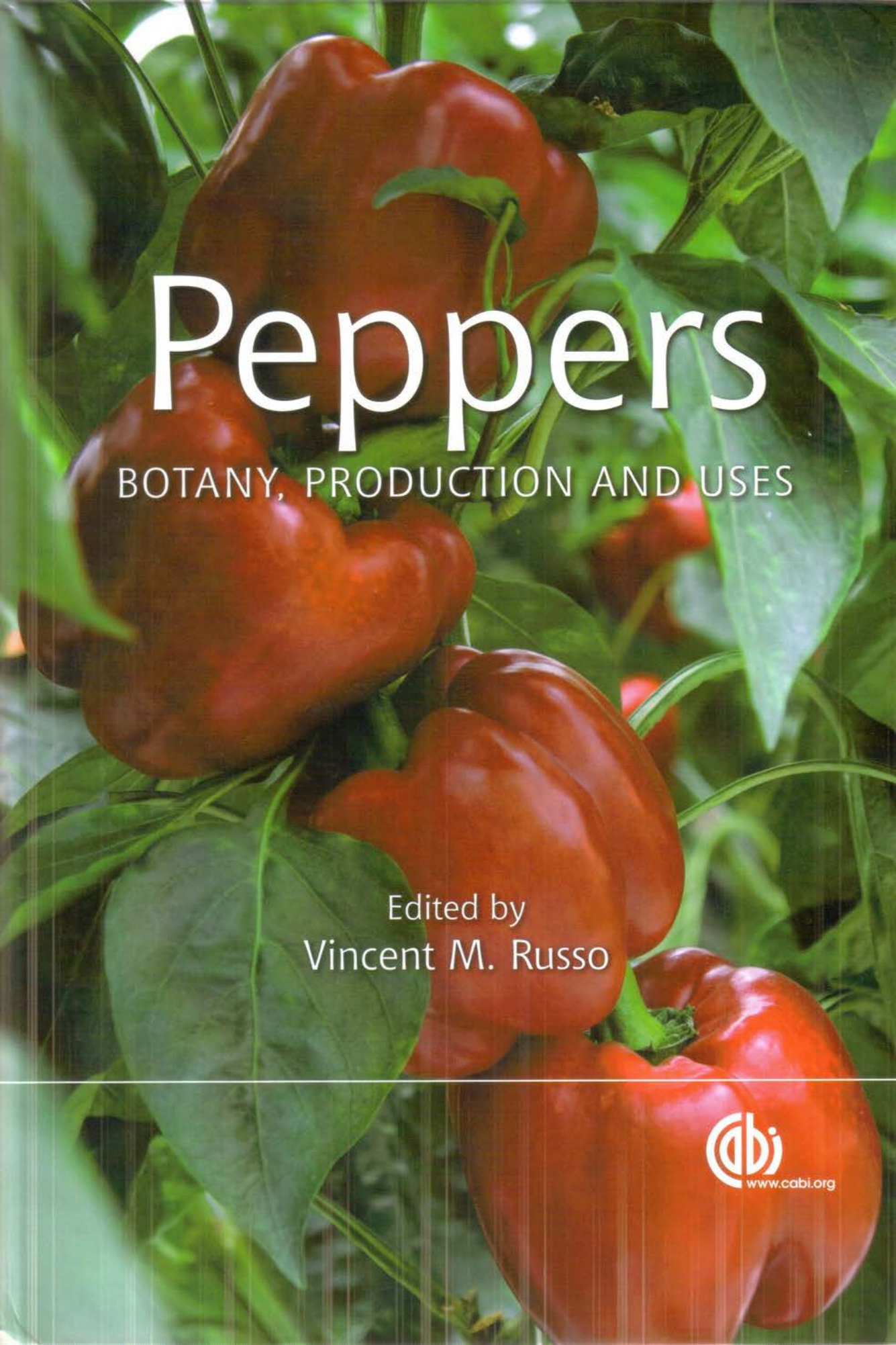
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5 Tissue Culture of *Capsicum* Species

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5.1 Introduction

Tissue culture is a key tool of plant biotechnology that exploits the totipotent nature of plant cells. This concept was proposed by Haberlandt (1902), and means that plant cells have the necessary genetic and physiological mechanisms to regenerate whole plants in aseptic conditions. Morphogenesis allows plant regeneration from cells, tissue, and organ culture, and is a fundamental process in the application of plant biotechnology to propagation and genetic improvement. Pepper plant regeneration is limited due to recalcitrance to *in vitro* manipulation of explants (Franck-Duchenne *et al.*, 1998; Steinitz *et al.*, 1999; Ochoa-Alejo and Ramírez-Malagón, 2001). The need for viable regeneration protocols to be applied in improvement and transformation of plants is necessary to understand the nature of recalcitrance in *Capsicum*.

5.2 Recalcitrance of *Capsicum* Genus

Recalcitrance can occur at all stages of a culture regime and little is known regarding its causal factors. *Capsicum* is a versatile plant, but lacks a highly efficient reproducible plant regeneration system. Other members of *Solanaceae*, e.g. potato, tomato, tobacco, and petunia, are frequently used as model systems in tissue culture. Despite this, research has been conducted to achieve a regeneration system for *Capsicum*. Fari (1986), Morrison *et al.* (1986), Ezura (1997), Ochoa-Alejo and Ramírez-Malagón (2001), and Kothari *et al.* (2010) published reviews in this area.

