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Foreword

The importance for humanity of peppers of the Capsicum genus began in the Americas thousands of years ago. Crops date back to 7000 years BC in Mexico, Peru, and Bolivia. With the discovery of America by Columbus, peppers, originated in Mexico and Central America, were spread throughout Europe via Spain, becoming a lucrative business for the Spanish. Around the same time, vendors in Mexico City traded different ways of preparing peppers, emphasizing fresh fruits and paprika used in many dishes from Aztec cuisine.

Among domesticated species, such as *C. annuum*, *C. pubescens*, *C. baccatum*, *C. frutescens*, and *C. chinense*, Brazil is the center of origin of peppers belonging to the *C. chinense* species. Several Latin American countries, including Brazil, are listed as first priority in the collection of *Capsicum* germplasm. Amazonia and southeastern Brazil are described as key areas when it comes to exploring the species of the genus. In these areas, there is great variability in fruits, for color, shape, size, and pungency, which still remain unexplored.

In the 1960s, the Universidade Federal de Viçosa (UFV) started a *Capsicum* seed collection program in different geographical regions of the country, on small farms and in the native forests of Brazil. From these collections, the Capsicum's Germplasm Bank was established at UFV, containing the cultivated species and many wild ones. Many of the collected accessions were morphologically and chemically characterized in various masters and doctoral theses at UFV. This collection offers unique and diverse material that helps to analyze the genetic diversity of the genus, which will take years of study in order properly to assess the potential of each accession in the improvement of cultivated peppers.

Currently, in addition to being used as a condiment, peppers are marketed as ornamental plants, opening new frontiers of improvement and selection of cultivars adapted to growing in pots. Ornamental peppers are generally of the *C. annuum* species, adapted to growing in pots by the producers themselves. The possibility of crossing with other cultivated species, in particular *C. baccatum*, *C. frutescens*, and *C. chinense*, opens the possibility of obtaining commercial cultivars that are properly suited to growing in pots. This book addresses cultivation, morphological

characteristics for growing in pots, as well as care and physiological factors that interfere with the postproduction of these plants.

In addition, it addresses several practical and theoretical aspects of the cultivation and breeding of peppers, with the participation of researchers and teachers who have been working in these areas. The book's chapters cover different subjects, such as importance and growth, cytogenetics, physiology, and postharvest of pepper fruits, genetics and plant breeding, molecular markers in pepper breeding, and tissue culture. The publication of this book adds new knowledge obtained through research conducted in recent decades.

Vicente Wagner Dias Casali

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Chapter 1
Pepper Importance and Growth (*Capsicum* spp.)

Cleide M. Ferreira Pinto, Izabel C. dos Santos, Fernanda Ferreira de Araujo, and Tania Pires da Silva

Abstract The cultivation of pepper has great importance in all regions of Brazil, due to its characteristics of profitability, especially when the producer and processing industry add value to the product, or its social importance because it employs large numbers of skilled labor. Peppers require monthly temperatures ranging between 21 and 30 °C, with an average of 18 °C. At low temperatures, there is a decrease in germination, wilting of young parts, and slow growth. Plants require adequate level of nitrogen, favoring plants and fruit growth. Most the cultivars require large spacing for adequate growth due to the canopy of the plants. Proper insect, disease, and weed control prolong the harvest of fruits for longer periods, reducing losses. The crop cycle and harvest period are directly affected by weather conditions, incidence of pests and diseases, and cultural practices including adequate fertilization, irrigation, and adoption of phytosanitary control measures. In general for most cultivars, the first harvest starts 90 days after sowing, which can be prolonged for a couple of months depending on the plant physiological condition.

Keywords Crop production • Cultivars • Fertilization • Insect control • Fungal and bacterial diseases

1.1 Socioeconomic Importance of Pepper

Approximately 89 % of total areas cultivated with peppers in the world are located on the Asian continent with the main growing areas located in India, China, Korea, Thailand, Vietnam, Sri Lanka, and Indonesia. The second most important region in

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the cultivation of peppers comprises the United States and Mexico with about 7 % of the total planted, and finally, 4 % of the cultivated area is in countries of Europe, Africa, and the Middle East (Rufino and Penteado 2006). The Thai and South Korean people are the biggest consumers of pepper in the world, consuming 5–8 g per person/day (Ribeiro et al. 2012).

In Brazil, the cultivation of peppers has great importance due to their characteristics of profitability, especially when the farmer adds value to the product, or due to social importance because employs large numbers of skilled labor. In addition cultivation of pepper allows the fixation of small farmers and their families in the countryside. The activity also allows seasonal hiring of labor during the harvest and the establishment of new processing industries, which is the key to the generation of new jobs. The major pepper-producing regions in Brazil are the Southeast and Midwest and, the main producing states are Minas Gerais, Goiás, São Paulo, Ceará, and Rio Grande do Sul. In 2012, the cultivated area with pepper in Minas Gerais was 264.7 ha with a production of 2467.3 metric tonnes. This year, the Central Supply Minas Gerais S/A (Ceasa-Minas), in all units, sold around 960 metric tonnes of fresh peppers (31 % total production), in the amount of US$ 450,000.

The Brazilian fresh pepper market presents various types, names, sizes, colors, flavors, and hotness of fruits. The Pimenta Cumari or Pimenta Passarinho (*Capsicum baccatum* var. *praetermissum*) is most common in the southeast. The Pimenta de Cheiro (*Capsicum chinense*) is the most widely cultivated especially in the northern part of the country, distinguished by a great variety of fruit colors ranging from yellow, milky yellow, light yellow, deep yellow, orange, salmon, red, and even black. Peppers with lower production, but very important, *Capsicum chinense*, is the Pimenta Murupi, whose major producers are located at Amazonas and Pará states. Pimenta de Bode is grown mainly in the western region of Brazil. The Pimenta Malagueta (*Capsicum frutescens*) is grown all over the country, but there are major production areas in the states of Minas Gerais, Bahia, and Ceará. In the latter state, there are large areas for production of Tabasco (Pinto et al. 2006a,b).

1.2 Climate Requirements and Growing Season

Climate factors have great influence on seed germination, development, and fruiting of pepper plants. The pepper requires high temperatures throughout the cycle and as a tropical plant it is sensitive to low temperatures and is frost intolerant. The ideal average monthly temperatures range between 21 and 30 °C, with an average of 18 °C. At lower temperatures, there is a decrease in germination, wilting of young parts, and slow growth. At temperatures above 35 °C, the fruit set is adversely affected, especially with low air humidity or dry winds. Temperature affects the fruit quality, especially the sugar content and vitamin C, as well as the intensity of the red and yellow colors, which are in general greater at high temperatures. Low temperatures can also affect the pungency of the fruit. Reseaches show that fruits of

pepper grown in spring and summer are more pungent than those plants cultivated in autumn–winter (Estrada et al. 1999; Kirschbaum-Titze et al. 2002).

In Brazil, the peppers are grown both in hot climates and under cold weather. The sowing season is contingent upon the local climatic peculiarities. In the mountain regions with an altitude above 800 m and mild temperature, sowing is done in the months from August to February; however, a more convenient season is from September to November due to the requirement of the species for high temperatures. In regions with mild winters, especially those of lower altitude (below 400 m), sowing can be done all year around (Pinto et al. 2006a, b).

In the pepper-producing regions of the south and southeast Brazil, from August to January are the months indicated for sowing. In the pepper-producing regions of Minas Gerais state with mild temperature, seeding occurs from August to February, although the most suitable period is from September to November. In the western part of the country, the cultivation of peppers can be done all year. Normally, sowing is done in November, but can extend up to the end of January. In the north and northeast of Brazil, one should avoid planting in the rainy season, which makes the tillage, cultivation, and pest control difficult.

1.3 Soil Preparation and Fertilization

Peppers do not grow well in heavy or compacted soils; the most suitable soils are those of medium (clay–sandy) texture, and sandy soil should be avoided.

Peppers are susceptible to attack by pests and disease; it is not advisable to plant peppers in areas that have been grown in the previous year with tomato, potato, eggplant, gilo, or peppers that belong to the same pepper family. Likewise, planting in areas with pumpkin, squash, and zucchini should be avoided, which can also be sources of pests and diseases. The planting of these crops in nearby areas, as well as planting continuously for years on the same site should be avoided. Crop rotation with corn or beans in alternate years is recommended.

Good soil preparation in needed to facilitate setting and rooting of plants. Sloppy areas should be delimited by contour lines spaced within 20–30 m of each other. Plantlets should be spaced from 1.20 to 1.50 m.

In almost all of Brazil, the soils are generally more acidic than ideal for the development of pepper, which requires pH ranging between 5.5 and 6.5. High acidity of the soil can cause a lot of problems for farming, such as high levels of aluminum or manganese, which drastically reduces production; in lower pHs there is a deficiency of calcium, magnesium, phosphorus, and other nutrients needed for proper growth, development, and yield of plants.

It is common for farmers to neglect proper soil pH correction by liming and they also do not do any chemical analysis of the soil. In contrast, it is common to apply heavy mineral fertilizers in acidic areas, jeopardizing the proper use of supplied nutrients, plant development, and increased cost of production.

It is necessary to apply lime to raise the base saturation to 70–80 % and minimum content of magnesium of 0.8–1.0 to $cmol_c/dm^3$ (Casali and Fontes 1999). The limestone should be applied on moist soil, about 15 days before planting, spread evenly over the area to be planted with incorporation at 15–20 cm deep. When doses are greater than 2 t/ha, it is recommended that 50 % of lime be applied before plowing and 50 % after plowing, and in doses less than 2 t/ha, the limestone must be applied before plowing. The finer the particle size and the higher the neutralizing value of the lime, the greater will be the relative power of full neutralization and the faster will be the effects in the correction of acidity.

1.4 Aspects of Mineral Nutrition and Fertilization in Pepper Plants

The visual symptoms of mineral deficiencies presented by pepper plants are important to diagnose nutritional status in field conditions. Some more specific symptoms of nutrient deficiency in pepper (Monnerat 1984; Balakrishnan 1999) are presented below:

(a) *Nitrogen* (*N*): Symptoms of N deficiency are uniform yellowing of leaves, including the veins, being more pronounced in older leaves. When the persistence deficiency occurs, plants show reduced growth and reduced leaf size. In addition, there is reduction of flowering and fruiting. Deficient plants also have poorly developed root systems. High levels of N can cause excessive growth of the plant canopy, inducing the appearance of apical rot in fruits, especially in the warmer periods.

