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Tissue culture techniques for clonal propagation, viral sanitation and germplasm improvement in strawberry (*Fragaria* x *ananassa* Duch.)

Scientific Review

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Abstract – Strawberry is the most important fruit plants for both fresh consumption and food processing in the temperate and subtropical countries. The establishment of large scale, stable and high yielding strawberry plantlets for cultivation, requires mass production of uniform and healthy plant material. In vitro tissue culture constitutes an efficient mean of propagation that can be employed for clonal multiplication, viral sanitation and germplasm improvement.
The in vitro propagation of Fragaria species using meristem culture followed by axillary bud proliferation, adventitious shoot regeneration and somatic embryogenesis, has been investigated in a number of studies. These organogenesis kinds are mainly dependent on culture media, especially plant growth regulators and on genotype.
Germplasm improvement contributes to promote existing cultivars and to create cultivars with new traits like higher productivity and resistance/tolerance to biotic and abiotic stresses.

Keywords : *Fragaria*, meristem culture, adventitious regeneration, viral sanitation, genetic transformation, somaclonal variation, *in vitro* selection

1. Introduction

Strawberry is a perennial and stoloniferous crop plant belongs to the family Rosaceae, subfamily Rosoideae and genus Fragaria. Some species are diploid, having two sets of chromosomes (2n=2x=14), others are tetraploid (2n=4x=28), hexaploid (2n=6x=42), octoploid (2n=8x=56) or decaploid (2n=10x=70) (Darnell 2003). Cultivated strawberry (Fragaria x ananassa Duch.) is an economically important berry crop with immense demand for fresh as well for fruit processing industry (Shulaev et al. 2008). It is one of the most popular fruits growing in the northern hemisphere in temperate and subtemperate environment (Biswas et al.2008). Berries are appreciated for their delicious flavor and fragrance; they are rich in natural antioxidants (Wang et al. 1996; Heinonen et al. 1998; Hannum 2004) including carotenoids, vitamins, phenols, flavonoids (Larson 1988). So, strawberry fruits exhibiting a high antioxidant capacity against free radical species: superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen, that may play an important role in human health protection (Wang and Jiao 2000).
Strawberry is traditionally propagated by vegetative organs (runners) (Biswas et al. 2008) that may transmit diseases through progenies. This mean is not suitable for commercial cropping that needs big quantities of healthy plants. As an alternative way to this conventional one, mass propagation through in vitro culture was initiated first in Europe (Boxus 1974) and then widely adopted in the world and especially in USA (Zimmerman 1981). This alternative provided a very efficient tool for sanitation (meristem tip culture) and mass production in a relatively short time (Mohan et al. 2005).
Strawberry breeding programs have been very active in the last few decades. Because of complexity associated with high strawberry heterozygosity and polyploidy, traditional breeding methods may be of a limited interest. Then, application of plant tissue culture and genetic engineering may hold special significance for strawberry improvement (Darnell 2003).

Indeed, these techniques are important tools for modern plant improvement programs including introduction of new traits into selected plants, multiplication of elite selections and development of suitable cultivars, in short time. However, it was accepted that complementarity between classical breeding methods and new technologies would be better in quick and efficient achievement in genetic improvement (Cao and Hammershlag 2000).
The present review aims to give some highlights on (i) in vitro propagation via meristem culture for mass production and viral sanitation, (ii) regeneration via organogenesis and somatic embryogenesis and (iii) genetic improvement through somaclonal variation, in vitro selection and genetic transformation in strawberry (Fragaria x ananassa Duch.).

2. Micropropagation

Plant micropropagation is an "integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized and incubated in a growth-promoting aseptic environment to produce many clone plantlets". The technique of cloning isolated single cells in vitro, demonstrated the fact that somatic cells, under appropriate conditions, may be able to differentiate into a whole plant. This potential of cells or tissues to form all cell types and regenerate a whole plant is the basic principle of plant cell totipotency (Steward et al. 1958).
Concerning strawberry, vegetative propagation is usually carried out via conventional method using runners to obtain true-to-type plant. When, this technique was used alone, it presented several limitations due to slowness, limited number of runners/plant/year and disease transmission through progenies. Coming of tissue culture techniques for strawberry propagation provided an alternative and novel possibility of producing a large number of virus-free plants from a single meristem-tip in a relatively short time and space (Fatemeh 2010).
The establishment of reliable and efficient protocols for plant micropropagation through meristem culture or through adventitious organogenesis (adventitious shoots or somatic embryos) from various explants could also make a significant contribution in improving strawberry culture (Fatemeh 2010).