In *Capsicum*, organogenic capacity differences have been observed in various pepper genotypes (Christopher and Rajam, 1996), cultivars (Ezura *et al.*, 1993; Szasz *et al.*, 1995; Ramírez-Malagón and Ochoa-Alejo, 1996), and species (Christopher and Rajam, 1996), using different explant sources (Ochoa-Alejo and Ireta-Moreno, 1990; Ezura *et al.*, 1993; Szasz *et al.*, 1995; Ramírez-Malagón and Ochoa-Alejo, 1996) and culture media (Ochoa-Alejo and Ireta-Moreno, 1990; Ezura *et al.*, 1993; Szasz *et al.*, 1995; Ramírez-Malagón and Ochoa-Alejo, 1996; Venkataiah *et al.*, 2003; Sanatombi and Sharma, 2008). Most reports agree on the low efficiency and reproducibility of most regeneration systems. Recalcitrance is a complex phenomenon, involving the whole plant physiology of the donor, *in vitro* manipulation of the regenerative capacity of cells and plant tissues, and other factors relating to *in vitro* stress physiology (Benson, 2000). Santana-Buzzy *et al.* (2005) observed that, when habanero pepper explants (*C. chinense* Jacq.) were grown in closed containers

without ventilation and without growth regulators, callus formation was on the upper side of the leaves and along the stems (Fig. 5.1a). Plants presented etiolated stems, leaf chlorosis, and early shedding of leaves (Fig. 5.1b), and plantlets flowered inside culture vessels (Fig. 5.1c). Shoots and plants grown in ventilated containers showed normal development (Fig. 5.1d).

Santana-Buzzy *et al.* (2006) evaluated effects of silver nitrate and cobalt chloride on ethylene production during *in vitro* development of habanero pepper plantlets. Cobalt chloride partially inhibited production of ethylene during *in vitro* culture. Silver nitrate did not inhibit ethylene production, but did inhibit effects of this hormone on plantlets. Further studies are required to achieve a better understanding of the role ethylene plays during growth and development and its relationship with recalcitrance in *Capsicum*. Differential gene expression was studied in *C. chinense* shoots cultivated in nonventilated and ventilated vessels (Santana-Buzzy, unpublished). Bello-Bello *et al.* (2010) reported obtaining an efficient protocol of direct organogenesis in *C. chinense*. However, shoot elongation was only possible under conditions of temporary immersion in a bioreactor type BioMint (Robert *et al.*, 2006).

Several reports, the majority on *C. annuum* (Agrawal *et al.*, 1989; Ochoa-Alejo and Ireta-Moreno, 1990; Valera-Montero and Ochoa-Alejo, 1992; Ramírez-Malagón and Ochoa-Alejo, 1996; Husain *et al.*, 1999; Venkataiah *et al.*, 2003; Santana-Buzzy *et al.*, 2006; Bello-Bello *et al.*, 2010) report relative success of shoot morphogenesis in *Capsicum*. A limiting factor for regeneration of *Capsicum* in cultures is formation of ill-defined leafy shoots which do not elongate, or resist elongation, and which limit development rate of shoots. Recalcitrance of *Capsicum* has been observed in somatic embryogenesis. The protocols result in low efficiency and low reproducibility, high frequency of deformed embryos, and poor capacity of embryos to develop into plants (Harini and Lakshmi Sita, 1993; Buyukalaca and Mavituna, 1996; Kintzios *et al.*, 1998; López-Puc *et al.*, 2006; Zapata-Castillo *et al.*, 2007). The *in vitro* regeneration of *C. chinense* through

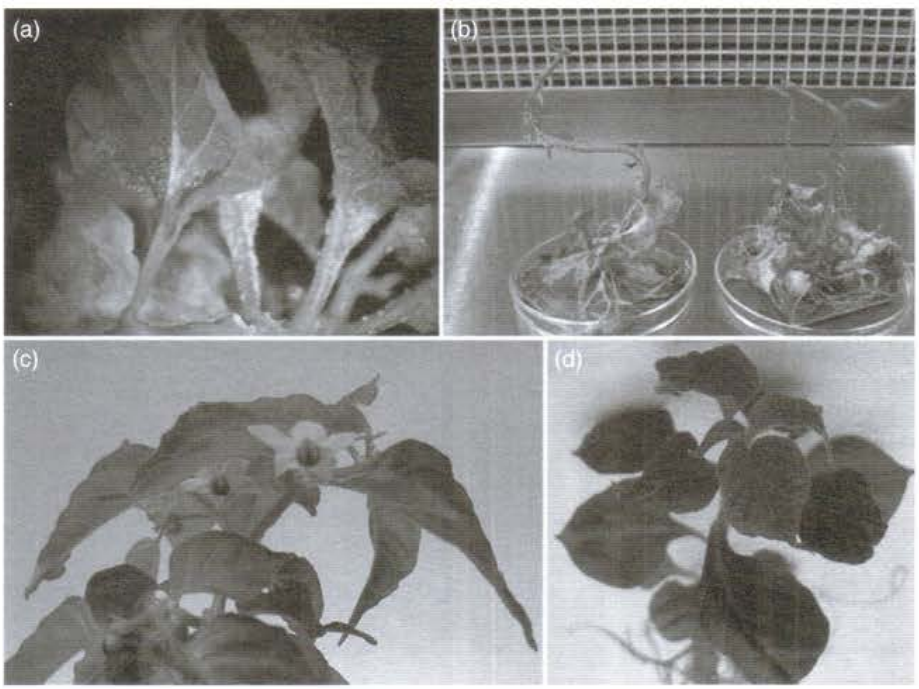

Fig. 5.1. Behavior of habanero pepper during *in vitro* culture in ventilated and nonventilated vessels without growth regulators: (a) *in vitro* callus formation over all tissues of plant, (b) *in vitro* early plant defoliation, (c) *in vitro* floral induction, and (d) normal plant grown in ventilated vessel.