(b) *Phosphorus* (*P*): Leaves with deficiency can be reduced in size and deeper green color (blue-green) with intervein necrosis in the middle part of fully developed leaves. Browning at the lower portion of the plants and reduced root growth can also occur. Due to the advancement deficiency, irregular interruptions appear on the leaf surface, the edges of older leaves become chlorotic, and severe leaf fall occurs. There are few fruits because of the dropping of flowers.

(c) *Potassium* (*K*): The symptoms of K deficiency are smaller plants with fewer leaves. The upper third of leaves becomes compactly arranged. There are also chlorosis and necrotic scores between the leaf veins. Due to the advancement deficiency, necrosis arises at the edges of the youngest leaves, expanding to the petiole, causing leaf drop. The excess of K reduces the absorption of calcium and magnesium, which makes them more susceptible to the apical fruit necrosis.

(d) *Magnesium* (*Mg*): Plants with Mg deficiency have reduced size. The fully developed upper third leaves become chlorotic and the younger with twisted blades. Due to the advancement deficiency, necrosis can be found between the veins. The fruits have reduced number and size. The root system does not develop normally.

(e) *Calcium* (*Ca*): Plants with Ca deficiency are small, compact, and with a reduced number of leaves. The young leaves have reduced development, becoming wrinkled and curled, and chlorotic in the base and between the veins. Due to the advancement deficiency, the leaves become necrotic and fall because of necrosis of the peduncle. Total drop of flowers may occur and, consequently, no fruiting. There is reduced formation of small fruit and brown staining on the bottom of the fruit.

(f) *Sulfur* (*S*): Plants with S deficiency are short and show yellowing of leaves starting at the base, gradually expanding to the tip. Due to the advancement deficiency, all leaves become yellow and there is no formation of fruits. The leaf blade has a wavy appearance, as if there were an uneven growth of veins and leaf blade. The fruits exhibit a pale green color.

(g) *Boron* (*B*): Plants with B deficiency are small and compact, due to the death of the growing apex. Old leaves become curved inwards and the new leaves are reduced in size, wrinkled at the base, translucent, and apparently thicker. The stems and leaves become brittle. Complete drop of flowers can occur and not fruit formation. The root system is severely affected, being undeveloped, with necrosis in the extremities.

(h) *Iron* (*Fe*): Plants with Fe deficiency show yellowing of younger leaves like the deficiency of S and N, however, the veins of the leaf blade are greener and the plants have higher height and number of leaves than plants with a deficiency in those nutrients. Due to the advancement of deficiency of Zn, all leaves become chlorotic. The fruits develop a pale yellow color.

(i) *Zinc* (*Zn*): Plants with Zn deficiency are small with a narrow leaf blade with discoloration between the veins. Extreme deficiency causes rosette-like plants.

The diagnosis of the symptoms of nutritional imbalance in the field, using the visual method is not always easy and accurate. Due to lack of equilibrated fertilization, more than one symptom often occurs. Leaf analysis of the nutrients helps to solve the nutritional problems (Fontes 2001). Some references on the levels of nutrients in pepper are presented in Table 1.1.

It is common to find, especially in Zona da Mata of Minas Gerais state, most of the pepper farms apply certain nutrients in excess or use unbalanced fertilizer, which can cause loss of fertilizer as well as environmental problems. Additionally, several foliar fertilizers containing nitrogen, which has caused great nutritional imbalances, are applied on the crops (Pinto et al. 2006a, b).

Fertilizer requirements for the culture of pepper should be based on the chemical characteristics of the soil, the type of irrigation used, and expected productivity. Regarding the latter, one should take into account the quantities of nutrients extracted by the crop. As such, amounts extracted are most often unknown; recommendations are usually based on various authors used as references for the addition of nutrients. Often, the amount of fertilizer to be applied at planting is determined based on published reports from some Brazilian states or regions (Table 1.2). Most of these reports are used for sweet pepper (*Capsicum annuum*). The total fertilizer indicated should be evenly distributed in the row.

Table 1.1 Interpretive data of foliar nutrients levels suggested for pepper

	Level		
Nutrients	Low	Normal	High
Macronutrients	dag/kg		
N	3.0–3.49	3.5–5.0	>5.0
P	0.18–0.21	0.22–0.7	>0.8
K	3.0–3.49	3.5–4.5	>4.5
Ca	1.0–1.29	1.3–2.8	>2.8
Mg	0.26–0.29	0.3–1.0	>10
Micronutrients	mg/kg		
B	23–24	25–75	>75
Cu	4–5	6–25	>25
Fe	50–59	60–300	>300
Mn	40–49	50–250	>250
Zn	18–19	20–200	>200

Source: Junior Jones et al. (1991)
Note: Period of sampling: at the third end of cycle
Number of leaves: 25 (collecting leaves of alternate plants, walking zigzag field within homogeneous areas). Location and type of leaf: the upper canopy and fully expanded leaf

Table 1.2 Doses of N, P_2O_5 and K_2O recommended for fertilization of pepper in the Zona da Mata of Minas Gerais, São Paulo, and Distrito Federal

	N	Level[a]	P_2O_5	K_2O
State or region	Dose (kg/ha)			
(a) Zona da Mata of Minas Gerais state	60	Low	300	240
		Medium	240	180
		Good	180	120
(b) São Paulo state	40	Low	600	180
		Medium	320	120
		Good	160	60
(c) Distrito Federal	150	Low	400–600	240
		Medium	200–400	180
		Good	100–200	120
		Very good	50	–

Source: (a) Pinto et al. (1999), (b) Raij et al. (1996), (c) EMATER-DF (1987)
[a]Level of P and K according to soil analysis

In the Zona da Mata of Minas Gerais, it is suggested to apply 20 t/ha of bovine manure or 5 t/ha of chicken manure at the planting row. For mineral NPK fertilizer, should use the recommendation in Table 1.2 (Pinto et al. 1999). Certain nutrients, such as K, and especially N, are subject to losses in the soil after application. Therefore, it is recommended to split N and K fertilization. Nitrogen fertilization should be done with adequate humidity in the soil, applying 60 kg/ha N each time, which corresponds to 300 kg/ha of ammonium sulfate or 140 kg/ha of urea, in the following periods: (a) at flowering, (b) the maturation of the first fruits, (c)

after 30–45 days of ripening of the first fruits, and (d) 30–45 days after the third application; the latter can be suppressed if the plants present good development and no yellowing of older leaves. Apply 50 kg/ha of K_2O, which corresponds to 80 kg/ha of potassium chloride together with the first N fertilization at flowering.

In São Paulo state, fertilizers are applied 10 days before the transplanting, at row, based on the recommendations on Table 1.2. Apply 10–20 t/ha of bovine manure or 1/4 of the quantities of chicken manure, 1 kg/ha of boron, 3 kg of zinc, and 10–30 kg/ha of sulfur. During plant growth, it is recommended to apply 80–120 kg/ha N and 80–120 kg/ha K_2O, parceling out four to six times. Smaller or larger quantities depend on the soil analysis, leaf analysis, cultivar, expected yield, and crop system (greenhouse or open field) (Raij et al. 1996).

At the Distrito Federal region, organic fertilization is recommended with 30 t/ha of bovine manure or 10 t/ha chicken manure in the row and mineral fertilization adopts the recommendation in Table 1.2 as recommended by the soil analysis (Emater-DF 1987). Also 2–4 kg/ha of boron, 2–3 kg/ha of zinc, and 10–30 kg/ha of sulfur are suggested. Up to the flowering stage, fertilization with nitrogen fertilizer is made during fruit set with a mixture of N and K at intervals of 30–45 days. Normally 20–50 kg N/ha and 20–50 kg/ha K_2O are used (Fontes and Ribeiro 2004).

Deficiencies of zinc and boron, if any, can be corrected by foliar fertilization, and application of 300 g of zinc sulfate for each 100 L of water or 100 g of boric to 100 L of water is recommended.

1.5 Seedling Production

Seeds are preferably sown in trays of 128 cells, filled with commercial substrate, containing two or three seeds per cell. The trays should be placed in a protected environment to prevent the entry of insects. Trays are maintained above the ground (about 80 cm). In addition to facilitate the realization of cultural practices, the suspended trays favor pruning of the root system by dry air (natural pruning), which occurs when the main root reaches the bottom of the cells and stops its growth. Because of the main root death, there is the emission of secondary roots, providing balance between the shoot and root system. Moreover, the lack of roots below the tray facilitates removal of the seedlings during transplanting, avoiding injury that facilitates infection by fungi and bacteria from the soil (Andriolo 2000; Minami 1995; Pinto et al. 2004). When the seedlings have at least two true leaves, the less vigorous plantlets are cut off, leaving only one seedling per cell.

Although polystyrene trays are still the most used, polyethylene plastic is already used in the production of tomato seedlings; they have good market acceptance and there is an increasing tendency to replace the polystyrene for pepper plantlet production (Faria Júnior 2004). Such trays have the advantage of being nonporous, preventing chemical and biological contamination. Also, the trays can be washed with high pressure water with greater efficiency. The trays can even be sterilized with hot water, avoiding chemicals, as is currently done.

Some producers still perform the production of seedlings in plastic bags with a volume between 150 and 250 cm^3, filled with a mixture of three parts soil previously treated with limestone, if necessary, after chemical analysis and a portion of bovine manure.

The production of seedling nursery beds is one of the oldest and cheapest methods, with less farmers still using it. The beds are 1.0–1.2 m wide and 0.20–0.25 m high. They use an average of 2–3 g of seeds per square meter of bed. After the distribution, the seeds should be covered with a thin layer of soil. Placing a cover of dry straws or grass prevents the impact of water drops or rain to reduce germination and seedling emergence. The seedlings should be protected from strong sunlight, reducing the incident radiation by 50 %. It is necessary to remove this cover gradually, as the seedlings grow, so they can adapt to full sun. Cultural practices such as irrigation, fertilization, and other practices should be performed as recommended for seedlings produced in trays or plastic bags.