2.1. Meristem culture

In vitro propagation through meristem culture is a secure clonal propagation method for true-to-type plant regeneration that guarantee stable transfer of desired traits into commercial genotypes without sexual recombination. It is also a powerful tool for large-scale propagation of plants (Kumar and Reddy 2011) or as a source of plant material for regeneration and transformation experiments (Boxus 1992). The explant used for strawberry micropropagation has been the meristem excised from runner tip (Sowik et al. 2001). A meristem with no leaf primordia or at most with 1-2 leaf primordia is excised and cultured in appropriate medium (Boxus 1974; Kumar and Reddy 2011). The ability of an apical shoot meristem to regenerate whole plant is the cornerstone of the micropropagation process (Debnath and Da Silva 2007).
Shoot tips are picked from young runners and meristems are removed and cultured ona nutrient medium with low level of cytokinins. A meristem consists in a dome of actively dividing cells, about 0.1 mm in diameter and 0.25 mm in long (Sowik et al. 2001). Regenerated explant is then cultured on a medium containing higher level of cytokinins to promote axillary budding without callus formation. Cytokinins are used to overcome apical dominance and enhance the branching of lateral buds from the leaf axis. Additional shoots are produced through further cycles of axillary bud branching (Debnath 2003). Cultures can be initiated and maintained on Boxus (Boxus 1974) medium containing Knop's macronutrients and Murashige and Skoog (1962) micronutrients and organic components, or MS medium supplemented with BAP, IBA and GA3 (Cerović and Ružić 1989; Mozafari and Gerdakaneh 2012) (Table 1).
Several studies have attested that micropropagated plants show more advantages than those produced by conventional method in terms of fruit yield, vigor, number of runners per plant (Zebrowska et al. 2003) and number of leaves per plant (Karhu and Hakala 2002; Zebrowska et al. 2003). However, despite its extraordinary potential, this technology is still faced to some problems. For example, occurrence of morphological and yield variation in regenerated plants has been reported (Graham 2005). Variations consisted in dwarfism, chlorosis or white striping of leaves, multiapexing and stem fasciations, intensified vigour and abnormal flowering (hyper-flowering) accompanied by increased production of smaller fruits (Boxus 1999; Graham 2005).

These variants that caused problem for production of uniform, true-to-type plants, did not derive from meristems. Reported variations derived either from callus induced at the base of shoots (Nehra et al. 1994) or from stipular buds (without callus) (Jemmali et al. 1992, 1994). These stipular buds occurred spontaneously in vitro on strawberry regenerated from meristem culture and axillary proliferation. These stipular buds were cloned and their implication in hyper-flowering abnormality has been proved on cv. Gorella (Jemmali et al. 1992, 1994). Boxus (1999) underlined the risks caused by adventitious regeneration to the mass propagation systems of strawberry. To ovoid this, using lower BA concentration, shortening subculture duration and limiting number of subcultures were recommended (Jemmali et al. 1992; Graham 2005). Moreover, it has been proved that the application of GA3 delay the development of adventitious shoots (Boxus 1999; Litwińczuk and Zubel 2005). Gibberellic acid improved axillary shoot elongation and reduced callus growth as well as roots formation and adventitious shoots development (Litwińczuk et al. 2009).
Such practices may increase the costs of micropropagtion without full guarantee of plant conformity because adventitious proliferation is not easily detected by the in vitro procedures (Litwińczuk et al. 2009).