organogenesis has occurred (Santana-Buzzy *et al.*, 2005, 2006; Montalvo-Peniche *et al.*, 2007; Bello-Bello *et al.*, 2010), and by direct and indirect somatic embryogenesis (López-Puc *et al.*, 2006; Zapata-Castillo *et al.*, 2007; Santana-Buzzy *et al.*, 2009). A system of direct somatic embryogenesis in a highly efficient liquid medium for *C. chinense* was developed (Fig. 5.2a–e) (Santana-Buzzy, unpublished). Analysis with SDS-PAGE and 2-D electrophoresis indicated that endogenous protein content diminished in somatic embryos of *C. chinense* as development advanced (Fig. 5.3). Santana-Buzzy (unpublished) determined that some proteins with low molecular weights present in the proteic profile of zygotic embryos of the species, were absent in the band profile of somatic embryos. Also endogenous contents of polyamines during somatic embryogenesis were determined. Additionally, cadaverine was present at levels above spermine and spermidine, and with RAPD and ISSR markers a high frequency of somaclonal variants was found in regenerants from somatic embryogenesis and organogenesis. Attempts to regenerate chilli *in vitro* have used a number of approaches (Table 5.1).

The genotype and explant in regeneration

Genotype and explant type are important factors limiting regeneration of *Capsicum* plants *in vitro*. Existence of strong genotype and explant specificity in regeneration capacity of different species of the genus, and cultivars of the same species, is a limiting factor for development of standard regeneration protocols in *Capsicum*. Gunay and Rao (1978) reported successful regeneration of pepper plants from two *Capsicum* cultivars and from a *C. frutescens* hybrid ('Baratha'), using cotyledon and hypocotyl explants with cotyledons being the most responsive. Similar results were reported using cotyledon explants from *C. annuum* (Sripichit *et al.*, 1987; Agrawal *et al.*, 1989; Ebida and Hu, 1993; Binzel *et al.*, 1996b), and from *C. praetermissum*, *C. baccatum* (Christopher and Rajam, 1996), *C. frutescens*, and *C. chinense* (Kumar *et al.*, 2007; Sanatombi and Sharma, 2008). Cotyledons, hypocotyls, leaves, shoot tips, zygotic embryos, embryonal leaves, stems, internodes, and mature seeds have been used as explants for *in vitro* regeneration of chilli plants (Agrawal and Chandra, 1983; Agrawal *et al.*, 1989; Alibert, 1990; Ebida and Hu, 1993;

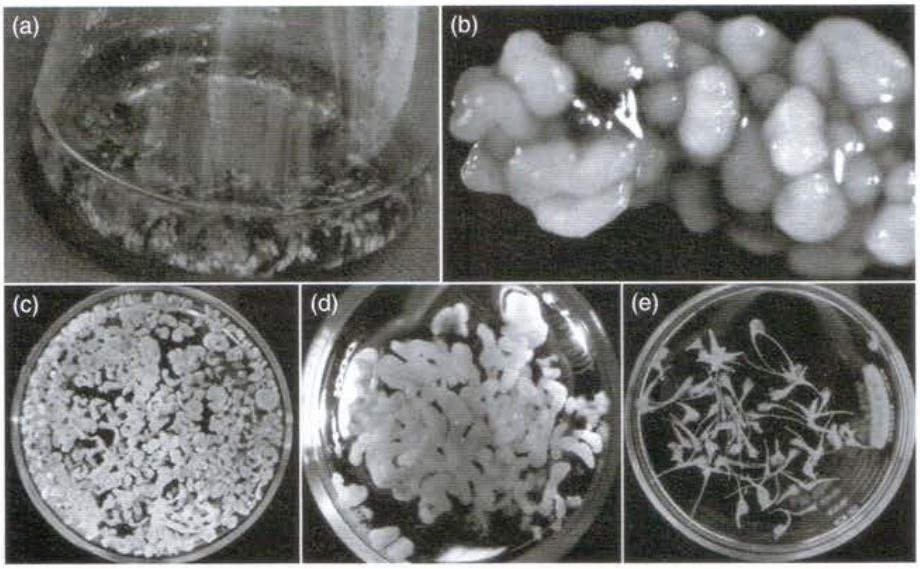

Fig. 5.2. Highly efficient direct somatic embryogenesis in liquid culture of habanero pepper: (a) direct somatic embryogenesis from habanero pepper hypocotyls in liquid culture, (b) efficient multiplication of somatic embryos from hypocotyl segments, (c) adventitious somatic embryogenesis from somatic embryos in liquid culture, (d) torped and cotyledonary embryos, and (e) somatic embryo germination.