1.6 Planting Seedlings In Situ (Transplant) and Spacing

Hardening of the seedlings is conducted 7–10 days before transplanting, with gradual reduction of applied water and, if possible, increased insulation to reach full light of the sun. This procedure aims to adapt the seedling to the local conditions in the field. The reduction of irrigation increases the dry matter content in the plant, which favors the development of stress resistance to transplanting. The decrease in turgor also facilitates handling and reduces mechanical damage. However, irrigation should be done just before transplanting.

Transplanting is done when the seedlings have developed six to eight leaves, and are about 10–15 cm high, which occurs approximately 50–60 days after sowing for most species of peppers. The seedlings are removed from the trays by holding the neck and pulling it out. The entire substrate must be adhered to the roots. For seedlings grown in plastic bags, cut off the plastic before planting.

The planting of pepper can be done either in small pits or rows. However, planting in rows allows easier application and incorporation of fertilizers. The planting in rows helps to control soil erosion.

At transplanting, seedlings must be placed at the same depth they were before transplanting, relative to the soil surface. The spacing of planting rows or pits is defined depending on the type of pepper, region, or time of planting and crop cycle (Table 1.3).

Several pepper plant cultivars can be conducted as semi-evergreen shrubs with cycles greater than 12 month, requiring spacing ranging from 1.20 to 1.50 m between rows and 0.70 to 1.00 m between plants. Very narrow spacing between plants retains more moisture and decreases the incidence of pests, mainly mites. On the other hand, this facilitates the attack of diseases and cultivation practices and harvesting. At Zona da Mata of Minas Gerais State, spaces of 1.20–1.50 m are used between the rows and 0.80–1.00 m between plants for Pimenta Malagueta (Pinto et al. 2006a, b).

Table 1.3 Spacing, time of planting, and growing cycle of the main types of peppers in different regions of the country

Region	Type of pepper	Spacing (m×m)	Population (plants number/ha)	Time of sowing	Crop cycle (months)
São Paulo	Girl's finger pepper	1.50×1.00	6,500	December to January	12
Goiás and DF	Smell pepper, Goat pepper	1.20×0.80	10,400	November to January	12
Goiás and DF	Cumari pepper	1.20×0.80	10,400	November to January	12
Goiás and DF	Chili pepper	1.50×1.00	6,500	November to January	12
Catalão-GO	Jalapeño pepper	1.00×0.33	30,000	February to March	6 or 7
Paraopeba—MG	Chili pepper	1.00×0.80	12,500	December	12
Pelotas—RS	Girl's finger pepper	0.80×0.50	25,000	August	8
Ceará	Tabasco pepper	1.20×0.60	13,889	August to March	8

Source: Cruz (2004)

1.7 Irrigation

The use of irrigation is a determining factor in the commercial production of pepper in regions with little or poorly distributed rainfall. The total water requirement for the crop is variable, because in addition to the weather conditions, it depends on the type of pepper and the duration of the development cycle. In general, ranges from 500 to 800 mm, and may exceed 1,000 mm long cycle cultivars. The daily need for water varies from 3 to 10 mm/day in peak demand of the crop (Marouelli and Silva 2008).

From sowing to the point of transplanting in trays, watering must be done preferably within hours of middle temperatures, from one to three times per day. The amount of water for irrigation should be sufficient when the water starts running off at the bottom of the cell. In a bed nursery, irrigation should be lighter and more frequent, daily or on alternate days. From transplanting until the recovery of growth lasts about a week, and watering should be daily or every three days depending on soil texture (Marouelli and Silva 2008). Water stress during the period between the initial plant establishment and full flowering (vegetative stage) has a negative effect on the production of pepper (Bosland and Votava 1999), even when the water supply at the fruiting stage is appropriate. Excessive irrigation favors higher occurrence of diseases and increase of nutrient leaching, especially nitrogen as nitrate. During fruiting from full bloom until the beginning of fruit maturation is the most critical period regarding the deficiency of water, especially during the full flowering and

fruit set stages. Deficiency of water may cause abortion of flowers and fruits, and reduce the size of ripe fruit (Nuez Viñals et al. 1996). Furthermore, the lack of water during the initial stage of fruiting can restrict the translocation of calcium and favor the occurrence of blossom-end rot (Bosland and Votava 1999).

In addition to supply water to plants at the time and in the correct amount, the application form is crucial to the success of the crop (Marouelli and Silva 2008). Pepper crops in Brazil are mainly irrigated by sprinklers and on a much smaller scale by drip irrigation. The choice of system should be based on the analysis of factors such as: initial cost and maintenance of the system, soil type, topography, climate, plant productivity, quality and quantity of water available, use of hand labor, water and energy consumption, and incidence of pests and diseases.

The main advantage of sprinkling compared to surface irrigation systems is the possibility of being used in various types of soil and topography, as well as having lower cost compared to drip irrigation systems. However, it favors higher incidence of foliar diseases, as well as washing off the pesticides, and provides high humidity on the canopy, especially when irrigation is frequent (Nuez Viñals et al. 1996).

Among the systems of surface irrigation the most appropriate is in the row, and is used mainly by small producers. One of the main advantages is the low initial cost, much less than the cost of sprinkler systems and drip irrigation. Another benefit is the wetting only the soil surface reducing foliar diseases. As a disadvantage, the system is not suitable for soils with high permeability such as sandy soils or with steep slope.

Some producers of pepper in the state of Ceará have adopted the use of drip irrigation. The main advantage of the system is the application of water in a localized manner in the root zone, without reaching the leaves and fruits, reducing the occurrence of foliar diseases and evaporation losses. The conservation of water and energy (20–40 %) and drip fertilization make an attractive system for the cultivation of pepper. Fertilizers such as nitrogen and potassium may be applied through irrigation water, minimizing nutrient losses and maximizing fruit yield. The two main disadvantages of drip irrigation are the high cost and the risk of clotting. The cost is directly related to the row spacing, which determines more or less what is spent on the drip lines. Thus, the system is more suitable for peppers grown with row widths above 100 cm. The presence of solid particles in the water and organics as well as carbonates, iron, and bacteria and the formation of insoluble precipitates within the tubing, are the main causes of clotting. These problems can be overcome by using filtering systems and performing chemical treatment of the water.

1.8 Weed Management

In addition to hosting insects and plant pathogens of pepper plants, weeds compete for growth factors such as light, water, and nutrients. To control weeds, it is usual to use a combination of management methods that can be preventive, cultural and mechanical control, actions that interfere with the life cycle of the weeds, and

changing the competitive balance in favor of the crop. Chemical control is not recommended for pepper, because of the lack of registration of herbicides by the Brazilian governing authorities.

Preventive management should avoid establishment species such as *Cyperus rotundus*, whose population rises dramatically (Santos et al. 2006). It is recommended to use seeds of good origin and free seedlings of weeds, and clean shipping trucks, agricultural machinery, and implements. Also one must be sure of the origin of organic fertilizers, especially manure from cows. Avoid the use of organic material from areas infested with *Cyperus rotundum* or pastures treated with Tordon herbicide (picloram + 2,4-D). Picloram residue present in manure can affect the initial development of pepper. The practice to use green mulching at off-season periods is also an important tool to prevent premature establishment of weeds. In ecological or organic farming systems, these methods should be privileged because they have less impact on the environment. All cultural practices that encourage rapid establishment of the pepper crop with good soil preparation, fertilization, proper spacing, directed irrigation, use of healthy and vigorous seedlings, and crop rotation contribute to weed management.

Soil tillage and irrigation stimulate germination and development of weeds. It is recommended to prepare the soil 2–3 weeks before transplanting the pepper, to allow germination and growth of weeds in the area (four to six true leaves), and post-emergent control through the application of herbicides nonselective of contact action, such as diquat and paraquat or systemic glyphosate (Pereira 2008).

Because pepper plants present a long cycle with wide spacing between rows of planting, intercropping with mulch is an alternative for weed management. Mulching used at earlier stages of flowering can provide nutrients in the same cycle of cultivation, especially nitrogen if a species with rapid decomposition is used. Santos et al. (2004a) found that *Pueraria phaseoloides* and *Calopogonium mucunoides* are promising for use in consortium with pepper, by offering a greater diameter of the plant canopy and higher fruit yield of chilli in relation to *Crotalaria breviflora*, *Dolichus lablab*, and the dwarf bean *Stizolobium deeringianum*.

Mechanical weed control is the most widely used by small farms all over the country. The critical period of weed completion is two-thirds of the crop cycle of pepper, which is 12 months for Chilli and Pimenta de Cheiro and 8 months for Tabasco and Dedo de Moça pepper.

Among the mechanical methods periodic cleaning causes less harm to the environment, because in addition to not disturbing the soil surface, it results in the formation of a mulch layer that protects the soil from the direct impact of sun and rain. During the decomposition of vegetation, allelochemical compounds have a beneficial effect on the control of some weed species. The management of weeds by mowing plants is especially interesting in organic farming, because it keeps the diversity of vegetation, one of the precepts of organic agriculture (Santos et al. 2004b).

Annual and perennial weed species grown from seeds can be controlled mechanically with rototillers, which basically break the intimate contact of the seedling with soil, causing their death or delaying their initial growth. Seedlings that are more developed with greater accumulation of reserves can survive the impact of

cultivation and growing back. Thus, this operation should be performed at the right time to ensure efficient control (Alves and Pitelli 2001). Perennial species that propagate in a vegetative way, like *Commelina* spp., require more attention because they can be favored by mechanical cultivation. The effectiveness of mechanical control will depend on the amount of reserves stored by the weeds. Therefore, the effectiveness of the first control must be accompanied in order to decide which strategy should be adopted, which can be based on two principles: (a) conducting mechanical successive crops in short intervals of time, which forces new growth, depleting the most of the reserves of the plant and preventing regrowth (Alves and Pitelli 2001); and (b) withdrawal of propagation material from the area through manual labor, which applies only to small areas.

1.9 Pests and Management Strategies

Mites and insects associated with pepper can cause direct and indirect damage. Indirect damage occurs when mainly aphids and trips are vectors transmitting viruses. Direct damage occurs when pests damage the roots, stems, flowers, and fruits such as whitefly, sweet pepper fly, caterpillar weevils, and others. The use of preventative control measures must be considered from planting and, when appropriate, additional pest control should be used.

