from three-leaved (rootless) explants (Nhut *et al*. 2006). In this system the authors found that the use of Micropore^{TM} 3M, added at 4 sheets (approx 2 cm^2) per box,

would increase the ventilation to the vessels and enhance the development of shoots and their subsequent acclimatization.

Bhatt and Dhar (2000) micropropagated wild strawberry (*F. indica* Andr.) using node culture. Nodal segments (2.5-3 cm), treated with Savlon (an antiseptic containing 3% antimicrobial agent centrimide + detergent) for 15 min, were immersed in 80% ethanol for 30 sec prior to surface sterilization in $HgCl_2$ (0.05%) containing few drops of Tween 20 for 5 min and then cultured on MS medium supplemented with 4 µM BA and 0.1 µM α-naphthalene acetic acid (NAA). The same medium was used for shoot multiplication.

Adventitious shoot regeneration

There have been a number of reports for adventitious bud and shoot regeneration from leaves (Nehra *et al*. 1988; Jones *et al*. 1988; Lui and Sandford 1988; Nehra *et al*. 1989, Sorvari *et al*. 1993; Passey *et al* 2003; Debnath 2005; Qin *et al*. 2005a, 2005b; Yonghua *et al*. 2005; Debnath 2006), petioles (Foucault and Letouze 1987; Rugini and Orlando 1992; Passey *et al*. 2003; Debnath 2005, 2006), peduncles/peduncular base of the flower bud (Foucault and Letouze 1987; Lis 1993), stems (Graham *et al*. 1995), stipules (Rugini and Orlando 1992; Passey *et al*. 2003), stolons (Lis 1993), roots (Rugini and Orlando 1992; Passey *et al*. 2003), runners (Liu and Sanford 1988), mesophyll protoplasts (Nyman and Wallin 1988), anther cultures (Owen and Miller 1996) and from immature embryos (Wang *et al*. 1984) of strawberries. Shoot regeneration directly from field- (Nehra *et al*. 1989) or greenhouse-grown strawberry plants (Barceló *et al*. 1998; Debnath 2005, 2006) or from *in vitro*-grown shoots (Sovari *et al*. 1993; Passey *et al*. 2003; Yonghua *et al*. 2005) have been reported. Explants taken from field-grown plants are difficult to sterilize to establish *in vitro* cultures due to high degree of contamination. It is usually recommended to take explants from plants grown under controlled conditions such as growth room or greenhouse, or from buds which flush from dormant shoots stored indoors.

Plant regeneration is a crucial aspect of plant biotechnology methodology and tissue culture that facilitates the production of genetically engineered plants and somaclonal variants, and the rapid multiplication of difficult-to-propagate species. Adventitious plant regeneration from strawberry explants can be divided into the following steps:
(i) formation of viable adventitious buds on the explant;
(ii) elongation of the buds into shoots;
(iii) rooting of the shoots to form whole plants.

A number of factors such as genotype, culture medium (including growth regulators and their combinations) (**Table 1**), physical environment, explant development stage, etc. affect adventitious shoot regeneration.

Thidiazuron (TDZ), a the substituted phenylurea (*N*-phenyl-*N'*-1,2,3-thidiazol-5-ylurea) with its cytokinin- and auxin-like effects, is now among the most active cytokinin-like substances for plant tissue culture and has been used to induce shoot organogenesis of strawberries (**Table 1**). TDZ alone (Debnath 2005) or in combination with 2,4-dichlorophenoxy-acetic acid (2,4-D) (Passey *et al*. 2003) or IBA (Yonghua *et al*. 2005) was found to be effective for shoot regeneration from strawberry leaves. While leaf explants were used in most of the early studies for bud and shoot regeneration, sepals (**Table 1**), a floral leaf or individual segment of the calyx of a flower that forms the outer protective layer in a bud, have been tested for shoot regeneration of *in vitro* strawberry cultures (Debnath 2005). Shoot regeneration was obtained from sepal, leaf and petiole explants by incorporating TDZ (2-4 µM) in the culture medium and a dark treatment for 14 d before incubating the explants under a 16-h photoperiod. A dark treatment similar to those used for strawberry (Barceló *et al*. 1998) leaf generation was used to achieve the highest response. Such TDZ-induced shoots were transferred to 2-4 µM zeatin-containing medium for elongation (Debnath 2005). Callus regeneration and shoot formation depended not only on the explant orientation and polarity, but also on genotype (Passey *et al*. 2003). Young expanding sepals with the adaxial side touching the culture medium produced the best results. Qin *et al*. (2005b) reported that 'Toyonoka' strawberry leaf explants cultured for 10 days in shoot regeneration medium in the presence of $AgNO_3$ not only enhanced shoot regeneration efficiency but also expedited the inhibition of adventitious buds. Being an ethylene inhibitor, $AgNO_3$ can markedly promote organogenesis in strawberries.

Protoplast culture and somatic hybridization

The plant protoplast, a "naked" cell, consisting of the cytoplasm and nucleus with the cell wall removed, provides a unique single cell system to underpin several aspects of

Table 1 Examples of basal media[1] and plant growth regulators (PGR, mg/l)[2] used for adventitious shoot regeneration of strawberries *in vitro*.