2.2. Adventitious organogenesis

Plant regeneration via adventitious shoots is a crucial aspect of plant biotechnology methods and tissue culture that facilitate the production of genetically engineered plants and somaclonal variants, and the rapid multiplication of difficult-to-propagate species (Debnath and Da Silva2007). This process is therefore a complex phenomenon, influenced by a number of genetic and environmental factors. Each species has its own specific requirements for in vitro regeneration. Fortunately, strawberry can be easily managed under in vitro conditions and efficient protocols for micropropagation and in vitro regeneration have been developed for many cultivars. This process is influenced by many factors such as ratio between plant growth regulators, culture conditions, genotype and explant type (Sorvari et al. 1993; Zhao et al. 2004; Hussaini and Abdin 2007).
Adventitious regeneration through direct or indirect regeneration has been obtained in many strawberry cultivars, using a board range of explants such as leaves (Jones at al. 1988; Nehra et al. 1989; Sorvari et al. 1993; Passey et al. 2003, Debnath 2005, 2006; Biswas et al. 2007; Karim et al. 2011), petioles (Foucault and Letouze 1987; Passey et al. 2003; Debnath 2005, 2006), flower buds (Foucault and Letouze 1987; Lis 1993), stipules (Rugini and Orlando 1992; Passey et al. 2003), roots (Rugini and Orlando 1992; Passey et al. 2003), runners (Liu and Sanford 1988; Lis 1993) (Table 1).
Growth and in vitro morphogenesis are regulated by the balance between the growth regulators supplied in the medium and the growth regulators produced endogenously (George et al. 2008). A balance in favor to cytokinin is often required for the formation of adventitious shoots.
In strawberry, most of regeneration protocols use Murashige and Skoog (1962) medium supplemented with 6-benzylaminopurine (BA) combined with auxins such as indole-3-butyric acid (IBA) (Quesada et al. 2007) or α-Naphthaleneacetic acid (NAA) (Karim et al. 2011). In recent years, there has been an increased interest on the use of thidiazuron (TDZ) instead of BA for strawberry regeneration. TDZ has been shown to be very effective on shoot regeneration of many species (Huetteman and Preece 1993), and its effect is not only due to its cytokinin like activity but also to its role as a modulator ofendogenous auxin levels (Quesada et al. 2007). Landi and Mezzetti (2006) evaluated the effect of TDZ on shoot regeneration from leaf tissue of several strawberry cultivars. A MS medium supplemented with TDZ and IBA in a temporary immersion bioreactor system has been used by Hanhineva et al. (2005) to optimize a protocol for shoot regeneration of five strawberry cultivars.
Genotype is also one of the most important factors affecting regeneration (Passey et al. 2003). The variation in the regeneration capacity among different cultivars has also been reported in some works (Jones et al. 1988; Nehra et al. 1989, 1990; Passey et al. 2003), indicating a strong genetic component determining the success of adventitious regeneration. It could be due, in part, to differences in levels of endogenous hormones, particularly cytokinin during the induction period; although the precise mechanism remains unclear (Schween and Schwenkel, 2003).
Type of explant is also one of the important factors in optimizing the tissue culture protocol. It significantly affects tissue culture process of plants (Kumar and Reddy 2011). In general, leaf tissue has the greatest regeneration capacity of all strawberry plant tissues in F. x ananassa (Passey et al. 2003; Jones et al. 1988; Popescu et al. 1997) and in wild strawberry F. vesca (El Mansouri et al. 1996; Alsheikh et al. 2002). Shoot regeneration rates using this explant are therefore very genotype-dependent.

Apart from growth regulators balance, genotype and type of explant, other factors can influence strawberry regeneration. Thus, Landi and Mezetti (2006) reported a stimulatory effect of continuous darkness incubation of explants on shoot regeneration. Dark incubation reduces tissue browning by inhibiting the enzymatic activity responsible for tissue oxidation (Titov et al. 2006). Besides this, it seems that a dark treatment for several weeks (Barceló et al. 1998; Hussaini and Abdin 2007) or even continuous darkness incubation (Landi and Mezetti, 2006) enhances organogenesis in strawberry leaf explants.

The addition of silver nitrate to the regeneration medium accelerates the initiation of adventitious buds and enhances shoot regeneration rates (Qin et al. 2005). This chemical has a significant stimulatory effect on chlorophyll and soluble protein contents as well as antioxidant enzyme activities. Tian et al. (2003) have found a correlation between endogenous antioxidant and morphogenetic process in strawberry.