1.9.1 Main Mites and Insects Causing Damage

The mite, *Polyphagotarsonemus latus* Banks (Acari: Tarsonemidae), is a major pest of hot peppers, with frequent occurrence in most areas where this vegetable is cultivated in Brazil's producing regions (Venzon et al. 2011). The mite preferably attacks the bottom face of the first leaf and at the main gowning buds of the plants. The attacked plants have bent-down leaves, dry and bronzed color, and finally the leaves may fall prematurely. Moreover, the plants usually have deformed fruits and flowers. These symptoms can manifest rapidly, indicating that a small number of mites is enough to cause economic damage, because the *Capsicum* species have low tolerance to mite attack. The symptoms can be confused with phytotoxicity, viruses, or micronutrient deficiencies, especially the lack of boron, which causes death of the apical growing bud. The development of the mite is favored by the combination of high temperature and humidity, associated with low luminosity. The mite disperses into the field by the wind and the contact between leaves of infested and healthy plants.

Other species of mites that occur in the culture of peppers, but with secondary importance (Venzon et al. 2011), are the spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) and red mite *Tetranychus ludeni* Zacher (Acari: Tetranychidae) and *Tetranychus evansi* (Baker & Pritchard). The spider mite can

appear in white, green, orange, and red colors and has two black spots on the back. The red mite has a very deep red color. Both species live on the bottom side of leaves, where they spread their webs and lay their eggs. The damage caused by these species is similar and causes widespread discoloration of leaves (veins being maintained green). High temperatures and low humidity favor the development of spider and red mites.

The green aphid *Myzus persicae* Shulzer (Hemiptera: Aphididae) and the cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) are the main species of aphids that attack pepper plants (Venzon et al. 2011). The adults of *Myzus persicae* measure approximately 2 mm in length with the apterous form in bright green color and the winged form with green color, head, antenna, and black thorax. The cotton aphid color can vary from light yellow to dark green, depending on the nutritional quality of the plant or environmental factors. The two aphid species are frequently found on the underside of leaves and attack the leaves and young branches of pepper plants, and the cotton aphid also attacks the flower buds and flowers. The leaves become curled and wrinkled, and shoots are curved and flattened. Due to continuous suction of the sap, retardation of plant growth can occur.

The continuous suction of aphids causes elimination of sugary sap, which the leaves turn sticky. In this environment, development of fungi occurs, especially from the genus *Capnodium* that can cover leaves and branches giving a dusky dark appearance, known also as dark mold. This mold compromises plant photosynthesis and the commercial value of fruit or makes them improper for marketing. In addition to these damages, aphids can transmit several viruses for pepper plants (PVY/PepYWV, CMV). Virus-infected plants show reduced growth, wrinkled leaves, chlorosis, and, consequently, reduced fruit quality, and yield losses can occur.

The main species of thrips associated with the culture of pepper are *Thrips palmi* Karny and *Frankliniella schultzei* Trybom (Thysanoptera: Thripidae). *Thrips palmi* is a species of insects yellowish to light brown that measure about 1 mm in length. The *Frankliniella schultzei* species have varying color from brown to black and measure approximately 1.4 mm in length; young forms have a lighter color than that of adults.

Thrips prefer young plants and grow best during low rainfall periods. The insect sucks the sap in the leaves, shoots, and buds, using your appliance scraper. The attack causes yellowing, deformation of leaves, and fall of flowers. The leaves may exhibit deformities with several white spots scattered across the surface. The scraped leaves favor the entry of air and become necrotic and brittle. Thrips also feed on the flowers, which may compromise the production of fruits.

The infested fruits become deformed and dull. Thrips may also cause indirect damage by transmitting tomato spotted wilt virus (Tospovirus). The most common symptoms of this viral disease occur mainly in growing buds: yellow spots on leaves forming mosaic, green strips in the veins, concentric rings on the leaves, stopped growth, and deformation of fruits (França et al., 1984).

The whitefly *Bemisia tabaci* (Gennadius; Hemiptera: Aleyrodidae) despite being called flies are sap-sucking insects, the same as aphids (Venzon et al. 2011).

Generally, whiteflies prefer to attack younger plants. These insects fly fast and can be carried by wind current infesting other crops.

Direct damage is due to excessive suction of the sap, when high insect populations occur in the field. However, when whitefly inserts its mouthparts into the leaf, the saliva can cause physiological changes in the plant, which can compromise its development and productivity. There are no reports of damage to pepper fruits as occur in tomato. Another type of direct damage is the growth of soft mold on all parts of the plant, including fruit.

However, the main damage caused by whiteflies is indirect and related to the transmission of viruses to the plants (Villas Bôas and França 2008; Venzon et al. 2011). The tomato severe rugose virus (ToSRV) from the genus begomovirus and Geminiviridae family was detected in pepper plants. These viruses can cause up to 100 % loss of culture and the more compromising infection occurs in the first weeks after transplanting. Plants can present different symptoms, such as yellowing of the leaf veins, shriveling leaves, and stunted plants. Because of this, when a severe infection occurs, the plant cannot produce or even has premature death.

The whitefly acquires the virus while feeding on a previously infected plant. Then, the fly can infect other plants and contribute to other individuals acquiring the virus. Therefore, it is important to remove the plants when the presence of the virus is confirmed.

The *Symmetrischema dulce* (Polvoný; Lepidoptera: Gelechiidae) is one of the most important pepper plant pests (Venzon et al 2011). The adult moths are gray-dark with a light-brown head whose length can reach up to 6 mm. Eggs are deposited in immature fruits, buds, and stems, alone or in groups of 4–6 eggs. The larvae have pinkish color, with a darker head and measure 5–7 mm in length; they live inside growing buds and fruit. In fruits, the larvae feed on the seeds until the last larval instar, when they feed on the pulp of the fruit, then open a hole for their departure. This promotes deterioration of the fruit by fungi, bacteria, and maggots. In the pupa stage, the pepper-fruit-borer usually remains in the soil until reaching adulthood. Their life cycle can last 60–65 days (Venzon et al. 2011).

The flies *Neosilba* sp. (Diptera: Lonchaeidae) oviposit in healthy fruits or on the exit holes of the larvae of the pepper-fruit-borer *Symmetrischema dulce*. The adult flies are black with shiny color and transparent wings and measure 4–6 mm in length. The larvae are white, with no apparent head, vermiform appearance, and measure 7–9 mm in length. The larvae feed inside the fruits, favoring their decay.

At the beginning of the attack, the fruits have yellowish stains resulted from the galleries formed due to the pulp removal by the borers. After maturation, fruit shows dark spots and galleries. In severe attacks, many fruits fall on the ground. When the output of the caterpillar of the pepper-fruit-borer occurs, there is a formation of a hole in the fruit. In addition, this enables the entry of decomposing microorganisms and the hole is usually used by sweet pepper fly for oviposition inside the fruits.

(a) *Biological control*—undertaken by various species of natural enemies (predators). The species *Cycloneda sanguínea* L., *Harmonia axyridis* (Pallas), *Eriopis conexa* (Germar), and *Hippodamia convergens* (Guérin-Méneville) (Coleoptera:

Coccinellidae) are the most common ladybugs in the pepper crop. These species are predators that preferentially feed on aphids, but also prey on mites and other insects.

Predatory mites of the family Phytoseiidae are the main natural enemies of plant mites (Moraes 2002). In the culture of pepper, one of the main natural enemies associated with mites is *Amblyseius herbicolus* (Chant) commonly found in plantations in the Zona da Mata of Minas Gerais (Venzon et al 2011). This predator has a high consumption capacity of all stages of mite development and prey on isolation up to 35 adults, 65 larvae, 75 pupae, and 64 eggs per day (Venzon et al. 2011).

Species *Chrysoperla externa* (Hagen) and *Ceraeochrysa cubana* (Hagen; Neuroptera: Chrysopidae) are commonly found in pepper crops. These are predators only at the young stage, when feeding on various arthropods, such as lepidoptera, mites, aphids, mealybugs, whiteflies, and others present in the culture of pepper.

Syrphid are fly predators belonging to the family Syrphidae, order Diptera, consisting of several important species of predators of aphids. These insects have been found associated with aphids *M. persicae*, *A. gossypii*, and *M. euphorbiae*, often in the pepper crop at Zona da Mata of Minas Gerais, especially where the use of pesticides is absent (Venzon et al. 2011). The association of syrphid with weeds was also verified, whose inflorescences can provide pollen and nectar in the areas of pepper fields. Therefore, selective weeding, which keeps away some species of weeds able to provide food for the adults, can help in pest control of the pepper crop.

The species of predatory bugs belonging to the families Anthocoridae, Geocoridae, Nabidae, Miridae, and Reduviidae (Hemiptera) can be considered among the most abundant and important for the culture of pepper. In families Anthocoridae and Geocoridae are species of tiny predators responsible for the control of mites, thrips, whiteflies, and aphids. The Anthocoridae family, the main predator species that occurs belongs to the genus Orius, also known in some regions as the pirate bug. For the Geocoridae family, bugs of genus Geocoris are the most common prey.

The families Nabidae, Miridae, and Reduviidae can contribute to the control of pests such as caterpillars from medium to large, stinkbug pests, and eggs of moths, flies, and other insects. All these bugs are abundant predators in the pepper crop and are often mistaken as pests by farmers.

The biological control by parasitoids is done by several species of microhymenoptera parasitoids that are associated with pests of pepper crops. Among these, the most important are associated with aphids *Aphidius colemani* (Viereck) and *Lysiphlebus testaceipes* (Cresson; Hymenoptera: Braconidae) (Rodrigues and Bueno 2001; Sampaio et al. 2001).

The genus *Encarsia* spp. (Hymenoptera: Aphelinidae) is one of the main natural enemies of whitefly. The species of the families Ichneumonidae, Braconidae, and Pteromalidae are parasites of the caterpillars of pepper.

Generally, the parasitoids are wasps with length from 1 to 3 mm and coloration that can range from light yellow to dark metallic green. The adults lay their eggs in or on the body of another insect, using it as a host for the development of their larvae.

The adult parasitoids feed on nectar and/or pollen, maintaining vegetation that provides these features contributing to the establishment of populations of parasitoids in the cultivated area.