Cultivar	Explant source	Basal medium (mg/l)	Shoot regeneration	Reference
'Redcoat', 'Veestar', 'Bounty', 'Kent', 'Micmac', 'Glooscap', 'Honeoye', 'Hecker', 'Fern', 'Selva'	Leaf disk	MS salts + B-5 vitamins	2.3 BA + 1.8 IAA	Nehra *et al.* 1989
'Hiku', 'Jonsok'	Leaf disk	MS salts + 39 Fe(III)Na-EDTA + 2000 KNO_3 + 400-600 CH	3 BA + 0.1 IBA	Sorvari *et al.* 1993
'Chandler'	Leaf disk	$N_{30}K$ macrosalts + MS microsalts and vitamins	2 BA + 0.5 IBA	Barceló *et al.* 1998
'Calypso', 'Pegasus', 'Bolero', 'Tango', 'Elsanta'	Leaf disk, petiole, root, stipule	MS	1 TDZ + 0.2 2.4-D, 2 BA + 0.5 TDZ + 0.2 2,4-D, 1 TDZ + 0.2 NAA or 2 BA + 0.2 2,4-D	Passey *et al.* 2003
'Sweet Charlie', 'Pajaro'	Leaf disk	½MS	1 BA + 1 IAA	Singh and Pandey 2004
'Senga Sengana'	Leaf petiole	Boxus (1974)	0.5 BA + 0.1 GA_3 + 0.1 IBA	Litwińczuk 2004
'Hecker', 'La Sans Rivale', diploid accessions (FRA197, FRA198)	Leaf, petiole	MS salts + B5 vitamins	2.2 TDZ + 0.3 IBA	Zhao *et al.* 2004
'Bounty'	Sepal, leaf disk, petiole half	BM-D	0.44-0.88 TDZ	Debnath 2005
'Toyonoka'	Leaf disk	MS	1.5 TDZ + 0.4 IBA	Qin *et al.* 2005a
'Toyonoka'	Leaf disk	MS + 1 $AgNO_3$	1.5 TDZ + 0.4 IBA	Qin *et al.* 2005b
'Bounty', 'Jonsok', 'Korona', 'Polka', 'Zephyr'	Leaf	MS	2 TDZ + 0.5 IBA	Hanhineva *et al.* 2005
'LF9'	Leaf, petiole, stolon	MS	0.11 BA + 0.011 2,4-D + 1 TDZ	Folta *et al*. 2006

[1] Media: BM-D = Debnath and McRae (2001); MS = Murashige and Skoog (1962); $N_{30}K$ = Margara (1984).
[2] PGR: 2,4-D = 2,4-dichlorophenoxy-acetic acid, BA = 6-benzyladenine, GA_3 = gibberellic acid, IBA = indole-3-butyric acid, IAA = 3-indolyl-acetic acid, NAA = α-naphthalene acetic acid, TDZ = thidiazuron.

modern biotechnology (Davey *et al*. 2005). Although reliable procedures are available to isolate and culture protoplasts from a range of plants (Davey *et al*. 2005), there have been few reports in strawberry. Fong *et al*. (1889) had limited success for protoplast isolation but failed to culture them. The need for protoplast technology for gene transfer, somatic hybridization and for somaclonal variation (Larkin and Scowcroft 1981) in strawberry breeding has been indicated by several authors (Wallin *et al*. 1993). Leaf- and petiole-derived protoplasts (Nyman and Wallin 1988, 1992) and protoplasts from callus (Wallin 1997) have been isolated and cultured with a 3.3 × 106 protoplasts/ g fresh weight of viable protoplasts yield (Nyman and Wallin 1992). Several parameters, particularly the source tissue, culture medium and environmental factors, influence the ability of protoplasts and protoplast-derived cells to express their totipotency and to develop into fertile plants. Protoplasts can be isolated mechanically by cutting or breaking the cell wall, and by digesting it away with enzymes or by a combination of mechanical and enzymatic separation (George 1993). Isolated strawberry protoplasts can be cultured on 8P medium (Glimelius *et al*. 1986) with either 1 mg/l 2,4-D and 0.5 mg/l BA (Nyman and Wallin 1992) or 2 mg/l NAA and 0.5 mg/l TDZ (Wallin *et al*. 1993). Shoot organogenesis is induced on MS medium with 2% sucrose and 2 mg/l BA and 0.2 mg/l NAA. Measurement of the DNA content of these plants has revealed a range of ploidy levels. Infante and Rosati (1993) isolated protoplasts and regenerated plants in wood strawberry (*F. vesca*). Plants grew normally in the greenhouse and did not show any visible abnormality compared to the original clone multiplied through micropropagation.

Protoplasts may be fused together to create plant cell hybrids. Isolated protoplasts do not normally fuse together because they carry a superficial negative charge causing them to repeal one another. The two most successful techniques of protoplast fusion are (i) the addition of polyethylene glycol (PEG) in the presence of a high concentration of calcium ions and a pH between 8-10 and (ii) the application of short pulses of direct electrical current (electro-fusion). Somatic hybridization offers the possibility of genetic exchange between the diploid *F. vesca* with cultivated octoploid strawberry. Wallin (1997) regenerated plants from calli originating from protoplast fusion between *F.* × *ananassa* and *F. vesca*. Protoplasts of *F.* × *ananassa* resistant to hygromycin were also fused to protoplasts of *F.* × *ananassa* resistant to kanamycin. Protoplast fusion-derived plants that deviated morphologically from the parents had >56 chromosomes which might be due to somaclonal variation (Wallin 1997).

Anther culture and haploid recovery

Anther culture involves the aseptic culture of immature anthers to generate fertile haploid plants from microspores. The production of haploid plants through anther culture is widely used for breeding purposes, as an alternative to the numerous cycles of inbreeding or backcrossing usually needed to obtain pure lines in conventional breeding. Chromosome doubling of haploids could result in immediate establishment of homozygosity for all loci. The success achieved with anther culture has led to the development of microspore culture to regenerate homozygous plants. Furthermore, isolated microspores are very attractive for protoplast isolation and applications aiming at transformation as they are unicellular and transgenic homozygous plants could be provided in a comparatively short time (Dhlamini *et al*. 2005; Germanà 2006).

Haploid recovery in strawberry through aseptic anther culture was unsuccessful in some early reports (Rosati *et al*. 1975), Niemirowicz-Szczytt *et al*. 1983). Owen and Miller (1996) obtained haploid plants from cultured anthers of 'Chandler', 'Honeoye' and 'Redchief' strawberries. The highest shoot regeneration across cultivars (8%) was obtained when a semi-solid MS medium contained 2 mg/l IAA, 1 mg/l BA and 0.2 M glucose. Chromosome counts of root tip cells from *ex vitro*-grown plants confirmed that haploid plants were obtained from all three cultivars.