2.3. Somatic embryogenesis

Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of somatic embryos (Quiroz-Figuera et al. 2006). The induction of somatic embryogenesis for in vitro plant regeneration provides more advantages than organogenesis (Wang et al. 2004). Somatic embryogenesis plays an important role in mass propagation, elimination of viruses, production of synthetic seeds (Swapnil et al. 2017) and conservation of germplasm via cryopreservation (Litz and Gray 1995; Merkle et al. 1995). When integrated with conventional breeding programs and molecular and cell biological techniques, it provides a valuable tool to enhance the genetic improvement of commercial crop species (Stasolla et al. 2003). Because somatic embryo resembles the zygotic embryo in various aspects (Feher et al. 2003), it may be used to study diverse subjects related to the embryogenesis process. Due to the presence of well-developed root and shoot primordia, somatic embryos germinated easily to produce plantlets without an additional step of rooting (Laux and Jugens 1997). Somatic embryos are defined as bipolar structures, which do not have a vascular connection with the mother tissue at any time of their life (Haccius 1978) and develop following the classical stages of embryogenesis (Williams and Maheswaran 1986).

Although plant regeneration through direct and indirect organogenesis has been reported in strawberry, only a few studies have so far focused on somatic embryogenesis (Donnoli et al. 2001; Biswas et al. 2007; Hussaini and Abdin 2007; Hussaini et al. 2008; Gerdakaneh and Zohari, 2013; Swapnil et al. 2017).

Several factors such as plant growth regulators combination, culture conditions influence embryogenic capacity of strawberry tissues. Plant growth regulators play a key role in the induction of somatic embryogenesis (Jimenez and Bangerth 2001). Particularly, auxins and cytokinins have been used to induce this phenomenon in strawberry. The following auxins 2,4-D (Stasolla et al. 2003; Kordestani and Karimi 2008), 4-amino-3,5,5-trichloro-2-pyridinecarboxylic acid (picloram) (Kordestani and Karimi 2008), indol-3-butyric acid (IBA) (Donnoli et al. 2001; Kordestani and Karimi, 2008) and α-naphtalene acetic acid (NAA) (Stasolla et al. 2003) used alone or in combination with the cytokinins BAP (Laux and Jugens 1997; Stasolla et al 2003) or thidiazuron (TDZ) (Pallavi et al. 2011) have been reported to induce embryogenesis (Table 1). Raemakers et al. (1995) estimated that in dicotyledonous species 2,4-D and BAP were the most used PGR for somatic embryo induction. Morphologically normal plants were obtained from somatic embryos that were transfered to MS medium containing NAA, GA3 and BA. Maintenance of the embryogenic cultures was, however, unsuccessful. Donnoli et al. (2001) reported somatic embryogenesis in 8% of the induced embryogenic calli in strawberry on MS medium supplemented with BA and IBA.

Light is an important culture condition that affects somatic embryogenesis during induction phase. Photoperiod has been implicated in the regulation of cytokinin levels as well as photoconversion of phytochromes (Torné et al. 1996).In strawberry, a negative effect of light on embryogenesis induction has been reported (Donnoli et al. 2001; Biswas et al. 2007). In chandler cultivar, a dark treatment significantly increased the number of somatic embryos on the leaf explants. Moreover, cold stress increased the embryogenic potential of strawberry (Hussaini and Abadin 2007).

Results of somatic embryogenesis in strawberry are still unsatisfying and more efforts are required to improve this technology (Swapnil et al. 2017).

Table 1. Main published results and corresponding references on strawberrymicropropagtionperformed techniques