(b) *Alternative products*—Herbal extracts with potential insecticide have been used in production systems where the use of pesticides is not allowed. One of the most researched plant species for pest control is the *Azadirachta indica* A. Juss (Meliaceae), known as neem. The azadirachtina, found mainly in the seeds of neem, is the main component responsible for the toxic effects on insects. The effects of azadirachtina on insects include repellency, feeding inhibition, growth arrest, interference with metamorphosis, sterility, and anatomical abnormalities (Martinez 2002; Venzon et al. 2011). Neem has the advantages of being practically nontoxic to humans and can be rapidly degraded in soil and plants. Neem-based products can be used to control some pests of pepper such as aphids and whitefly and also have potential for the control of pepper-fruit-borer (Venzon et al. 2011). On the other hand, it is noteworthy that the use of these products must follow the technical recommendation, avoiding the continuous use of products and concentrations above those recommended, because neem may negatively affect some natural enemies present in the agro-ecosystem of pepper. There are several neem products available on the market. However, few are standardized and contain the amount of active ingredient reported on the product label (Venzon et al. 2011).

(c) *Alternative products—phytoprotective syrup*. The phytoprotective syrup can be used to control pests and diseases. For the culture of pepper, lime sulfur, obtained from heat treatment of sulfur and quicklime, is indicated for the control of white mites. Results of experiments conducted at Agricultural Research of Minas Gerais—EPAMIG Zona da Mata, showed the efficiency of this syrup at a concentration of 1.0 % for white mite control in pepper (Venzon et al. 2011).

(d) *Cultural and mechanical control—crop rotation*. Whenever possible, crop rotation should perform the alternate planting of crops preferentially with nonsolanaceae species. This practice aims to make it difficult for pests to complete their life cycle successive times in the same area.

(e) *Cultural and mechanical control*—Collection and disposal of damaged fruit. An effective measure to reduce the populations of pepper-fruit-borers can be achieved by scavenging and destruction of fruit with symptoms of attack and those found under the plants. It is recommended to bury fruits collected at least 30 cm deep.

(f) *Cultural and mechanical control—elimination of plants with signs of viruses*. When the presence of plants with symptoms of viral disease is detected, the

plants should be removed from the cultivated area. As there is no effective measure control for viruses, the removal of infected plants can prevent the presence of insects carrying the infestation to healthy plants from diseased plants that serve as sources of virus.

(g) *Cultural and mechanical control—destruction of crop residues*. A common practice performed in the Zona da Mara of Minas Gerais, is the maintenance of pepper plants in the cultivation area, even after the harvest period. In general, these plants become the focus of the multiplication of insects and mites that may attack the cultivation of the next crop. After the end of harvesting, it the incorporation of crop residues to at least 20 cm deep is recommended.

(h) *Cultural and mechanical control—use of natural barriers*. To prevent or delay the entry of arthropod pests on pepper crops, natural barriers may be used. The barriers must be perpendicular to the prevailing wind direction and where it is possible, surrounding the field. For this, plants such as sorghum, corn, and sugarcane should be used (Villas Bôas and França 2008).

(i) *Cultural and mechanical control—consortium of plants*. Planting of other species near or between the rows of pepper field will also provide an alternative income and can reduce the attack of insects and mites. In this type of management, it should avoid cultivation species from the same family such as tomatoes, sweet peppers, eggplant, and gilo, because they host similar diseases and pests of pepper.

(j) *Cultural and mechanical control—maintenance of areas with natural vegetation*. Areas with weeds growing near the pepper field should be preserved. These plants provide shelter, alternative prey, pollen, and nectar for the natural enemies of pepper insects (Venzon et al. 2011). An example is the *Ageratum conyzoodes*, a species that can provide food resources for predators, especially ladybugs.

(k) *Chemical control*—It is noteworthy that for pepper varieties no insecticides and miticides are registered in the Brazilian Ministry of Agriculture, which is responsible for the control of pesticides in the country. Therefore, the adoption of sustainable pest management for peppers is necessary, incorporating various strategies to obtain satisfactory control.

1.10 Diseases

Among the diseases of fungal etiology, figure *Cercospora* leaf spot, powdery mildew, *Phytophthora* wilt, and anthracnose. Among the bacterial etiology highlight the leaf spot and bacterial wilt. Among the viruses are PVY and PepYMV, TMV, CMV, and tospoviruses.

(a) *Cercospora leaf spot* (*Cercospora capsici*)—Disease whose symptoms occur mainly in the leaves, in the form of brown circular spots with light gray center,

which sometimes can tear or detach the lesion, leaving spread round holes. Spots can reach a diameter greater than 1 in. Older leaves may turn yellow and fall due to the attack of the disease. The development of the disease is favored by temperatures above 25 °C and humidity above 90 %. Plants with nutritional stress are more susceptible to the disease. Disease control should involve preventive measures, including the use of healthy seedlings, crop rotation, removal of crop debris, and spraying with products registered in MAPA (Carmo et al. 2006; Lopes and Henz 2008).

(b) *Powdery mildew*—Important disease in plants of the genus *Capsicum*, especially in greenhouse-grown crops. It is favored by prevailing environmental conditions such as relative humidity of 50–70 %, temperature 20–25 °C, and irrigation methods that do not promote leaf wetness, such as drip or ground irrigation. The disease is initially perceived by the occurrence of chlorotic spots on the upper surface of the leaves. These spots become necrotic or have black marks with little defined format. The underside of the leaves is covered with whitish structures ("white powder") of the fungus, which can lead to a general chlorosis of the leaf. However, chlorosis and necrosis can occur without clear detection of the "white powder,", which complicates the diagnosis (Carmo et al. 2006; Lopes and Henz 2008) Attacked leaves may fall and no symptoms occur at fruits. In young plants of *Capsicum*, infection is absent and more severe symptoms occur in adult plants at the fruiting phase (Café Filho et al. 2001).

After the onset of symptoms, it is necessary to take steps by applying fungicides based on sulfur. Alternative products such as potassium dihydrogen phosphate, calcium bicarbonate, and sodium bicarbonate are also reported to be effective in controlling the disease, but in excess can cause symptoms of phytotoxicity (Café Filho et al. 2001). Sprinkler irrigation can also be used as a complementary strategy to control the disease by promoting washing and removal of surface structures of the pathogen. Preventive measures to control powdery mildew are: avoid planting of susceptible species, crop rotation, and diversification with nonhost plants and having balanced fertilization.

(c) *Phytophthora wilt or Blight* (*Phytophthora capsici*)—a major disease of peppers in Brazil (Lopes and Henz 2008). The fungus causes rotting of the cortical region at the bottom of stems and roots, resulting in wilting and death of the plants in a few days after the initial wilting (Berke et al. 2005; Carmo et al. 2006; Lopes and Henz 2008). The necrotic roots slough off easily from plants and lesions in dark-brown color may occur with frequent presence of white mycelium and sporangia of the pathogen. Under high humidity, infection can also occur at the aerial parts of the plant with dark and softened spots in the leaves and stems.

The disease is favored by environmental conditions that predominate in temperatures between 22 and 29 °C and high soil moisture (Matsuoka et al 1996). Rainfall or irrigation for prolonged periods, especially in heavy soils, contributes to the disease development, because saturation of water in the soil occurs,

which promotes the development, survival, and spread of the pathogen. A high density of plants favors the development of infection at the lower parts of the plants.

The control of blight should be based on a series of preventive measures including: (a) avoid planting in soil with a history of disease occurrence; (b) planting in well-drained soils, not subject to flooding; (c) adopt greater spacing between plants to facilitate air circulation of the culture; (d) proper management of irrigation, avoiding excess water, especially in contact with the stems; (e) crop rotation preferably with grasses; (f) avoiding the use of mulch covering the crown portion of the shoot.

(d) *Anthracnose* (*Colletotrichum gloeosporioides* (*Penz*) *Sacc*)—The importance of this disease is recognized almost exclusively as causing lesions on fruits, in the field or after harvest (Lopes and Henz 2008). The disease begins with small round and depressed areas, which grow rapidly and can reach all the fruit. Under high humidity, the center of the lesion is covered by a mass of pink color, formed by the fungus spores. Important measures to minimize the damage are to use seeds and healthy plants, and also to reduce plant density, because this facilitates the flow of fresh air at the culture. In addition, preventive measures include the destruction of crop residues and rotation with nonhost species. Chemical control is done by applying copper-based fungicide. The pulverization can be preventative, at the beginning of fruit set.

(e) *Bacterial spot and bacterial pustule—Xanthomonas axonopodis* pv. vesicatoria (Doidg) *Dye* (Jones et al. 1998) (=*Xanthomonas campestris* pv. vesicatoria). The disease is common in places with high temperatures and humidity. Symptoms are more visible on adult plants and in older leaves. The lesions are irregularly shaped, of dark green color, and soggy appearance. Under favorable conditions, the lesions coalesce and form large "treacle" spots on the leaves. The diseased leaves turn yellow and fall. In fruits, the disease induces spots similar to blisters, initially with a white appearance evolving to darkened centers (Lopes and Henz 2008). In rainy periods, infections are more abundant and lesions develop more rapidly in number and size, which leads to severe and early defoliation of the plant (Carmo et al. 1996). It is difficult to control the developments of the disease in the field. In sweet pepper, the application of copper fungicides or organic copper is usually recommended (Aguiar et al. 2003). However, variation in the efficiency of these products occurs according to region and time of year (Carmo et al. 2001). Other important control measures are the use of healthy seedlings and seeds, destruction of crop residues, and rotation with grasses.

(f) *Bacterial wilt* (*blight*) (*Ralstonia solanacearum*)—A disease that occurs only when the temperature and humidity are both high, a situation that is more common in the north and northeast regions of Brazil and even in some centers of production in the lowland of the southeast (Lopes and Henz 2008). Affected plants may not wilt, but have limited growth. When wilted, symptoms first

appear during the hottest hours of the day. New leaves wilt first, sometimes on one side of the plant. The stem base of the plant appears wilted brownish. Most often, the disease is perceived only at the start of fruiting. For its control a series of preventive measures must be adopted, such as planting in an area that has no history of the disease with other species from the same family or other host for the bacteria. For growth in soils not subject to flooding, do not irrigate in excess, avoid injury to roots and the base of the plant, and restrict the transit of people and machines from contaminated areas. In addition, remove plants with early symptoms of wilting and avoid the use of black plastic as a ground cover during the summer, because the plastic cover keeps the temperature and humidity very high in the ground.