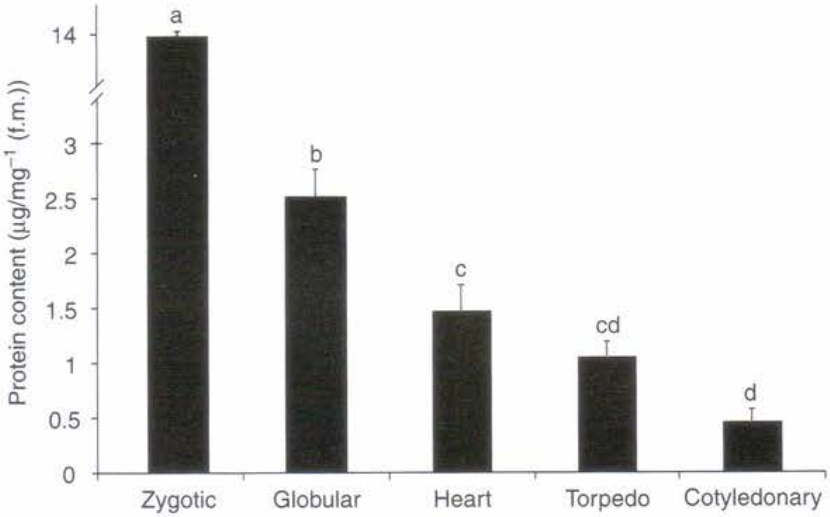

Fig. 5.3. Protein content in developmental phases of somatic embryos produced *in vitro* and in the mature zygotic embryo of habanero pepper. There are differences between the cotyledonary somatic embryo and the mature zygotic embryo.

Ezura *et al.*, 1993; Ramírez-Malagón and Ochoa-Alejo, 1996; Berljak, 1999; Santana-Buzzy *et al.*, 2005; López-Puc *et al.*, 2006; Bello-Bello *et al.*, 2010). Christopher and Rajam (1996) reported that leaf explants consistently generated more shoots than hypocotyls or cotyledons. Similar results were reported by Dabauza and Peña (2001) for explants from cotyledons, leaves, and cotyledonary node shoot tips, and embryonic cotyledons, hypocotyls and from wounded seedlings. Venkataiah *et al.* (2003) reported that leaf explants were superior to cotyledons for shoot morphogenesis, while Santana-Buzzy *et al.* (2005) used different aged nodal and internodal segments of aseptic plants to evaluate regeneration capacity of *C. chinense*. Golegaonkar and Kantharajah (2006) investigated shoot forming capacity of leaf and cotyledon explants and found that regeneration frequency was influenced by explant, culture media, and cultivar. Velichka *et al.* (2006) studied callugenesis and regeneration ability of cotyledon and hypocotyl explants in MS basal medium supplemented with BAP, IAA, and GA_3. High levels of callugenesis and organogenesis were produced in both types of explants from all varieties. The highest percentage of plant-regenerants was established in cotyledon explants. Other factors influencing organogenic responses are position of the explant in the plant or the physiological age of the seedlings used. Sanatombi and Sharma (2008) studied effects of different explants in cultivars of *C. annuum*, *C. frutescens*, and *C. chinense* and found leaf and cotyledons to be most responsive compared to hypocotyls. Ashrafuzzaman *et al.* (2009) evaluated hypocotyl, cotyledon, and shoot tip explants of *C. annuum* to determine regeneration potential, and observed greater callus formation and shoot initiation in hypocotyls.

Embryogenic capacity of *Capsicum* explants has been much more limited. Most reports on somatic embryogenesis have been restricted to use of immature or mature zygotic embryos (Harini and Lakshmi Sita, 1993; Binzel *et al.*, 1996a; Buyukalaca and Mavituna, 1996). Immature zygotic embryos made induction of somatic embryos possible in plant species which had been considered recalcitrant (Ahloowalia, 1991; Raemakers *et al.*, 1995; Arnold *et al.*, 1996). Hypocotyls (López-Puc *et al.*, 2006; Zapata-Castillo *et al.*, 2007) could be used for the efficient direct and indirect induction of somatic embryos in *C. chinense*.

Table 5.1. *In vitro* plant regeneration of chilli pepper (*Capsicum* spp.).

Species	Explant	System of regeneration	PGRs[a]	References
C. annuum	Hypocotyl, cotyledon	Organogenesis	BA+IAA	Gunay and Rao (1978)
C. frutescens				
C. annuum	Zygotic embryo	Organogenesis	BA	Agrawal and Chandra (1983)
C. annuum	Seedling explants	Organogenesis	BA	Phillips and Hubstenberger (1985)
C. annuum	Hypocotyl, cotyledon, stem, leaf, root, shoot-tip, embryo	Organogenesis	BA+IAA	Agrawal *et al.* (1989)
C. annuum	Hypocotyl	Organogenesis	AIA, 2iP	Ochoa-Alejo and Ireta-Moreno (1990)
C. annuum	Cotyledon, hypocotyl	Organogenesis	BA+IAA	Arroyo and Revilla (1991)
C. annuum	Hypocotyl	Organogenesis	AIA, BA	Valera-Montero and Ochoa-Alejo (1992)
C. annuum	Seedling explants	Organogenesis	BA+NAA	Ebida and Hu (1993)
C. annuum	Mature seeds	Organogenesis	MS without GRs	Ezura et al. (1993)
C. annuum	Immature zygotic embryos	Direct somatic embryogenesis	10% CW, 2,4-D	Harini and Lakshmi Sita (1993)
C. annuum	Shoot tip	Axillary meristem	BA	Madhuri and Rajam (1993)
C. annuum	Shoot tip	Axillary meristem	BA	Christopher and Rajam (1994)
C. praetermissum				
C. annuum	Mature zygotic embryo	Indirect somatic embryogenesis	2,4-D	Buyukalaca and Mavituna (1996)
C. annuum	Immature zygotic embryos	Direct somatic embryogenesis	2,4-D, TDZ	Binzel *et al.* (1996a)
C. annuum	Cotyledon	Organogenesis	BA, IAA+$AgNO_3$	Hyde and Phillips (1996)
C. annuum	Hypocotyl, cotyledon, leaf	Organogenesis	BA+IAA	Christopher and Rajam (1996)
C. praetermissum				
C. baccatum				
C. annuum	Hypocotyls	Organogenesis	IBA	Ramírez-Malagón and Ochoa-Alejo (1996)
C. annuum	Cotyledon	Organogenesis	BA, IAA+EBr	Franck-Duchenne *et al.* (1998)
C. annuum	Cotyledon	Organogenesis	BA+PAA	Husain *et al.* (1999)
C. annuum	Zygotic embryos	Organogenesis	BA+NAA	Arous *et al.* (2001)
C. annuum	Seedling explants, embryonal explants	Organogenesis	TDZ	Dabauza and Peña (2001)