Tissue culture technique	cultivar	Explants source	Culture medium	references
micropropagtion via axillary shoots	Kurdistan, Merk	meristem	N6 medium + BAP + IBA + GA_3	Mozafari and Gerdakarch (2012)
	Chandler	Shoot bud	MS +KN+BAP+GA3	Kaur et al. (2005)
	Sweet Charlie	Runner tips	MS+TDZ	Rekha et al. (2012)
	camarosa	Runner tips and Nodal segments	MS + BAP + NAA	Tanziman et al. (2012)
	Bari	Tip runner	MS+BAP	Ashrafuzzaman et al. (2013)
	Camarosa	Shoot tips	MS+BAP+TDZ	Fatemeh et al. (2010)
	Senga Sengana, Elsanta	Crown shoot and tips	MS+BAP+BA+GA3	Litwińczuk et al. (2009)
	Chandler, Oso-grande, Camarosa	Nodal segment	MS+BAP+NAA	Mir et al. (2010)
	Iranian cultivar	Nodal segmnet	MS+BAP+KN	Moradi et al. (2011)
	AOG, JP-2, JP-3, Camarosa, Sweet Charli, Grant, Mountain, Festival	Runner tips	MS+BAP+KN	Tanziman et al. (2013)
micropropagation via adventitious shoots	Chandler, Camarosa, Sweet Charlie	Leaf disc, Petiole, Stipule, Root segment	MS+BAP+TDZ+2,4-D	Passey et al. (2003)
	Bounty	sepal	MS+TDZ+2,4-D	Debnath et al. (2005)

	Chandler x Totem	cotyledons	MS+BAP/NAA	Miller and Chandler (1990)
	JP-2	Immature leaves	MS + BAP + NAA	Karim et al. (2011)
	Iranian cultivars	Leaf discs	MS+BAP+NAA	Mahmoud and Kosar (2013)
	Bounty	Leaf discs, sepal, petiole	MS+TDZ	Debnath (2006)
	Gorella, Redgault	Petiole, flower buds	MS+2,4-D+BAP	Faucault and Letouze (1987)
	Addie, Dana, Gea, Santame	Stipule, roots	MS+BAP+IBA	Rugini and Orlando (1992)
	Chandler	Lefa discs	MS+TDZ	Hussaini and Abdin (2007)
	Camarosa, Gaviota, Seascape	Leaf discs	MS+TDZ	Mohamed et al. (2007)
	Pbgel-2000	Nodal segment	MS+BAP+2,4-D+proline	Biswas et al. (2007)
		leaf		
	Sweet Charlie	Leaf disc	MS +BAP+2,4-D	Omar et al. (2013)
	chandler	petiole		
	Camarosa	Leaf, nodal segment, petiole, stamen	MS+picloram	Gerdakaneh and Zohari (2013)
	Paros			
Micropropagtion via somatic embryogenesis	kurdistan			
	Selva, camarosa	leaf	MS+picloram	Kordestani and Karimi (2008)
	camarosa	Leaf disc	MS+2,4-D+BA	Swapnil et al. (2017)
	Sweet Charlie	Petiole segments	MS+TDZ+2,4-D	Pallavi et al. (2011)
	Chandler	Leaf discs	MS + TDZ	Hussaini and Abdin (2007)

3. Virus elimination

Vegetative propagation of Fragaria sp. is traditionally carried out using stolons. This system of propagation is slow facilitate the spread of plant diseases, particularly viral pathogens (Mozafari and Gerdakaneh 2012; Quiroz et al. 2017). Many of viruses can greatly reduce yield, rapidly spread in the field without exhibiting evident symptoms (Martin and Tzanetaki 2006). The yield reduction caused by some viruses may be up to 80% (Thompson and Jelkman 2003).In some cases, individual virus strains may not constitute an economic problem but multiple virus infections are reputed to be more severe.

Initially, many of the virus strawberry diseases were described based upon symptoms. More recently, significant progress has been made in the molecular characterization of the aphid-borne viruses, the identification and characterization of several whitefly-borne viruses, and the molecular characterization of viruses associated with several of the "graft transmissible virus-like diseases" of strawberry. Currently, detection methods based on molecular reverse transcription–polymerase chain reaction (RT-PCR) are developed for the most viruses infecting strawberry. Seven aphid-transmitted viruses are reported in strawberry: Strawberry crinkle virus (SCV), Mild yellow edge disease virus (SMYEV), Strawberrymottle virus (SMoV), Strawberry vein banding virus (SVBV), Strawberry pseudo mild yellow edge virus (SPMYEV), Strawberry chlorotic fleck virus (SCFV) and Strawberry latent C virus (SLCV). Among these SCV, SMYEV, SMoV, and SVBV have been considered the four most economically important viruses (Martin and Tzanetakis 2006).