Rooting and acclimatization

Both *in vitro* and *ex vitro* methods have successfully been used to root and acclimatize micropropagated strawberry shoots. Proliferated shoots can be rooted *in vitro* on Boxus (Borkowska 2001; Sowik *et al*. 2001), half-strength MS (Yue *et al*. 1993) or modified cranberry (Debnath 2005) medium without growth regulators, or on half-strength MS with activated charcoal (0.6 g/l) and IAA (5.7 µM) (Moore *et al*. 1991). The *in vitro*-formed roots are thick, posses no hairy roots, grow horizontally, and are fragile and easily damaged. *In vitro*-grown plantlets have low photosynthetic activity, poor water balance and their anatomy and morphology are far from being optimal (Grout and Millam 1995; Borkowska 2001). Kozai (1991) and Hayashi *et al*. (1997) developed a photoautotrophic micropropagation system for plant multiplication by simultaneously increasing the CO_2 concentration and light quality. Plantlets that are rooted *ex vitro* have a larger root system and more runners than those formed by *in vitro*-rooted strawberry plants (Borkowska 2001). Similar results were observed by Nhut *et al*. (2000, 2003, 2006) when using either CO_2-enrichment with or without light-emitting diodes, or even when a simple improved aeration system was implemented in mixotrophic culture.

When moved from *in vitro* culture conditions, microcuttings must be acclimatized gradually to ambient conditions to avoid mortality that might otherwise occur under an abrupt change in relative humidity, temperature or irradiance. For *in vitro*-rooted plantlets, standard procedure is to wash the plantlets and transfer to pots containing ProMix BX (Premier Horticulture Limited, Riviére-du-Loup, Québec, Canada) (Debnath 2005, 2006) or 1 peat : 1 vermiculite (Zhou *et al*. 2005), and maintained in a humidity chamber and acclimatized by gradually lowering the humidity over 2-3 weeks (temperature 20 ± 2°C, humidity 95%, PPF = 55 µmol m^{-2} s^{-1} , 16 h photoperiod). Hardened-off plants can be maintained in the greenhouse (temperature 20 ± 2°C, humidity 85%, maximum PPF = 90 µmol m^{-2} s^{-1}, 16 h photoperiod) (Debnath 2005, 2006).

Rooting can be induced *ex vitro* with complete success (Hayashi *et al*. 1997; Borkowska *et al*. 1999; Borkowska 2001) by transferring microshoots directly in rockwool (Borkowska *et al*. 1999). After one month, the plantlets rooted *ex vitro* are planted in 1 peat : 1 rockwool (v/v) and grown in the greenhouse for acclimatization (Borkowska 2001). *In vitro*-derived strawberry shoots were rooted *ex vitro* in granulated, water-repelling and water absorbing mineral wool (Grodan) mixed in a proportion of 1:1 (v/v) by Sowik *et al*. (2003). Fertilizer (10% N, 52% P, 10% K) are applied daily at the rate of 250 mg/l during the first week, followed by 800 mg/l during the next three weeks with alternating every second day. Rooting is done at 21-23°C under a PPFD of 75 µmol $m^{-2}s^{-1}$. The rooted shoots are planted in sand, soil substrate and leaf compost (0.3:5:1.3, v/v) medium (Sowik *et al*. 2003).

Debnath (2006) developed a protocol that enables strawberry micropropation in one step, i.e. multiplying shoots and having them rooted in the same culture medium. The use of microcuttings, giving both root and shoot growth in a medium containing cytokinin, is emerging as a better choice for micropropagation of strawberries than multiple shoot proliferation, (using a cytokinin supplemented medium) with subsequent rooting of shootlets. *In vitro*-derived strawberry shoots can be proliferated, elongated and rooted on zeatin-containing medium. Zeatin alone at very low levels (1-2 µM) produced two to three shoots per explant, averaging 88% rooting incidence in a single medium in 'Bounty' strawberry. Furthermore, the protocol did not use auxin in the culture medium, which lowers the cost and reduces the probability of somaclonal variation among

the proliferated plants. The main advantage of this protocol is that all the shoot tips of the *in vitro*-grown plantlets can be used for shoot proliferation and rooting, whereas basal rooted nodal segments can be transferred to the potting medium and acclimatized in the greenhouse. The protocol can eliminate stage II of micropropagation and can increase both multiplication rate and rooting rate; this translates into a faster micropropagation of strawberry. The technique is now routinely used at the Atlantic Cool Climate Crop Research Centre in St. John's, NL, Canada.

Somatic embryogenesis

Somatic embryogenesis involves the development of bipolar embryos from embryogenically-competent somatic cells *in vitro*. In contrast to organogenesis, where microshoots and roots develop on different media, somatic embryogenesis is apparently a one-step procedure involving the development of embryos having both a shoot and a root pole, as in the zygotic embryos. The initiation and development of embryos from somatic tissues was first observed by Steward *et al*. (1958) and Reinert (1958) in cultures of carrot tissues. Wang *et al*. (1984) reported somatic embryogenesis from strawberry cotyledons on MS medium supplemented with 22.6 µM 2,4-D, 2.2 µM BA and 500 mg/l casein hydrolysate where few of the embryogenic tissues developed into somatic embryos. Morphologically normal plants were obtained from somatic embryos that were transferred to MS medium containing 2.89 µM GA_3 or 2.22 µM BA + 0.54 µM NAA. Maintenance of the embryogenic cultures was, however, unsuccessful. Donnoli *et al*. (2001) reported somatic embryogenesis in 8% of the embryogenic calli in strawberry cultivar 'Clea' on MS medium supplemented with 4.88 µM BA and 4.90 µM IBA. Somatic embryogenesis research with strawberries is still in a preliminary stage and some more efforts would be required to develop the technology (Graham 2005).

Bioreactor micropropagation

Automated bioreactors for large scale production of tissue culture plants are important for commercial success of the micropropagation industry. Bioreactors are self-contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and outflow systems, designed for intensive culture and control over microenvironmental conditions (aeration, agitation, dissolved oxygen, etc.) (Paek *et al*. 2005). The use of large-scale liquid cultures and automation has the potential to resolve the manual handling of the various stages of micropropagation and decreases production cost significantly. Bioreactor systems have been introduced for mass propagation of horticultural plants (Levin and Vasil 1989) and have proven their potential for large-scale micropropagation. Different types of bioreactors developed for optimal mixing of oxygen, nutrients and culture without severe shear stress are generally two types: (i) mechanically agitated bioreactors and (ii) pneumatically agitated and non-agitated bioreactors (Paek and Chakrabarty 2003).