Table 5.1.

Species	Explant	System of regeneration	PGRs[a]	References
C. annuum	Leaf cotyledon	Organogenesis	TDZ	Venkataiah *et al.* (2003)
C. annuum	Zygotic embryos	Somatic embryogenesis	2,4-D, centrofenoxina	Steinitz *et al.* (2003)
C. annuum	Leaf, meristem	Organogenesis	BA, AIA+($AgNO_3$), benzoic acid	Kumar *et al.* (2005)
C. annuum	Microspores	Somatic embryogenesis	2,4-D	Bárány *et al.* (2005)
C. annuum	Hypocotyl	Somatic embryogenesis	TDZ, IBA	Khan *et al.* (2006)
C. annuum	Anthers	Somatic embryogenesis	2,4-D, IAA	Koleva-Gudeva *et al.* (2007)
C. annuum	Nodal segments of seedling plants	Organogenesis	TDZ	Ahmad *et al.* (2006)
C. chinense	Meristems	Minimal growth of shoot tips	Osmoregulators (mannitol and sorbitol)	Montalvo-Peniche *et al.* (2007)
C. chinense	Leaf, cotyledon, hypocotyls, zygotic embryo	Somatic embryogenesis	2,4-D	López-Puc *et al.* (2006)
C. frutescens	Shoot tip	Axillary proliferation	BA+Kin	Sanatombi and Sharma (2008)
C. annuum	Cotyledon	Organogenesis	BA+PAA	Joshi and Kothari (2007)
C. annuum	Leaf, cotyledon, hypocotyl	Organogenesis	BA+IAA	Sanatombi and Sharma (2008)
C. frutescens *C. chinense*	Leaf, cotyledon, hypocotyls, zygotic embryo	Somatic embryogenesis (Indirect)	2,4-D	Zapata-Castillo *et al.* (2007)
C. chinense	Hypocotyls	Somatic embryogenesis and histological analysis	2,4-D	Santana-Buzzy *et al.* (2009)
C. chinense	Nodal segments of seedling plants	Direct organogenesis, multiple shoots	TDZ+PAC	Bello-Bello *et al.* (2010)

[a]PGRs – Plant Growth Regulators: BA, 6-benzylaminopurine; IAA, indole-3-acetic acid; 2iP, 2-isopentenyladenine; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; EBr, 24-epi-brassinolide; PAA, phenyl acetic acid; Kin, kinetin; PAC, paclobutrazol.

5.3 *In vitro* Morphogenesis of *Capsicum* Genus

The creation of new form and organization, where previously lacking, is termed morphogenesis. Tissues or organs that have the capacity for morphogenesis are said to be morphogenic(morphogenetic).Organogenesis and somatic embryogenesis can be used to achieve plant regeneration, and can occur directly and indirectly from explants. Formation of monopolar structures, shoots and/or roots, that maintain vascular connection with the tissue from which they

originated, is organogenesis. Somatic embryogenesis is the process from which bipolar structures originate, with their caulinar and radical apex well defined and perfectly distinguishable, without vascular connection with the tissue from which they originated.

Organogenesis

Organogenesis is a complex morphogenetic process involving formation of monopolar structure to form shoots or roots. Gunay and Rao (1978) were first to report successful regeneration of shoots and plants from cotyledon and hypocotyl explants in *Capsicum*, using *C. annuum* cvs Pimento and California Wonder and a hybrid of *C. frutescens* ('Bharath'). Fari and Czako (1981) studied the relationship between position and morphogenetic responses of hypocotyl explants of *C. annuum* cv. T. Havani. Shoot regeneration was only from apical segments. Agrawal and Chandra (1983) reported differentiation of multiple shoot buds and plantlets in cultured embryos of *C. annuum* cv. Mathania. Numerous shoot buds were produced on margins of expanded cotyledons of embryos grown on medium with BA. Similar results were obtained by Sripichit *et al.* (1987), who found BA more effective than kinetin (Kin) to induce shoot formation in cotyledon explants cultured on MS medium.