In vitro meristem culture and establishment of micropropagation protocols are important tools for solving the problem of viral infection. In recent years, considerable efforts have been made to develop in vitro propagation of the commercial strawberry in order to produce virus-free plants of high quality (Mozafari and Gerdakaneh 2012; Quiroz et al. 2017). Regeneration of virus-free plants is inversely correlated to the meristem size. It's recommended to associate this technique with thermotherapy for better results of sanitation. However, when meristem size is less than 1 mm, the majority of viruses may be eliminated from the regenerated plants by meristem culture without thermotherapy.

This means that, more the meristem is smaller, more sanitation rate is high (Isac et al. 2010).

Regenerated healthy plants may serve as a mother germplasm that should strictly protected against virus vectors to ovoid recontaminations. Greenhouses with insectproof films are used for their purpose under strict controls (Martin and Tzanetaki 2006). However, nurseries planted with in vitro plants as mother source are subjected to certification measures that take into account the generation number (F0, F1, F2…) regarding the tolerated rate.

Improved methods of strawberry micropropagtion contributed to the successful eradication of the most important viruses from the frequently commercialized varieties. Those viruses are latent A strawberry virus, strawberry vein-yellowing, strawberry crinckle virus, strawberry mild yellow-edge virus, strawberry mottle virus (Coman and Neculae 1981 cited by Isac et al. 2010).

4. Germplasm improvement

Traditional germplasm improvement is based on the identification of the most suitable genetic resources from either cultivated or wild germplasm. Starting from wild germplasm, progeny from strawberry crosses with F. virginiana glauca as a common parent have shown significant increases in fruit quality and nutritional features. More recently, tissue culture techniques have been exploited to create genetic, to improve the state of health of the planted material and to increase the number of desirable germplasms available to the plant breeder. Cell culture has produced somaclonal variants with crop-improvement potential. Tissue culture techniques, in combination with molecular techniques, have been successfully used to incorporate specific traits through gene transfer (Brown and Thorpe 1995). A number of molecular studies have targeted strawberry for gene cloning, genetic transformation, fingerprinting and mapping for important agronomic traits such as disease resistance and fruit nutritional quality. These achievements are expanding the potential applications of DNA recombinant technology for the transfer and validation of target genes that control important traits (Mazzetti 2013).

4.1. Somaclonal variationand *in vitro* selection

Somaclonal variation is defined as the "occurrence of variation in plants regenerated from in vitro cultures" (Larkin and Scowcroft 1981). This variation may be spontaneous (accidentally) or induced. Whatever the way of its occurrence, variation may be desirable or not.

In the case of spontaneous variation, no case of desirable characters was reported in micropropagated strawberry. All of them have been considered as morphological abnormalities including leaf variegation,

consisting in a narrow white streak in the leaf blade, chlorosis of the leaves, chlorosis of the leaves and growth changes including dwarfs, compact trusses, lack of runner production, and female sterility (Swart et al. 1981; Adel and Sawy 2007). 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).

In the case of induced variations, selection pressure is generally applied at the collagenesis step and uses several factors of induction. This technology has been adopted as a powerful tool in selection program based in the acquired traits by some cell lines when placed under pressure. Some of those traits are obtained by exposing cells to stresses by phytotoxins from pathogens, herbicides, thermic stress and salinity or drought (Chaleff 1983).

Usually, cells subjected to a suitable in vitro selection pressure are able to recover any variant lines that have developed resistance or tolerance to the stress followed by regeneration of plants from the selected cell. Somaclonal variants 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 than phenotypic and karyologic analysis. Actually, there are various methods available which can be used to detect and monitor tissue culture-derived plants and varietal identification. ISSR (Inter Simple Sequence Repeats) and RAPD (Random Amplified Polymorphic DNA) are the molecular technique for identification of genetic variation (Palei et al. 2015). Strawberries (Fragaria x ananassa Duch.) have been extensively analyzed using randomly generated markers for clone identification and diversity studies. Polymorphism also appears in acid-phosphatase, glutamate-dehydrogenase and peroxidase of regenerated plants (Adel and Sawy, 2007).