(g) *Hollow stem* (*soft rot*) (*Pectobacterium* spp. *and Dickeya* sp.)—The disease is more severe in the summer under conditions of high humidity and can be restricted to the stem and fruits. As the bacteria penetrate through wounds, injuring plants and fruits at both pre- and postharvest of fruits should be avoided. We recommend measures such as avoiding excess moisture for the plant, especially in summer; adding correct fertilizer to plants; and spraying with copper fungicides, especially when there are injuries. After harvest, dry the fruits and keep in a well-ventilated place (Carmo et al. 2006; Lopes and Henz 2008).

(h) *Potyvirus—PVY/PepYMV*—Both viruses cause similar symptoms and are serologically related (Truta et al 2004). Molecular tests are the safest way to differentiate the two viruses. According to Trout et al. (2004) the PepYMV is widespread in the fields of Minas Gerais state and it is possible that the predominant potyvirus in pepper may currently be the PepYMV. Symptoms caused in pepper plants by PVY and PepYMV are curling of the leaves, the development of mosaic with yellow-green, and the overall reduction of the size of the plant and fruit (Truta et al. 2004).

Both viruses are transmitted by aphids of various species and through injury or cutting instruments. The presence of aphids and infected plants favors the spread of the disease within the field and between fields, because the aphids can fly to the neighboring fields or be transported by wind over long distances.

(i) *Tospovirus* (*TSWV*, *GRSV and TCSV*)—These viruses cause virtually identical symptoms as well as being very similar to the symptoms induced by potyvirus PVY and PepYMV. The tospoviruses are transmitted naturally by several species of thrips, especially *Frankliniella ooccidentalis*. The prevalence of the virus species in producing regions or crops is usually associated with the predominant species of thrips (Groves et al. 2001).

(j) *TMV*—The symptoms resemble those induced by other viruses that infect peppers. TMV has no insect vector and transmission occurs when an infected plant, tools, machinery, and/or hands contaminated by viruses that come in contact with a healthy plant.

(k) *Begomoviruses*—The symptoms are similar to those caused by PepYMV and isolated from TMV or CMV and are therefore difficult to distinguish in the field

(Carmo et al. 2006). The begomoviruses are transmitted by whitefly (*Bemisia tabaci*). In Brazil, the only species of begomoviruses detected in pepper is the ToSRV (Bezerra-Agasie et al. 2006). For management of viruses affecting peppers, it is recommended to produce or acquire seedlings grown in greenhouses from insectproof cages and away from the producing field, eliminate weeds around the crop field because such plants can be a source of inoculum, eliminate plants with symptoms from the field while the incidence is still low, and destroy crop residues after harvest.

1.11 Harvesting, Packaging, Marketing, and Commercialization

The crop cycle and the harvest period are directly affected by weather conditions, incidence of pests and diseases, and cultural practices such as fertilization, irrigation, and adoption of phytosanitary control measures. In general, the first harvest of ripe fruits starts 90 days after sowing for the earlier peppers such as "Murupi" and 120 days for the later ones such as chili pepper "Malagueta". The ideal harvest is visually determined when the fruits reach the maximum size and growth of the typical format of each species with specific color demanded by the market. Mature green fruits for "Cambuci" pepper, red for chili "Malagueta", yellow or red pepper to "Pimenta de Bode", yellow color for "Cumari", and light yellow to "Murupi" fruits.

Peppers are harvested by hand, tearing up the fruits of plants with or without stems, depending on the type of pepper and the target market. The Dedo de Moça for the fresh fruit market should be harvested with the stem, which improves the appearance and preservation of fruits. An additional operation to remove the stem for sauces and canning industries is needed. This operation is dispensable for Cumari pepper, Malagueta, Red Cumari (Bird pepper), and others whose stems are easily detached from the plant.

There is still no official classification standard and standardization for peppers in Brazil (Henz and Moretti 2008). Thus, virtually all processing operations are done by pickers, who must pick only well-developed berries and typical coloring for each type of pepper without malformed fruits. Harvested fruits must be handled carefully to avoid mechanical damage which reduces their postharvest durability.

Different packaging for commercialization of is used in Brazil, according to the size and type of fruit, region, and market demand. At The Society of General Warehouses of São Paulo (Ceagesp), pepper varieties with bigger fruits, such as Cambuci pepper, "Dedo de Moça" and sweet peppers are sold in plastic boxes or wooden type "K" box containing 12–15 kg, and the smaller fruits such as Malagueta and Cumari peppers are packed in carton boxes of 1–2 kg and plastic bags from 1 to 10 kg. In all wholesale markets, the peppers are also sold in smaller quantities using glass cups or cans from 250 to 1000 mL capacity, according to consumer demand.

At retail stores, the most common form of marketing is in bulk, where consumers select the amount to be purchased. In street markets, it is more common to find peppers in glass or cans of 250–300 mL, containing different types of peppers for the same price. In supermarkets and retail shops, peppers are also sold in perforated plastic bags of 50 g, trays containing 50–100 g of fruits covered with PVC film, and boxes of plastic PET with 250 mL capacity.

In most wholesale markets, the price quotations are not differentiated for the different types of peppers, but use the classifications "Pepper", "Red Pepper", and "Hot Pepper".

The Brazilian market for processed peppers is exploited by a large number of small and large companies. In the country, there are many companies already consolidated with considerable importance in processing sauces. The growth rate in sales of sauces is approximately 10.5 % per year (Ohara and Pinto 2012). In Latin America, Brazil is the second largest consumer market in the Tabasco® sauce brand, behind Mexico.

In addition to processed sauces, in Brazil there are several companies selling peppers processed as antipasti, jams, and other pepper-based products.

The market for processed pepper products for the international market is restricted to a few companies with some specific types of products. This is a market that is influenced by fluctuations of the dollar and the international competition between producers of peppers in countries including India, China, Thailand, and Korea (Ohara and Pinto 2012). Within the portfolio of products exported by the Brazilian Sakura, pepper sauces and other processed products are shipped to 15 countries, including Japan, Spain, the United States, and all of South America. "Kenko" and "Bravo" pepper sauces in various versions and sizes are also exported in small volumes (Ohara and Pinto 2012).

Unfortunately, Brazil is not recognized as an exporter of pepper-based products. There are few data available on exports of pepper-processed products.

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Chapter 2
Physiology and Postharvest of Pepper Fruits

Fernando Luiz Finger and Giselda Maria Pereira

Abstract The *Capsicum* genus comprises a large and diverse group of cultivated and nondomesticated plants producing flesh fruits that vary from sweet to hot spicy taste. Fruits from the domesticated species of peppers, *Capsicum annuum*, *C. frutescens*, *C. baccatum*, and *C. chinense* present a nonclimacteric behavior for respiration and ethylene production. Nevertheless, harvested fruits show different degrees of sensitivity to exogenous ethylene regardless of the species. With Fruits in the same species, ethylene induces different intensity of changes for color, chlorophyll degradation, and total soluble solids content. Postharvest loss of fresh weight has different intensities, which is associated with the thickness of the pericarp, surface/volume ratio, and composition of waxy epidermis. Fruits with a thicker pericarp are more susceptible to wounding but less susceptible to shrinking under intense water loss. Regardless the species, fresh fruits are susceptible to develop chilling symptoms when stored bellow 10 °C, which can be reduced by wrapping the fruits with plastic film. The intensity of chilling seems to be related to the stage of ripening of the fruits and variety.

Keywords Respiration • Ethylene • Shelf life • Pigments • Water loss • Temperature

2.1 Introduction

The genus *Capsicum* comprises a large and diverse group of plants producing flesh fruits varying from sweet to hot. Originating from Latin American tropical regions, spreading from Chile to the southeastern United States, the *Capsicum* species are cultivated and appreciated around the world. Due to the unique flavor, spice uses, and presence of hot taste of the fruits, they are consumed fresh and in different forms of processed products.

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There are five domesticated species in the genus, with distinct characteristics and distribution. *Capsicum annuum* is the most popular and diverse species. The other four species, C. *frutescens*, *C. baccatum*, *C. pubescens*, and *C. chinense*, usually have their cultivation restricted to particular countries or regions. In Brazil, *C. baccatum* and *C. chinense* are very popular, because they are quite adapted to the climate conditions present in the equatorial and tropical regions of the country. In addition, their fruits have good culinary characteristics for in natura consumption as part of dishes or as fresh vinaigrette.

Peppers belong to a group of botanical species with unique characteristics, producing flesh fruits with a wide range of hot flavors in addition to sweet fruits with no capsaicin. The plant is perennial but cultivated as an annual crop. The plant looks like a bush with height of 120 cm with many lateral shoots, although small plants are also cultivated for ornamental purposes. It is autogamous but presents cross-pollination within and between species (Araujo 2005).

The market for the fruits is enormous with a wide range of processed products, as fresh fruits for both in natura consumption and ornamental purposes. Processed products include sauces, pickles, paprika, dry cracked pepper, whole dry fruits, jams, and medicinal products.

The diversity of color, format, and flavor of fruits are some of the reasons for their appreciation but the most important feature is their hotness, due to the presence of capsaicin secreted by the glands present in the fruit placenta. Pepper consumption helps digestion and is an important source of antioxidant substances, including vitamin C, carotenoids, and vitamin E.

The market for in natura consumption of fresh fruits is small when compared to other vegetables, mainly because they are used in small amounts as part of sauces, although sweet pepper can be used in large quantities as occur with the Buiquinho pepper (*C. chinense*) in Brazil. Its fresh fruits are largely used as pickles and as an important ingredient in salads. The market for fresh fruit is locally driven and requires patronization and a high quality product.