Culture in liquid medium is advantageous for several plant species but often causes asphyxia and hyperhydricity, resulting in malformed plants and loss of material. The malformations are manifested in glossy hyperhydrous leaves with distorted anatomy. To overcome these problems, two major solutions for malformation control includes: use of growth retardants to control rapid proliferation and temporary immersion bioreactors (TIB, Ziv *et al*. 2003) in which the explants are alternately exposed to liquid cultivation medium and air. To avoid cell clumping in cell suspension cultures of cell line FAR (*Fragaria ananassa* R), Edahiro and Seki (2006) suggested the inclusion of 0.1 mM L-α-aminooxy-β-phenylpropionic acid, or AOPP, a specific inhibitor of phenylalanine ammonia lyase – the starting and key enzyme of the phenylpropanoid pathway.

Limited reports are available on *in vitro* bioreactor strawberry culture (Takayama and Akita 1998). Hanhineva *et al*. (2005) reported shoot regeneration from leaf explants of five strawberry cultivars in a commercially available TIB bioreactors (RITA®, VITROPIC, Saint-Mathieu-de-Tréviers, France) containing liquid MS medium with 9 µM TDZ and 2.5 µM IBA (**Table 1**). The TIB system proved to be well suited for shoot propagation and for subsequent subculture of the developing plantlets. Regeneration frequencies were 70 ± 8 to 94 ± 2% and 83 ± 5 to 92 ± 3% in the TIB system and on semi-solid medium, respectively. The labour time taken by the TIB system was less than half of the time required for handling plant material for cultivation on semi-solid medium.

Field evaluation of micropropagated plants

Increased branching and vigorous vegetative growth are often noted in plants produced through *in vitro* culture. Differences in performance of tissue-cultured and conventionally propagated plants have been investigated for strawberry (Swartz *et al*. 1981; Boxus *et al*. 1984; Cameron *et al*. 1989; Cameron and Hancock 1986; López-Arand *et al*. 1994; Szczygiel and Borkowska 1997; Szczygiel *et al*. 2002). Tissue culture-derived strawberry plants grow more vigorously producing more crowns and runners and increased petiole length, yield per area and number of inflorescences per crown than conventionally propagated plants (Swartz *et al*. 1981; Boxus *et al*. 1984; Cameron *et al*. 1989; López-Arand *et al*. 1994). Zebrowska *et al*. (2003) reported that in comparison to vegetatively propagated plants, 'Teresa' strawberry microplants produced more leaves, runners and inflorescences. Also, yielding and resistance to leaf scorch of these plants were much higher. Litwińczuk (2004) compared strawberry plants of cv. 'Senga Sengana' obtained *in vitro* from axillary and adventitious shoots with their runner progeny and with standard runner (control) plants under field conditions. In the planting year, *in vitro* obtained plants developed significantly more crowns and runners while compared to other groups. Such differences, especially in runners' number were not observed in the next two years. In the planting year, all *in vitro* propagated plants and about 80% their runner progeny flowered contrary to control (the only 3% plants). Every year tissue culture plants developed significantly more inflorescences than other studied groups. Plants obtained *in vitro* produced bigger fruits and higher yield than other groups in the first two years. However, a reduction of berry yield for tissue culture plants in contrast with control was observed in third year only. The primary effects, increased vigour and axillary bud activity, are possibly related to the forced proliferation *in vitro* through hormonally induced crown branching (Swartz *et al*. 1981). Micropropagated strawberry 'Gorella' showed higher resistance to frost damage than did standard runner plants, when injury was evaluated in the field in spring (Rancillac and Nourrisseau 1989). Similarly, Dalman and Malata (1997) found that micropropagated 'Senga Sengana' strawberry plants overwintered better than did the plants produced from runners, although for 'Mari' the opposite was observed, and for 'Jonsok' no differences between the two types of plants occurred.

Palonen and Lindén (2001) compared cold hardiness and overwintering of three types of strawberry plants of cultivars 'Senga Sengana' and 'Jonsok': (i) micropropagated virus-free elite plants, (ii) certified plants (runner plants from elite plants) and (iii) ordinary plants (runner plants of conventionally propagated plants from a strawberry farm). No consistent differences in cold hardiness among the three types of plants were detected during winter. Field evaluation did not reveal any differences in their winter survival, either. Micropropagated plants flowered more freely than did the plants produced through runners.

SOMACLONAL VARIATION

Clonal fidelity is one of the main concerns in commercial micropropagation, true-to-type propagules and genetic stability are prerequisites for the application of strawberry propagation *in vitro*. The occurrence of variation in plants regenerated from *in vitro* cultures was named as 'somaclonal variation' by Larkin and Scowcroft (1981) and has been reported for morphological and yield variation in micropropagated strawberries (Graham 2005). There are concerns about genetic changes resulting from strawberry micropropagation. Discrete morphological variants have been observed in micropropagated strawberry plants, e.g., leaf variegation consisting of a narrow white streak in the leaf blade (Swartz *et al*. 1981), chlorosis of the leaves (Swartz *et al*. 1981), and growth changes including dwarfs, compact trusses, lack of runner production, and female sterility (Swartz *et al*. 1981). Moore *et al*. (1991) observed variability among micropropagated subclones of 'Olympus' which were most likely transient responses to the micropropagation environment, not genetic. Generally, micropropagated plants have greater vigor, runner production, and yields than runner-propagated plants (Swartz *et al*. 1981). However, not all cultivars exhibit a yield increase (Cameron *et al*. 1985).

Genetic stability during micropropagation is controlled by numerous factors including genotype, presence of chimeral tissue, explant type and origin, media type, types and concentrations of growth regulators, culture conditions (temperature, light, etc.) and duration of culture (Graham 2005). Neither somatic embryogenesis nor shoot organogenesis is widely used in commercial strawberry micropropagation as adventitiously regenerated plants may give rise to somaclonal variation. Somaclonal variations can be distinguished by their morphological, biochemical, physiological and genetic characteristics. Molecular markers are powerful tools in genetic identification of somaclonal variation with greater precision and less effort than phenotypic and karyologic analysis.