Direct and indirect *in vitro* plant regeneration from chilli pepper, cv. Soroksari, was reported by Berljak (1999). Direct shoot regeneration was achieved only from basal parts of shoot-tip explants cultured on media with BA or zealone, and with BA and IAA. Reddy *et al.* (2002) studied effects of triacontanol (TRIA) on shoot multiplication and rooting of *in vitro* derived shoot tips of *C. frutescens* and *Decalepis hamiltonii*. Kumar *et al.* (2005) obtained *in vitro* direct multiple shoot formation from seedling explants of Indian highly pungent *C. annuum* cvs Arka Abhir and Arka Lohit. Santana-Buzzy *et al.* (2005) induced multiple shoots from habanero pepper. Explants were cultivated in MS medium supplemented with varying concentrations of kinetin, BA, and thidiazuron (TDZ), the latter being the key growth regulator in the process. Velichka *et al.* (2006) studied callugenesis and regeneration ability of cotyledons and hypocotyls of pepper plantlets (Santana-Buzzy *et al.*, 2006). Khan *et al.* (2006) induced multiple shoots by culturing nodal explants excised from 1-month-old aseptic seedlings of *C. annuum*, cv. Pusa Jwala, on MS medium supplemented with TDZ. Peddaboina *et al.* (2006) developed a procedure for *in vitro* propagation of *C. annuum*, cv. CA960, *C. baccatum*, *C. frutescens*, and *C. praetermissum* using shoot meristem explants, and employing a revised medium for rapid growth and bioassays with a tobacco tissue culture medium. Montalvo-Peniche *et al.* (2007) studied effects of nitrate, sucrose, and osmotic regulators (mannitol and sorbitol) on growth of habanero pepper germplasm for *in vitro* conservation. Mannitol 2% had a better effect on minimal growth of plantlets and did not affect plant physiology and quality. Sanatombi and Sharma (2008) reported *in vitro* regeneration from leaf, cotyledon and hypocotyl explants of *Capsicum* cultivars by direct organogenesis. Valadez-Bustos *et al.* (2009) developed a protocol for *in vitro* regeneration of jalapeño and serrano, *C. annuum* var. *glabriusculum/aviculare* (Piquin), and habanero by direct organogenesis. Ashrafuzzaman *et al.* (2009) developed an efficient *in vitro* propagation protocol for clonal propagation of cultivars of chilli using cotyledon, hypocotyl, and shoot-tips from *in vitro* regenerated plants. Shoot elongation was accelerated using supplementation of GA_3 and $AgNO_3$. Bello-Bello *et al.* (2010) evaluated performance of nodal segments from habanero pepper during shoot induction and elongation, with different semisolid and liquid culture. Temporary immersion bioreactor (BioMINT™) was used for multiplication and elongation of isolated shoots. The authors reported an efficient protocol for *in vitro* propagation of habanero pepper that produces plants with a high survival rate when transplanted to soil (Fig. 5.4a–c).

Somatic embryogenesis

Somatic embryogenesis (SE) is the developmental pathway by which somatic cells

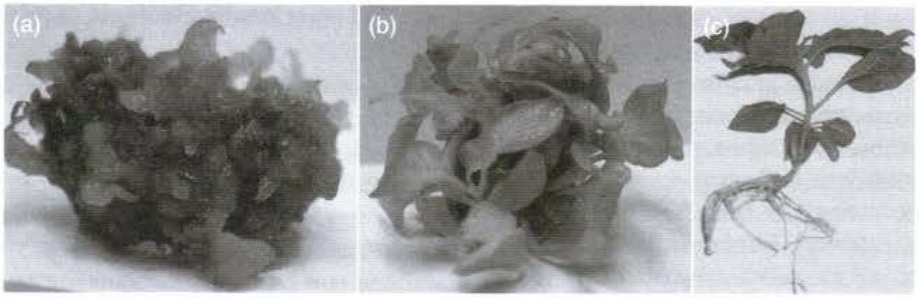

Fig. 5.4. Direct organogenesis of habanero pepper: (a) direct shoot induction from nodal segments, (b) shoot development, and (c) rooted shoots.

develop into structures that resemble zygotic embryos (i.e. bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes. Somatic embryos are used for studying regulation of embryo development, but also as a tool for large scale vegetative propagation. Studies on factors controlling *in vitro* plant morphogenesis are desirable, not only for development of improved regeneration systems, but for analysis of molecular mechanisms underlying plant embryogenesis. Somatic embryogenesis in *C. annuum* has been induced mainly from immature zygotic embryos (Harini and Lakshmi Sita, 1993; Binzel *et al.*, 1996a; Jo *et al.*, 1996) and mature zygotic embryos (Buyukalaca and Mavituna, 1996). Kintzios *et al.* (2001) used leaf explants for somatic embryogenesis in *Capsicum*. Khan *et al.* (2006) and López-Puc *et al.* (2006), working with different species, reported production of embryos using somatic bud and stem segments and hypocotyls. Harini and Lakshmi Sita (1993) developed an *in vitro* regeneration protocol from immature zygotic embryos of *C. annuum* via direct somatic embryogenesis. Buyukalaca and Mavituna (1996) reported the first protocol for regeneration of *C. annuum* cv. Ace, through somatic embryogenesis in liquid media using mature zygotic embryo explants for embryogenic callus formation. Embryos were matured and converted into plants *in vivo* and *in vitro* with an efficiency of up to 97%. Plants of *C. annuum* have been regenerated from immature zygotic embryos via direct somatic embryogenesis (Binzel *et al.*, 1996a). Histological examination indicated that secondary embryogenesis occurred directly from primary somatic embryos. Differentiation of embryos was non-synchronous, and some embryos were swollen and distorted with fasciations. More than 70% of mature normal somatic embryos germinated readily on MS medium containing GA_3 or TDZ, alone and in combination, and developed into normal plants. Mavituna and Buyukalaca (1996) induced somatic embryogenesis of pepper in an airlift and a magnetically stirred bioreactor, reporting that oxygen demand of cultures can be different at each stage of embryogenesis. Kintzios *et al.* (2000), working with young leaves of chilli pepper, observed that globular embryo proliferation depended on leaf position on the donor plant, and the somatic embryo induction was significantly affected by initial culture incubation under illumination or in darkness. A protocol for separation of somatic embryos from embryogenic suspension cultures has been reported (Buyukalaca *et al.*, 2003).