All around the world the market for pepper is growing because of its culinary acceptance in many dishes, industrial uses, medical properties, and in more recent years as an ornamental plant. The flesh fruits have relatively short shelf life because of the presence of several abiotic and biotic stresses:

(a) Losses due to mechanical injury or presence of insects or diseases
(b) Water loss by transpiration and respiration
(c) Losses by exposition to low or high extremes of temperature, causing freezing or excessive dehydration
(d) Development of physiological disorders such as chilling symptoms induced by low temperatures in the field or during storage
(e) Losses of dry matter by the respiration process
(f) Breakdown of vitamins

The expansion of the fresh market requires the improvement of postharvest handling, but little is known regarding the physiological reaction of the fruits to long-term storage and quality changes that occur during transportation and display at retail stores.

2.2 Physiology of Fruit Growth and Ripening

The flesh fruit shows a simple sigmoid pattern of growth and at the end of its increase in fresh matter, the fruits develop their ripe color, ranging from purple, yellow, orange, or red. The intensity of color in ripe fruit depends on the variety, stage of development at harvest, and climate conditions at the field. Mutations also can change the color of ripe fruits, usually in genes responsible for the synthesis of carotenoids.

The initial phase of growth is characterized by intense cell division followed by cell enlargement due to the uptake of water and photoassimilates. The later phase is responsible for the thickening of the pericarp giving the final fruit succulence and hardiness. Fruits with thick pericarp are more suitable for fresh consumption because they are more hydrated than those with thinner pericarp. On the other hand, peppers with thinner pericarp usually have more soluble solids, thus are more suitable for the production of dry products such as paprikas, due to a faster dehydration and allowing less contamination by postharvest diseases (Lannes et al. 2007).

At the end of the growing phase the mature green fruits start to change color and develop some softening of the pericarp. The degradation of chlorophyll starts at the breaker stage of fruit color, after fruits reach the mature green phase, revealing the carotenoids or purple colored anthocyanins. In climacteric fruits, ripening is induced by a low concentration of ethylene (system I) simultaneously to the induction of climacteric respiration and autocatalytic production of ethylene by the fruit. Nevertheless, in nonclimacteric fruits, ethylene usually induces changes in both skin and flesh color without inducing ripening or autocatalytic synthesis of ethylene (system II). The response of nonclimacteric fruits to ethylene seems to be related to the advance on senescence instead the beginning of fruit ripening. Usually, the harvest of ripe peppers extends for several months, because the fruits are present at different stages of development, with immature, mature green, and ripe fruits in the same plant (Fig. 2.1). This fact can be evident in plants of *C. frutescens* known as Pimenta Malagueta, a common variety grown for fresh consumption and for sauce production.

Based on respiratory pattern and ethylene evolution during the ripening, peppers behave as nonclimacteric fruits. Following the growth and ripening of different domesticated *Capsicum* species no increase in respiration and ethylene production occurred when fruits were attached or detached from the plant. Pereira (2004), studying 16 accessions of the peppers from *C. annuum*, *C. chinense*, *C. baccatum*, and *C. frutescens*, applied 1000 mg L^{-1} ethephon solution in mature green fruits. In the treated fruits, there was no increase in respiration and autocatalytic ethylene evolution. Nevertheless, in overripe fruits there were changes in respiration and ethylene production. These changes may reflect the advanced stage of senescence that follows fruit ripening. In previous works, Saltveit (1977) and Pretel et al. (1995) studied both respiratory and ethylene behavior in peppers treated with propylene, an analogue ethylene gas. Their conclusion was that the fruits responded like nonclimacteric fruits, inasmuch as no significant changes in respiration and ethylene production

Fig. 2.1 Maturity diversity of *Capsicum frutescens* variety Pimenta Malagueta fruits during plant production

were detected. In another experiment, Gross et al. (1986) evaluated the biochemical changes in the ripening of *C. annuum* cv. Choorachong harvested at the mature green stage. They determined a climacteric-like increase in respiration during ripening but ethylene production up to 18 h after being kept at 20 °C in closed flasks was not detectable. Also, the ethylene detected was not similar to an autocatalytic evolution, which is characteristic of any climacteric fruit. The peak of respiration occurred when the fruits had 50 % red color, which seems a symptom of senescence and not a climacteric peak of respiration.

Barrera et al. (2005) determined that pepper fruits of *C. annuum* and *C. frutescens* from the germplasm bank of the Amazon SINCHI (Instituto Amazônico de Pesquisa Científica—Colômbia) kept ethylene evolution always below 0.01 μL L^{-1} (a typical behavior of a nonclimacteric fruit) and absence of system II or autocatalytic ethylene production throughout the ripening. In another study, the related *C. annuum* sweet pepper treated with 100 μL L^{-1} ethylene determined the elevation of mitochondrial respiration in green sweet pepper, but the internal concentration of carbon dioxide within the fruits decreased when the ethylene was removed. This result clearly indicates that the increment in respiration depends on the presence of ethylene, a response that is present in nonclimacteric fruits.

Ethylene nevertheless was able to induce changes on some characteristics of fresh harvested fruit peppers. Dipping mature green fruits of different species of peppers for 30 min in a solution at concentration of 1000 mg L^{-1} ethephon, an enhancement to the development of red or yellow ripe fruit was observed (Table 2.1).

At the first stages of fruit development in both climacteric and nonclimacteric fruits, high rates of ethylene production are related with cell growth, followed by a period of sharp drop parallel to the cell expansion. The earlier and elevated production

Table 2.1 Means followed by the same letter, in a row and within each accession, are not significantly different according to F test (p 0.05)

Species	Color of ripe fruit	Control	Ethephon[1]
		(Days to ripe fruit color)	
C. annuum			
Mirassol	Red	21.5a	11.8b
New Mexican	Red	20.3a	17.7a
C. chinense			
BGH 1716	Orange	7.8a	7.3a
BGH 1723	Red	14.8a	18.8a
C. baccatum			
BGH 4366	Red	13.0a	9.0a
BGH 6029	Yellow	5.3a	3.5b
C. frutescens			
BGH 4179	Red	12.8a	8.0b
BGH 4708	Red	16.3a	17.5a

of ethylene is related to cell division and radial growth of cells regardless of the climacteric or nonclimacteric nature of the fruit.

Two ethylene systems of production act at the same biosynthetic pathway. System I is responsible for the production of small quantities of ethylene, which is present at the preclimacteric phase of climacteric fruit or flowers, just before the increase in respiration. This system is also present in all vegetative tissues of plants. System II produces massive quantities of ethylene acting during ripening of climacteric fruits or senescence of climacteric flowers. The autocatalytic production of ethylene is due to the increase of RNA transcription and translation of the key enzymes ACC synthase and ACC oxidase. Some pepper fruits present an increase in ethylene and respiration but the climacteric nature of the rises remains to be determined. Villavincencio et al. (2001) suggest that some cultivars of *C. annuum* may present an intermediate climacteric pattern.

The importance of the fruit respiratory pattern is related to the strategies for handling the fresh produce after harvest. Little is known about the physiological behavior of pepper fruits, related to ethylene production and sensitivity in particular. Responses to ethylene treatment help to understand the changes in fruit metabolism and potential for storage. As mentioned previously, Pereira (2004) analyzed the responses of 16 pepper accessions under ethylene treatment. Ethylene reduced the number of days for the mature green fruits to reach the full red, orange, or yellow ripe color (Table 2.1). However, the response was dependent on the accession treated with ethephon, which showed different sensitivity to the hormone ethylene, enhancing the development of the ripe fruit color or having no effectiveness whatsoever. Two accessions of *C. chinense* were insensitive to ethylene, regarding the development of changes on carotenoid pigment accumulation.

In the same extended study of several accessions of hot peppers, conducted by Pereira (2004) in fruits from the species *C. annuum*, *C. chinense*, *C. baccatum*, and *C. frutescens*, it was found that the submersion of fruits in 1000 mg L^{-1} ethephon

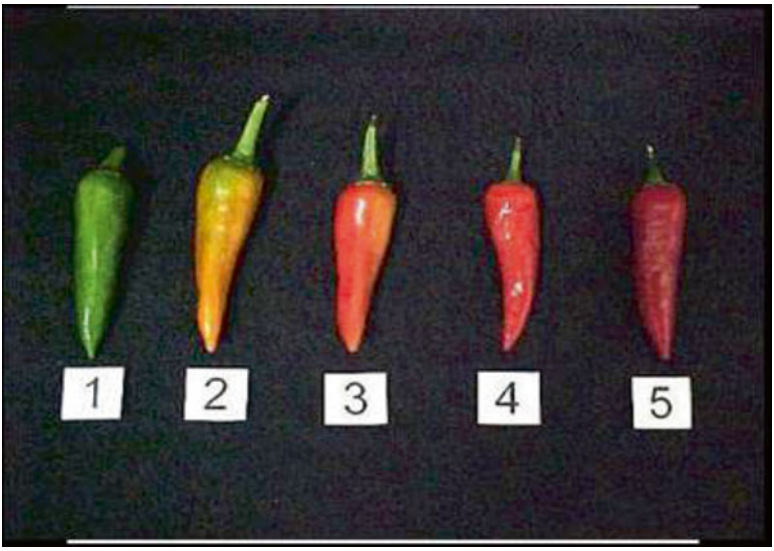

Fig. 2.2 Ripening of cultivar Ca 6 (*C. annuum*) fruit. (1) Mature green; (2) 25 % red; (3) 50 % red; (4) 75 % red; (5) 100 % red

harvested at the mature green stage induced changes in total soluble solids in only two accessions: the BGH 4366 (*C. baccatum*) and BGH 4708 (*C. frutescens*) held at the germplasm bank BGH/UFV. Nevertheless, the remaining 14 accessions did not respond to ethylene regarding the accumulation of total soluble solids. Furthermore, the changes in color were affected by the action of ethephon (Table 2.1), This work showed that the response to ethylene varies among the genotypes within the same species of pepper. This is an important characteristic, because the development of intense red color is important for the hot paprika industry production. The induction of carotenoid biosynthesis in this nonclimacteric fruit remains to be evaluated in future studies.

Hot peppers from *C. annuum* ripen well when attached to the plant as showed in Fig. 2.2. If detached from the plant at the mature green stage, the fruits must be treated with ethylene in order to induce more intense changes in color. In the absence of ethylene, color changes occur at a low rate and are related to an advanced stage of senescence.