Although somaclonal variation is not desirable for commer-cial micropropagation, it is a valuable tool in plant breeding wherein variation in tissue culture-regenerated plants from somatic cells can be used in the development of crops with novel traits. By applying selection pressure during tissue culture it is possible to develop somaclones resistance to biotic and abiotic stress (Jain 2001). Somaclonal variation has been associated with changes in chromosome number and structure, deamplification and amplification of genes, transposable element activation and alteration in DNA methylation (Phillips *et al*. 1994). Strawberry somaclonal variatnts have been produced through *in vitro* culture and selection. Although phenotypic variants might occur, even among plants regenerated from meristem (Sansavini *et al*. 1989), it is generally emphasized that genetic variation is associated with regeneration from callus culture (Popescu *et al*. 1997). Popescu *et al*. (1997) observed useful variation in plant and fruit characteristics in strawberry plants regenerated from leaf and petiole-derived callus. Both genotype and type of explant strongly influence the occurrence of somaclonal variation. A variant having a modified (white) colour of flesh for all fruits was induced from petiole-derived callus of cv. 'Gorella' (Popescu *et al*. 1997). Some regenerants evidence reduced susceptibility to soil borne fungi causing plant wilting (Battistini and Rosati 1991; Toyoda *et al*. 1991). Variants for earliness, calyx separation, mildew susceptibility and ploidy level were also found (Simon *et al*. 1987).

Sansavini *et al*. (1989) reported the chlorophyll-mutant white stripe, chlorosis and dwarfism, the variants most commonly associated with micropropagated strawberry. The seven cultivars tested showed differential susceptibility to these alterations and these somaclonal variations were easily transmitted to the runners but declined markedly over the growing season. Transmission also occurred sexually, the symptoms themselves being more marked when the mother plant was affected. In selfing, S_1 offspring had a 26.7% incidence of white stripe, 60% of dwarfism and semidwarf and 66.7% of chlorosis. In crossing of affected and normal plants, white stripe affected only 15.4% and dwarfism 56.3% of F_1 seedlings (Sansavini *et al*. 1989). Leaf chlorosis, white streak and dwarfism were also noted in 'Pajoro' strawberry somaclones regenerated from anther culture (Faedi *et al*. 1993).

Variations in callus and cell suspension growth rates and in isoenzyme patterns of acid phosphatase, peroxidase and glutamate dehydrogenase among the regenerants of strawberry cultivars were reported by Damiano *et al*. (1995). Brandizzi *et al*. (2001) found variation in DNA content among strawberry regenerants from callus cultures. These variations were, however, lost after transfer to the greenhouse in four of the five cultivars. A significant change in DNA methylation status was noticed in cryopreserved strawberry shoot-tips (Hao *et al*. 2002).

IN VITRO SELECTION

In vitro selection is a useful tool in identifying plants resistant or tolerant to stresses produced by phytotoxins from pathogens, herbicides, cold temperature, aluminium, manganese and salt toxicity (Chaleff 1983). Usually, cells are subjected to a suitable selection pressure *in vitro* to recover any variant lines that have developed resistance or tolerance to the stress followed by regeneration of plants from the selected cell. This approach presumes that tolerance or resistance operating at the unorganized cellular level can act, to some degree of effectiveness, in the whole plant. The trait can be transferred to other plants if the tolerance/resistance has a genetic basis.

In strawberry, research was done to obtain plants resistant/tolerant to *Alternaria alternata* (Takahashi *et al*. 1992), *Botritis cinerae* (Orlando *et al*. 1997), *Colletotrichum acutatum* (Damiano *et al*. 1997; Hammerschlag *et al*. 2006), *Fusarium oxysporum* (Toyoda *et al*. 1991), *Phytophthora cactorum* (Maas *et al*. 1993; Sowik *et al*. 2001), *P. fragariae* (Maas *et al*. 1993), *P. nicotianae* var. *parasitica* (Amimoto 1992), *Rhizoctonia fragariae* (Orlando *et al*. 1997) and to *Verticillum dahliae* (Sowik *et al*. 2001, 2003). Hammerschlag *et al*. (2006) used an *in vitro* screening system to evaluate strawberry cultivars, 'Chandler', 'Delmarvel', 'Honeoye', 'Latestar', 'Pelican' and 'Sweet Charlie' propagated *in vitro*, and shoots regenerated from leaf explants of these cultivars for resistance to Anthracnose (*C. acutatum*) isolate Goff (highly virulent). Regenerants with resistance were genotype specific, and the highest levels of anthracnose resistance (2 to 6% leaf necrosis) were exhibited by regenerants from explants of cultivars 'Pelican' and 'Sweet Charlie'.

Insufficient winter hardiness is one of the major constrains limiting strawberry production in cool climates. An *in vitro* screening technology was determined for cold resistant strawberry seedlings (Rugienius and Stanys 2001). Strawberry plants were regenerated from an isolated embryo axis on MS medium without phytohormones, and from rescued cotyledons on the medium with 1.0 mg/l BA and 0.5 mg/l NAA. The temperature interval, at which genotypes differentiated according to cold resistance *in vitro*, was -8 to 12°C. Differentiation of strawberry genotypes according to this character conformed to their differentiation *in vivo* with a strong correlation (r = 0.93) between cold resistance *in vitro* and *in vivo*.

GENETIC TRANSFORMATION

Genetic transformation of *Fragaria* has made notable progress. The primary focus has been on transformation using genes with potential for pest and herbicide resistance, cold tolerance and ripening. Transformation in diploid and octoploid strawberries has been well documented using different constructs in various genotypes (**Table 2**). The earliest report of *Agrobacterium tumefaciens*-mediated transformation

Table 2 Genetic transformation with plant regeneration in strawberry.