A study carried out by Steinitz *et al.* (2003) confirmed efforts to reduce persisting deficiencies in regeneration protocols reported for *Capsicum*. Embryos detached from explants and transplanted on to a growth regulator-free medium germinated; recovered regenerants were without a shoot, and some bore a single deformed cotyledon while others had no cotyledons. Regenerants lacking a shoot were generated irrespective of auxin type and across all responsive genotypes. Absence of a shoot, resulting from a failure in establishment of a normal functioning apical shoot meristem, was the principal developmental disorder precluding regeneration of normal

plants via direct somatic embryogenesis. Since stem cells of shoot meristems become established in globular and heart-stage embryos, they deduced that absence of a shoot in germinating embryos could originate from deviant differentiation at early stages of embryogeny. We agree with these. Results of others show that the majority of deformations of somatic embryos are located in the shoot meristem, and found more in the torpedo and cotyledonary stages (López-Puc *et al.*, 2006; Santana-Buzzy, unpublished). However, Khan *et al.* (2006) reported an efficient protocol of direct somatic embryogenesis from stem segments and shoot tips of *C. annuum*. All regenerated plants were normal with respect to morphology and growth characteristics. López-Puc *et al.* (2006), working with *C. chinense*, induced direct somatic embryogenesis in different explants with hypocotyls being best. Zapata-Castillo *et al.* (2007), working with different culture media and explant types induced somatic embryogenesis of *C. chinense* from embryogenic cell suspension. Ontogenesis of direct high-frequency somatic embryogenesis of *C. chinense* induced from hypocotyls was characterized by histological analysis (Santana-Buzzy *et al.*, 2009). Proembryogenic cells were originated from provascular hypocotyl cells.

5.4 Plant Tissue Culture for Pepper Crop Improvement

Expression of pre-existing variation in plants can be promoted and *de novo* variation can be induced. True to type deviations are observed in regenerated plants, this unpredictable phenomenon being termed somaclonal variation (Larkin and Scowcroft, 1981). Plant tissue culture provides techniques and procedures through which genetic variation can be generated or pre-existing variation in cells expressed. Brar and Jain (1998) reviewed the somaclonal phenomenon in plants and found that somaclonal variation is present in almost all species and affects many plant traits. *Capsicum* has not been the exception (Hossain *et al.*, 2003; Valadez-Bustos *et al.*, 2009).

Somaclonal variation in pepper

In vitro culture changes can occur in DNA of cell nuclei, extra-chromosomal or epigenetic, resulting in individuals exhibiting differences in trait levels: morphological, biochemical, or DNA sequences. The source of somaclonal variation and degree of variation in pepper depends on genotype, explant source, culture medium formulation, type and concentration of growth regulators, balance of growth regulators, morphogenetic pathway and other specific compounds, or a combination of all of them (Brar and Jain, 1998). The literature on somaclonal variations in chilli pepper is scarce. Christopher and Rajam (1994) regenerated plants of *C. annuum* and *C. praetermissum* by direct organogenesis; the cytogenetic analyses they performed produced chromosome aberrations, delayed chromosomes and anaphase bridges. Somaclonal variations can be distinguished by morphological traits (Maralappanavar *et al.*, 2000) and by random amplified polymorphic DNA (RAPD) analysis (Chen *et al.*, 1998). Tomaszewska-Sowa *et al.* (2002), working with sweet pepper genotypes, proved that direct shoot regeneration decreases probability of somaclonal variation as cytokinins present in the initial medium do not disturb mitosis or cause changes in ploidy of regenerants. DNA methylation is related to a number of heritable but potentially reversible epigenetic changes. The successful use of somaclonal variation to achieve crop improvement is, in part, dependent on its genetic stability in subsequent generations (Jain, 2001). Hossain *et al.* (2003) evaluated morphological and genetic variations in somaclones of chilli pepper derived from tissue culture. Genetic variations among somaclones were revealed by RAPD analysis. Valadez-Bustos *et al.* (2009) measured the R_0 generation obtained by organogenesis of genotypes of *C. annuum* and *C. chinense* on the basis of vegetative, phenological, and agronomic characters. A study on somaclonal variation generated from different regeneration methods, found that *C. chinense* regenerants obtained through somatic embryogenesis showed greater variability in DNA patterns analyzed with RAPD and ISSR molecular markers (Santana-Buzzy, unpublished).