In strawberry, research was done to obtain plants resistant/tolerant to biotic stress such as Alternaria alternata (Takahashi et al. 1992), Botritis cinerae (Orlando et al. 1997), Colletotrichum acutatum (Hammerschlag et al. 2006), Fusarium oxysporum (Toyoda et al. 1991), Phytophtora cactorum (Sowik et al. 2001), Rhizoctonia fragaria (Orlando et al. 1997), Verticillium dahlia (Sowik et al. 2003;Zebrowska 2010) and to abiotic stresslike salinity (Dziadczyk et al. 2003) and cold (Rugienius and Stanys 2001). Regeneration of resistant plants is genotype-dependant (Hammerschlag et al. 2006).

4.2. Genetic transformation

Genetic engineering of Fragaria has made notable progress (James et al. 1990; Nehra et al. 1990).The first work has been focused on transformation using genes for pest and herbicide resistance, cold tolerance and ripening (Hussaini and Srivastava 2006; Debnath and Da Silva, 2007).

Transformation was generally achieved viaAgrobacterium tumefaciens-mediated technology carrying the plasmid pBI121 (Ricardo et al. 2003; Folta et al. 2006). Transgenic nature of the shoots was confirmed by the GUS assay and PCR analysis.

Previous studies on strawberry have clearly demonstrated the importance of various factors such as genotype (Liu and standford 1988), type and source of explant (Nehra et al. 1989), hormonal balance and incubation conditions (Liu and Sanford 1988) on efficiency of transformation.

James et al. (1990) found that petioles of ‘rapella’ cultivation were transformed more efficiently than leaf discs. By contrast, the latter explant gave better results than stipules in F. vesca (Alsheikh et al. 2002). Matthews et al. (1998) observed highest transformation rates when using meristematic sections obtained from the base of in vitro proliferating plantlets, but a high percentage of regenerated plants were chimeras.

According to Khammuang et al. (2005), the optimal condition for regeneration of transformed shoots from leaf explants cv. Tiogar was MS medium containing BA (0.1mg/l) and 2,4-D (0.2 mg/l).

A first example concerns recombinant gene encoding antifreeze protein isoform HPLC 6 was successfully constructed using codons which were optimally expressed in the strawberry plant. The antifreeze protein genes, naturally in plasmid pSW1 and modified in plasmid pBB, were introduced instrawberry leaf explants by Agrobacterium tumefaciens LBA 4404. Polymerase chain reaction (PCR) indicated that the transgenes were integrated in the genome of transformants (Khammuang et al. 2005).

The second example concerning the gene encoding the taste-modifying protein miraculin (Toshiyuki et al. 2008). Miraculin was detected in leaves and fruits of the transgenic plants with variable levels ranged

from 0.5 to 1 mg/g miracle fruit. High levels of miraculin accumulation were detected in the mature fruits. The transgenic lines were subsequently propagated via runners for three vegetative generations, and miraculin was detected at equal levels in the leaves and fruits of the plants from each generation (Toshiyuki et al.2008).

5. Conclusion

In vitro propagation of strawberry finds its main practical application in mass propagation of virus-free plants. In association with breeding programs micropropagtion may have a significant impact, such as mass production of elite selections, accelerating the breeding process by in vitro selection and germplasm conservation. Meristem tip culture alone or combined with thermotherapy has been utilized to eliminate viruses from infected strawberries (Graham 2005). True-to-type propagules and genetic stability are prerequisites for the application of micropropagtion. In vitro organogenesis and somatic embryogenesis are alternative means of mass scale propagation. The development of somatic embryogenesis of strawberry has not progressed as far as other methods of regeneration. Further studies are then required for optimization of the culture conditions for the induction, development and germination of somatic embryos. Clonal fidelity of micropropagated plants can be monitored at morphological, biochemical, physiological and molecular levels. Molecular markers like ISSR and RAPD are powerful tools in genetic identification of somaclonal variation. In vitro selection, considered as an alternative to genetic transformation, may offer to plant breeders a new approach for strawberry improvement.

In vitro techniques are important tools for modern plant improvement programs to introduce new traits into selected plants and to multiply elite selections. Used in conjunction with classical breeding methods, an efficient in vitro shoot proliferation and regeneration system could accelerate cultivar development programs. Standardization of an efficient regeneration system for each strawberry genotype is generally a necessary pre-requisite for the successful development of transgenic plants.

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