Cultivar/genotype	Explant	Vector/strain	Marker gene(s)[1]	Functional gene/trait[2]	Reference
'Rapella'	Leaf, petiole	*Agro*/LBA 4404	*nos/nptII*	--	James *et al.* 1990
'Redcoat'	Leaf	*Agro*/M P90	*nptII/gus*	--	Nehra *et al.* 1990a, 1990b
'Chandler'	Leaf	*Agro*/LBA 4404	*nptII/gus*	--	Barceló *et al.* 1998
'Chandler'	Leaf	*Agro*/LBA 4404	*nptII/gus*	--	Harpster *et al*. 1998
'Totem'	Leaf	*Agro*/EHA 105	*nptII/gus*	--	Mathews *et al.* 1998
Diploid *F. vesca* 'Alpine' accession FRA 197	Leaf, petiole	*Agro*/LBA 4404	*nptII/gus*	--	Haymes and Davis 1998
'Chandler'	Leaf	*Agro*/LBA 4404, Biolistic	*nptII/gus*	--	Cordero de Mesa *et al.* 2000
'Gariguette', 'Polka', line no. 88312	Leaf	*Agro*/AGL0	*nptII/gus*	--	Schaart *et al.* 2002
'Chandler'	Leaf	*Agro*/LBA 4404	*nptII/gus*	*njjs25*	Jimenez-Bermudez *et al.* 2002
'Pajaro'	Leaf	*Agro*/LBA 4404	*nptII/gus*	--	Ricardo *et al.* 2003
'Joliette'	Stipules	*Agro*/LBA 4404	*nptII*	*pcht28*	Chalavi *et al.* 2003
'Induka', 'Elista'	Leaf	*Agro*/LBA 4404	*nptII/gus*	--	Gruchała *et al.* 2004
'Hecker', 'La Sans Rivale', diploid accessions (FRA197, FRA198)	Leaf, petiole	*Agro*/EHA 105	*nptII/gus*	--	Zhao *et al.* 2004
'Toyonaka'	Anther calli	Biolistic	*nptII*	LEA3	Wang *et al.* 2004
'Chambly'	Shoot	*Agro*/GV 3101	*nptII*	*wcor410a*	Houde *et al.* 2004
'Tiogar'	Leaf	*Agro*/LBA 4404	*nos/nptII*	*APF*	Khammuang *et al.* 2005
'Firework'	Leaf	*Agro*/CBE 21	*nptII*	*thau II*	Schestibratov and Dolgov 2005
'Anther'	Leaf	*Agro*/LBA 4404	*nptII*	*FagpS*	Park *et al.* 2006
F. vesca diploid accessions	Leaf	*Agro*/GV 3101, LBA 4404	Hygromycin/*gfp*	--	Oosumi *et al.* 2006
'Pájaro'	Leaf	*Agro*/LBA 4404	*nptII*	*ch5B*, *gln2*, *ap24*	Vellicce *et al.* 2006

[1]*gfp* = green fluorescent protein gene, *gus* = glucuronidase gene, *nos* = nopaline synthase gene, *nptII* = neomycin phosphotransferase gene.
[2]*ap24* = thaumatin-like protein gene from *Nicotiana tabacum*, *APF* = antifreeze protein gene from Antarctic fish, *ch5B* = chitinase protein gene from *Phaseolus vulgaris*, *FagpS* = antisense cDNA of ADP-glucose pyrophosphorylase (AGPase) small subunit, *gln2* = glucanase protein gene from *N. tabacum*, LEA3 = late embryogenesis abundant protein gene frm barley, *njjs25* = strawberry pectate lyase gene, *pcht28* = *Lycopersicon chilense* chitinase gene, *thau II* = thaumatin II protein gene, *wcor410a* = wheat dehydrin gene.

of strawberry were made by Jelenkovic *et al*. (1986) followed by James *et al*. (1990) and Nehra *et al*. (1990a, 1990b). Although a direct gene transfer method has been reported (Nyman and Wallin 1992; Wang *et al*. 2004), methods using leaf disks or crown sections as explants for *A. tumefaciens*-mediated transformation are generally employed (Jelenkovic *et al*. 1986; James *et al*. 1990; Nehra *et al*. 1990a, 1990b; El Mansouri *et al*. 1996; Haymes and Davis 1998; Barceló *et al*. 1998; Ricardo *et al*. 2003; Folta *et al*. 2006; Mezzetti and Constantini 2006). Although Abdal-Aziz *et al*. (2006) have cautioned that the use of *Agrobacterium* plasmids often results in the integration of non-T-DNA sequences (i.e. that lies outside T-DNA, in particular the *trfA* gene) in an average of 65.7% of transgenic plants (ranging from 40 to 90%). The availability of micropropagation and regeneration systems coupled with the susceptbility to infection by *Agrobacterium* species (Uratsu *et al*. 1991) makes the strawberry well suited for *Agrobacterium*-mediated genetic transformation studies using different constructs and various germplasm (**Table 2**). *Agrobacterium*-mediated transformation involves a co-cultivation of the *Agrobacterium* strain with the explants in an organogenic regeneration system on a nutrient medium containing BA or TDZ with an auxin (2,4-D, IAA) (Schaart *et al*. 2002; Zhao *et al*. 2004; Oosumi *et al*. 2006). Selection of regenerants occurred on medium with 25-75 mg/l kanamycin (Schaart *et al*. 2002; Chalavi *et al*. 2003) or 4 mg/l hygromycin (Oosumi *et al*. 2006). Cefotaxime, carbenicillin and/or ticaricillin are used to control *Agrobacterium* contamination after inoculation. The transfer of gene is generally confirmed by Southern blot analysis (James *et al*. 1990). Cordero de Mesa *et al*. (2000, 2004) reported a transformation protocol where gold particles were coated with *Agrobacterium* cells and used to bombard 'Chandler' strawberry leaf explants. Folta *et al*. (2006) developed a rapid regeneration and transformation system for genetic line 'Laboratory Festival #9' derived from self-pollination of a productive Florida cultivar, 'Strawberry Festival'. An effective method for the production of transgenic plants from which selectable marker genes have been removed has been reported by Schaart *et al*. (2004). The system combines a chemically inducible recombinase activity and a bifunctional selection system that allows the production of marker-free transgenic strawberry plants. Wang *et al*. (2004) transformed 'Toyonaka' strawberry calli from anthers by particle bombardment with plasmid pBY520 containing late embryogenesis abundant protein gene, *LEA3*, from barley (*Hordeum vulgare* L.). The bombarded calli were selected on 10 mg/l phosphinothricin (PPT) containing medium where 15.4% regeneration was observed. Hybridization signals detected by DNA dot blot analysis indicated foreign gene integration into the strawberry genome.