Haploid culture

Haploid plant breeding is more efficient than conventional plant breeding for generation of diploid homozygous pure lines. Culture of ovules, ovaries, microspores, or anthers is a useful tool for obtaining F_1 hybrids from diplohaploid lines in one step. Lines obtained in this way are homozygous for all genes (pure lines) and have applications in plant breeding (Bhojwani and Razdan, 1997). Wang *et al.* (1973) were the first to report chilli pepper anther culture and haploid plant regeneration. Anthers with microspores at the uninucleate stage were cultured on MS medium modified with micronutrients and vitamins, and supplemented with Kin, NAA, or 2,4-D. Others have reported regeneration of haploid plants from anther culture of *Capsicum* species and hybrids (George and Narayanaswamy, 1973; Kuo *et al.*, 1973; Novák, 1974; Wang *et al.*, 1981; Sibi, 1982; Morrison *et al.*, 1986; Kristiansen and Andersen, 1993; Gyulai *et al.*, 2000; Koleva-Gudeva *et al.*, 2007). Spontaneous haploids have been reported in *Capsicum* species (Christensen and Bamford, 1943; Pochard and Dumas de Vaulx, 1979).

Sibi *et al.* (1982) developed a more successful anther culture protocol in pepper, which was further optimized by Dumas de Vaulx *et al.* (1982). Regner (1996) studied directly isolated microspore culture of bell pepper, but was not able to develop a successful culture protocol. Kim *et al.* (2008) reported high frequencies of embryo production and plant regeneration through isolated microspore culture of hot pepper. Despite its importance, few studies have been carried out to obtain haploid plantlets in hot pepper genotypes which are less responsive to anther culture. However, Supena *et al.* (2006) established an efficient doubled haploid production method for breeding hot pepper.

Protoplast culture

Protoplasts are plant cells without cell walls on a liquid medium, isolated from tissue cells selected and cultured under special conditions to produce new individuals *in vitro*. It might be possible to recover somaclonal variants, pre-existing genetically changed somatic cells of tissue donor explants, or somatic hybrids in cases of sexual interspecific incompatibility and F_1 hybrid sterility. This could also be used as a genetic transformation method, or in the uptake of a foreign genome. Few studies are available on protoplasts from *Capsicum*, as reported by Ochoa-Alejo and Ramírez-Malagón (2001). The first report was by Saxena *et al.* (1981) on bell pepper, cv. California Wonder. *C. annuum* and *C. chinense* have been the peppers in which this technique has been most studied (Saxena *et al.*, 1981; Díaz *et al.*, 1988; Murphy and Kyle, 1994; Prakash *et al.*, 1997).

Genetic transformation of chilli pepper

The genetic transformation of plants has become a powerful tool for molecular research and for cultivar improvement. The most common methods of plant genetic transformation, those employing *Agrobacterium tumefaciens*, have been applied with the highest success to a large number of plant species, including mono- and dicotyledon crops. For pepper, they remain highly recalcitrant to both *in vitro* propagation and to the *A. tumefaciens*-mediated transformation. Even though the first results on their genetic transformation were published (Liu *et al.*, 1990), the methods reported in the scientific literature are rarely consistent and repeatable (Kothari *et al.*, 2010). Pepper "recalcitrancy" represents a significant obstacle not only for the application of modern biotechnology, but for development of functional genomics studies.

5.5 Perspectives

Research and development in biotechnology over the last two decades have provided practical results that prove the usefulness of cell and tissue culture in pepper breeding (Fari, 1995). *In vitro* cell and tissue culture has become an efficient means of breeding for resistance, while increasing yield and quality

of pepper. The connotation somatic embryogenesis acquired is a consequence of its usefulness as a tool in investigation of zygotic embryogenesis, as well as being an adequate system for mass-propagation of plants when integrated with a conventional breeding program and molecular and cell biology techniques. Somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement. The broad applications of somatic embryogenesis, in basic and applied research, have motivated studies to determine *in vitro* conditions for induction of somatic embryos and their conversion into plants in *Capsicum*. Some changes suffered *in vitro* can be exploited as a source of new variation that can subsequently be incorporated into breeding programs or used to develop new varieties with important agronomic or economic traits (Bridgen, 1996). Tissue culture, in combination with molecular techniques, has been successfully used to incorporate specific traits through gene transfer.

Genetic transformation holds great promise for alleviating major constraints to crop productivity. Genetic manipulation is an attractive proposition which involves recombination of an efficient cell or tissue culture regeneration system with recombinant DNA technology. This technology has proven very useful in transfer of specific genes from other taxa, or the modified expression of specific native genes. It will allow *Capsicum* to reach the comparative transformation efficiency values achieved for other important crops. The transient transformation using *A. tumefaciens* could be a valuable tool to study pepper gene functions in a homologous system.

Future prospects for application of biotechnological tools are promising in *Capsicum*, especially for new hybrids developed by tissue culture and biotechnology methods. There has been a growing expectation that the biotechnology industry will deliver a second generation of transgenic products for more challenging traits relating to yield and yield stability, which are under complex polygenic control. The severe recalcitrance of *Capsicum* genus to *in vitro* culture has been the main reason for limited advances in employment of these techniques. However, there are important advances in development of organogenic and embryogenic systems for *in vitro* chilli pepper plant regeneration. The progress achieved in recent years in *Capsicum* has contributed to a better understanding of the recalcitrance phenomenon. This knowledge will provide an unprecedented opportunity to identify regulatory genes and networks controlling events to efficiently produce normal *Capsicum* plants from *in vitro* propagation.

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