The transformation efficiency varies among cultivars and a given media formulation works well with a subset of cultivars. A transformation frequency of 0.95% has been reported for the cultivar 'Rapella' (James *et al*. 1990), 6.5% for 'Red Coat' (Nehra *et al*. 1990b) and 11% for 'Firework' (Schestibratov and Dolgov 2005) in *Agrobacterium*-mediated transformation system. Combining *A. tumefaciens* infection and biolistic bombardment on transformation, Cordero de Mesa *et al*. (2000) reported a transformation frequency of 20.7% in 'Chandler' strawberry. A number of risks are associated with strawberry transformation including escapes, formation of chimeric shoots (Mathews *et al*. 1998) and somaclonal variation. To minimize these risks, rapid shoot regeneration and stringent selection are required. Oosumi *et al*. (2006) used hygromycin instead of kanamycin to suppress the growth of untranformed cells, yet to allow efficient transgenic shoot formation and to minimize escapes and chimaeras.

Transformation has improved strawberries for many traits. Resent reports present evidence that cultivated strawberries may be engineered with specific pathogenesis-related (PR) genes that can decrease the severity of strawberry grey mold caused by *Botrytis cinerae* (Schestibratov and Dolgov 2005; Vellicce *et al*. 2006). Genes conferring strawberry mild yellow edge virus-coat protein (SMYEV-CP) (Finstad and Martin 1995), cowpea trypsin inhibitor (CPTI) (Graham *et al*. 1995), have been transferred into strawberry. Strawberry has been transformed with a rice chitinase gene (*RCC2*), and the resulting plants exhibit improved resistance to powdery mildew, imparted by the fungus *Sphaerotheca humuli* (Asao *et al*. 1997). The cowpea protease trypsin inhibitor gene (*CpTi*) was introduced to a strawberry cultivar and the CPTI overexpressing plants exhibited significantly higher root mass than control plants (Graham *et al*. 2002). Enhanced resistance to *Verticillium daliae*, the causal agent of verticillum wilt, was obtained in plants overexpressing

pct28 chitinase from *Lycopersicon chilense* relative to non-transformed controls (Chalavi *et al*. 2003). Herbicide tolerant strawberry plants expressing the phosphinothricin acetyl transferase gene have been produced by du Plessis *et al*. (1997). The CP4.EPSP synthase gene, which confers resistance to glyphoste, was introduced into the cultivar 'Camarosa' and 30 lines were selected based on their tolerance to glyphosate (Morgan *et al* 2002). Wang *et al*. (2004) reported increased salt tolerance in transgenic 'Toyonaka' strawberry shoots. Under 50 mmol/l and 100 mmol/l NaCl salt stress conditions, the wilting rates of controls were 62.2% and 96.0% respectively, compared with 18.6% and 42.8% for LEA3-transgenic shoots.

The antifreeze protein genes were transferred into strawberries by Khammuang *et al*. (2005). Houde *et al*. (2004) reported an improvement of the selection procedure and the transfer of the wheat *Wcor410a* acidic dehydrin gene in strawberry. The WCOR410 protein prevents membrane injury and greatly improves freezing tolerance in leaves of transgenic strawberry. In growth chamber studies, the transgenic strawberry plants have significantly higher resistance to *Verticillium dahliae* as compared to non-transgenic controls. Owens *et al*. (2002) observed Orthologs of *CBF1*, a cold-induced transcription factor important in the cold acclimation response in *Arabidopsis thaliana* were cloned from strawberry (*F.* × *ananassa*) and sour cherry (*Prunus cerasus* L.) with degenerate PCR primers. The putative orthologs [*F.* × *ananassa CBF1* (*FaCBF1*) and *Prunus cerasus CBF1* (*PcCBF1*)] have 48% amino acid identity to *CBF1* and mRNA levels were up-regulated in leaves of both crops following exposure to 4°C from 15 min to 24 hrs. However, mRNA of *FaCBF1* and *PcCBF1* was not detected in pistils of strawberry and sour cherry following 4°C exposure. *Agrobacterium*-mediated transformation of a *CaMV35S-CBF1* construct was conducted on *F.* × *ananassa* 'Honeoye' crown discs. Two transgenic lines regenerated form 'Honeoye' strawberry expressed the transgene *CBF1*, a cold-induced transcription factor at low levels. The freezing tolerance values of these lines were significantly greater than the value for the wild-type 'Honeoye' leaf discs of -6.4°C (Owens *et al*. 2002).

To control the strawberry fruit softening, Jimenez-Bermudez *et al*. (2002) developed transgenic plants that incurporate an antisense sequence of a strawberry pectate lyase gene. Transgenic lines were significantly firmer than control lines (Jimenez-Bermudez *et al*. 2002). Similar results were confirmed by Sesmero *et al*. (2007) in two lines, 'Apel 14' and 'Apel 23', in which pectate lyase mRNA transcript level was reduced 90% and 99%, respectively. Palomer *et al*. (2006) concluded that, following the insertion of two antisense divergent *endo*-β-(1,4)-glucanase (Cel1 and Cel2) genes, that fruit firmness was not affected, and that the *cel2* gene might play a pivotal role in fruit development prior to ripening. Transgenic *Fragaria vesca* ectopic expressing the FaGAST gene – which encodes a small protein with 12 cysteine residues conserved in the C-terminal region and similar to a group of proteins identified in other species having diverse functions such as cell division, elongation, or elongation arrest – under the control of the CaMV-35S promoter caused both delayed growth of the plant and fruits with reduced size, late flowering and low sensitivity to exogenous gibberellin, hinting at a role for FaGAST in the arrest of cell elongation during strawberry fruit ripening (de la Fuente *et al*. 2006).

To modulate the soluble sugar content of strawberry fruits, Park *et al*. (2006) generated transgenic strawberry plants that incorporated an antisense cDNA of ADP-glucose pyrophosphorylase (AGPase) small subunit (*FagpS*) under the control of the strawberry fruit-dominant ascorbate peroxidase (APX) promoter (cv. 'Anther'). Most transgenic fruit did not show significant differences in weight and hardness compared to control fruit although the starch content in fruit was decreased to 27-47% and the total soluble sugar content was increased to 16-37% in transgenic plants.

In a very recent study, hygromycin-resistant strawberries derived from *Agrobacterium*-mediated gene transfer could be developed in temporary immersion bioreactors and transgenes could be easily verified by TAIL-PCR (Hanhineva and Karenlampi 2007).

LOW-TEMPERATURE STORAGE AND CRYOPRESERVATION

Maintaining material *in vitro* under slow growth conditions could reduce cost associated with less frequent subculturing *in vitro* and with germplasm maintenance under field or greenhouse condition. Shoot-proliferating cultures of strawberry can be stored *in vitro* for 24 months (Lisek and Orlikowska 2001) or longer at 2-4°C on media without growth regulators. Storage of shoot cultures on filter paper bridges in liquid medium at 4°C in the dark with additional medium added at 3-month intervals is successful for many commercial strawberry genotypes (Mullin and Schlegel 1976). Strawberry cultures are stored for many years on a semi-solid basal medium at low temperature with annual transfer for rejuvenation (Jungnickel 1988). Cold storage of *Fragaria* germplasm in semi-permeable plastic tissue culture bags ranges from 9-24 months at 4°C between transfers, with an average storage interval of 15 months (Reed 2002). The addition of a 12-h photoperiod improves the storage condition (Reed 2002). Lisek and Orlikowska (2004) stored strawberry shoot tips cultures encapsulated in calcium alginate, *in vitro* at 4°C in the dark with 90-100% survival after 3 months but with reduced survival after storage for 6-9 months. Strawberry shoot tips can be preserved *in vitro* on half-strength MT-medium (Murashige and Turker 1969) with 0.5 mg/l BA, 0.05 mg/l NAA, 20 g/l sucrose, 20 g/l mannitol and 8 g/l agar for storage at 4°C with a 12-h photoperiod (11 μmoles $m^{-1}s^{-1}$) (Hao and Deng 2005). Amplified fragment length polymorphism (AFLP) analysis with 16 primer combinations did not detected any change in AFLP patterns between controls and strawberry plants recovered from slow-growth culture. However, methylation-sensitive amplified polymorphism (MSAP) analyses indicated that slow-growth storage induced changes in DNA methylation and DNA de-methylation patterns, while no *de novo* methylation was found (Hao and Deng 2005).

Zhao *et al*. (2006) cryopreserved *in vitro* cultured strawberry shoot tips using a one-step vitrification procedure. Excised shoot tips were pre-cultured for 12 days on solid MS medium with 2% di-methylsulfoxide, treated with a loading solution (20% glycerol and 0.4 M sucrose, pH 6.0) at room temperature for 20 min and then pre-treated in PVS4 (Sakai 2000) which contained 35% glycerol and 20% ethylene glycol in the liquid MS medium containing 0.6M sucrose (pH 6.0) at 4ºC for 50 min before being immersed in liquid nitrogen. The survival rate reached 82.1% and regrowth of shoot tips was 61.3%.

Medina *et al*. (2007) found that the agronomic performance (fruit production and quality) of two cvs. 'Andana' and 'Camarosa' were insignificantly different whether derived from *in vitro* tissue culture clones or from cryopreserved material.

CONCLUDING REMARKS

Strawberry micropropagation finds its main practical application in mass propagation of virus-free plants. In association with breeding programs micropropropagation can have a significant impact in: (i) mass production of elite selections and for analysis in a replicated trial of new releases, (ii) germplasm conservation, (iii) accelerating the breeding process by *in vitro* selection and haploid recovery and (iv) as a vehicle for molecular genetic research. Meristem tip culture alone or following thermotheraphy has been utilized to eliminate viruses from infected strawberries (Graham 2005). *In vitro* proliferation and regeneration systems have in fact been developed that use axillary shoot proliferation, adventitious bud induction and somatic embryogenesis. Cultures of axillary shoots allow successful propagation of

genotypes that have been established *in vitro* using materials from either juvenile or mature plants. Micropropagated, virus-tested strawberry plants are used to establish propagation fields and the runners are harvested and sold to commercial growers. However, micropropagation can profoundly alter the physiology of micropropagated strawberry plants. Many of the observed changes are related to rejuvenation; the causes of others are not yet understood. Somaclonal variation can arise from callus formed at the base of shoot clumps. The possibility of manipulating plants towards greater vigour, runner production and yield in strawberry micropropagules is of particular interest. The development of somatic embryogenesis of strawberry has not progressed as far as other methods of regeneration, and further studies are required for optimization of culture conditions for the induction, development and germination of somatic embryos of strawberries. In view of the enormous potential of somatic embryogenesis for production of genetically improved plants, these studies should be put in motion. Methods that utilize effective selection agents *in vitro* are not viewed with the antipathy of the public to genetic modifications, and may help to enhance those characteristics of strawberries that will be demanded for plant health in future. *In vitro* selection, perhaps as an alternative to genetic transformation, must be considered as one of the methods which, in combination with conventional breeding methods including biotechnological procedures, may offer plant breeder a new approach for strawberry improvement. However, for the purpose of more common and extensive use, the problems of high production cost and somaclonal variation in micropropagated plants should be overcome. The micropropagation system using embryogenic cell suspension culture and the bioreactor with a temporary immersion system will reduce the production cost. Molecular marker techniques applied for somaclonal variant detection will make quality control of the micropropagated plants easier. Combined with the powerful techniques of plant molecular biology and integrated with well established plant breeding practices, these closer collaborative ties should strengthen the efforts in achieving strawberry improvement.

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