As do other fruits, peppers have different degrees of response to ethylene, affecting the shelf life and quality. The handling of fruits requires knowledge of physiological changes during maturation and ripening, including changes in respiration and ethylene synthesis and sensitivity. The use of 1-methylcyclopropene (1-MCP), an ethylene action inhibitor, has profound effects on the ripening of climacteric fruits and reduces the rate of deterioration in some nonclimacteric fruits such as peppers. The use of 1-MCP in potted ornamental plants of *C. annuum* hot peppers prolongs the postproduction shelf life in indoor environments, indicating that vegetative tissues also respond to the action of ethylene in *Capsicum* plants. Thus, dur-

Fig. 2.3 Ripe fruits of *Capsicum chinense* genotypes from BGH/UFV

ing handling, shipping, and storage, pepper plants in wholesale and retail stores must be checked for the presence of concentrations of ethylene above 10 μL L^{-1}, which has proved to affect the abscission of leaves, flowers, and fruits if the exposure is more than 24 h at room temperature. In cultivars with high sensitivity to ethylene such as Calypso fumigation with 1 μL L^{-1} 1-MCP for 6 h was able to prolong the post production shelf life by inhibiting the abscission of leaves induced by ethylene (Finger et al. 2015).

One of the main desirable attributes in fruits is the color and it is related to the stage of fruit development and the cultivar. The BGH/UFV germplasm bank of *C. chinense* has fruits with different intensities of red, yellow, and orange colors when ripe (Fig. 2.3). Intense red ripe fruits usually are suitable for processing as dry powder because they have high amount of carotenoid pigments, which define the final quality of dry processed pepper products.

One of the main changes during fruit ripening is the degradation of the pulp chlorophyll, but during the fruit growth phase there is a large increase in the chlorophyll synthesis. Mattos et al. (2008) found differences in the chlorophyll content among several varieties, and the so-called Pimenta de Bode had 60 % more chlorophyll than Dedo de Moça. In the same study, the ratio between chlorophyll a/b varied from 1.19 to 2.33, depending on the cultivar, indicating different degrees of chloroplast development in mature green fruits. Menichini et al. (2009) found that the composition of carotenoids in immature and mature fruits of cultivar Habanero increase when the fruits were at the mature green stage compared to immature green stage, but the phenols had an opposite behavior.

Fruit pungency is one of the most important quality of peppers in both in natura consumption and processing, especially for condiments and sauces of ethnic dishes around the world. During fruit growth there is a substantial accumulation of capsaicin and dihydrocapsaicin distributed at the placenta of the flesh fruit. The placenta contains glands that are responsible for the production of these alkaloids, which usually spread to all other portions of the fruit, including the seeds and pericarp.

The varieties used for cooking and processing have different capsaicinoid contents. The dry powder of paprikas have different contents of capsaicinoids, varying from 0.003 to 0.01 % known as sweet products, 0.05 to 0.03 % considered light dry powders, and finally, the hot powders ranging from 0.3 to 1 % of capsaicin (Araujo 2005).

The content of capsaicinoids depends on several factors, including the genetic factor, cultivar, and stage of development of the fruit (Bosland and Votava 1999). Furthermore, environmental conditions affect the accumulation of these alkaloids, mainly the temperature, light intensity (Estrada et al. 1999a; Tewksbury et al. 2006), water stresses (Estrada et al. 1999b), and plant nutrition (Estrada et al. 1998). Lannes et al. (2007) evaluated 49 accessions of *C. chinense* for capsaicin and dihydrocapsaicin contents and determined a wide range of hotness. The highest content was 14 mg g^{-1} dry weight and the lowest content was 1.9 mg g^{-1} for the light hot fruits. In addition, fruits were found that had only traces of capsaicinoids. Thus, 27 % of the accessions could be included as light hot fruits, while 2.5 % of the accessions had 12–14 mg g^{-1} dry weight and were consider as hot paprikas.

Despite the fact that the biosynthesis of capsaicinoids is known, the degradation of capsaicin and dihydrocasaicin is not completely understood. The enzyme peroxidase seems to be involved in the oxidation of capsaicinoids, because there is an increase in peroxidation during fruit ripening. The increase of peroxidase activity is related to the increased demand for phenylpropanoid intermediates for the synthesis of cell wall suberin and lignin, competing with intermediates needed for the synthesis of capsaicin and dihydrocasaicin (Bernal et al. 1995). Pereira (2007) found a significant decrease for the capsaicinoids on the majority of hot *C. chinense* fruits during ripening. In the same work, it was observed that peroxidase activity was higher at the beginning of fruit ripening regardless of whether fruits were sweet or hot. Such behavior shows that during fruit ripening peroxidases are involved on the changes of cell wall metabolism as suggested before.

In addition to the contribution of capsaicinoids to the flavor, the presence of 102 different volatiles compounds when fruits of Habanero changed from mature green to ripe was observed. In a study, Pino et al. (2006) found alcohols, ketones, and aldehydes as the major volatiles that increased the flavor.

2.3 Postharvest Water Loss

The harvest interrupts the fruit water supply and the subsequent loss of water is responsible for the qualitative and quantitative losses on most of the varieties of fresh sweet and hot peppers. Excessive loss of water results on less shiny skin and

shrunken fruits, but the amount of water necessary to cause these problems depends on the variety and environmental conditions of storage. In addition to the above symptoms, the fruits are more susceptible to deterioration, including an increase in oxidative reactions, degradation of chlorophyll, and elevation of ethylene synthesis and action.

The total postharvest losses of fresh fruits results from both the water loss and the consumption of dry matter by respiratory activity, the former being the most relevant. Previous works have determined that weight losses from 3 to 5 % may cause shrinkage in most flesh fruits (Ben-Yehoshua 1987). Weight loss has a negative impact on shipping, storage, and sales at retail stores. Surveys on retail stores showed that loss of weight is the most important cause of postharvest losses of peppers in the majority of Brazil's local stores and large supermarkets. Unfortunately, most of the retail store owners do not apply any method to reduce the rate of water loss, which could be achieved by wrapping fruits with plastic bags and reduction of temperature. Because of that, the shelf life of most pepper varieties does not exceed three days.

The main barrier to determine the rate of water loss of fresh fruits is the waxy cuticle that covers the epidermal cells. This cuticle is formed by esterified lipids with a distinct composition and thickness, depending on the species and the variety. The integrity of the cuticle is a determining factor that affects the passage of water vapor and infection by pathogens. Previously, it was established that the cuticle chemical composition is more important than its thickness regarding the effectiveness in reducing the water loss. Lownds et al. (1994) determined a variation of 60 % on the rate of weight loss analyzing the influence of storage temperature and humidity on shelf life of several sweet pepper cultivars. The variation in the rate of weight loss among the cultivars was related to the permeability of the cuticle to water vapor.

Exchange of gases between the fruit and the storage environment is usually influenced by the surface/volume ratio (cm^2/cm^3). This ratio is determined by the size and format of the fruit. Higher losses of water are present on those fruits with elevated ratios. Assuming the fruit format is constant, the smaller fresh fruits are more susceptible to water loss than the larger ones during shelf life and processing as dry powder.

In ripe fruits of *C. chinense*, Cabral (2006) found a positive correlation between the surface/volume ratio and the rate of weight loss during storage at room temperature (Table 2.2). Fruits with elevated surface/volume ratio had a shorter shelf life at room temperature, due to a much higher rate of fresh weight loss. An increase of surface/volume ratio from 1.17 to 5.27 increased the weight loss from 0.9 % per day to 3.25 % per day, shortening the shelf life in these fruits by five days (Table 2.2).

In a previous analysis of several genotypes belonging to *C. chinense*, it was established that fruits with a thicker pericarp are more appropriate for fresh consumption due to the pericarp's higher resistance to wounding, firmness, and freshness. However, fruits with less thick pericarp had more dry matter and soluble solids, and were more suitable for processing as dry products.

During pepper fruit dehydration there is the activation of lipoxigenases changing the permeability of cell membranes. As a consequence of membrane damage, a

Table 2.2 Surface/volume ratio, weight loss, and days to chilled fruits in accessions of *Capsicum chinense* stored at 22 °C and relative humidity of 60 %

Accessions	Surface/volume ratio (cm^2/cm^3)	Weight loss (%/day)	Days to waste
BGH 6371	1.17	0.90	8
BGH 4213	2.78	1.73	5
BGH 1716	3.38	2.63	5
BGH 1723	5.27	3.25	3

cascade of catabolic reactions takes place, causing the deterioration of tissues. The leakage of electrolytes is an indicator that the membrane integrity is lost. Such behavior was observed in genotypes of hot peppers susceptible to elevated water loss compared to those cultivars less susceptible to dehydration during storage (Maalekuu et al. 2005).

In *C. annuum* cultivars, the activity of lipoxigenases is associated to the development of volatiles compounds and degradation of pigments throughout the different stages of ripening (Luning et al. 1995; Jaren-Galan and Minguez-Mosquera 1999).

Maalekuu et al. (2006) verified a negative correlation between the lipids content in the membrane, electrolyte leakage, and lipoxigenaes activity in 10 accessions of *C. annuum*. Thus, the genotypes susceptible to high rates of water loss presented lower quantities of total lipids in their membranes. Inverse behavior was observed in genotypes with low susceptibility to water loss. The differences among the genotypes seemed to be related to the cuticle wax composition, enzyme activity of cell wall degradation, and the integrity of cell membranes.

Lannes et al. (2010) found that hot peppers *C. chinense* with less thick pericarp had more dry matter and soluble solids. This fact may reflect a higher capacity of these plants to accumulate and translocate dry matter or due to dehydration of the fruit during ripening when attached to the plant. Such fruits would be more adequate to the paprika industry and dry pepper spices, because less water would be necessary to remove during processing, reducing the cost for dehydration. But fruits with thicker pericarp are more appropriate for fresh consumption due to higher resistance of the pericarp to the wounding, firmness, and freshness aspect of the fruits.

2.4 Temperature of Storage

Temperature is the most important factor regarding the postharvest shelf life of peppers. It is well known that length of shelf life is inverse to the elevation of temperature. However, the *Capsicum* species fresh fruits are sensitive to chilling injury determining their handling and extent of storage.

In general, the symptoms of chilling in fleshy fruits are the development of depressions and/or discolorations and further browning on the surface. In addition, the development of chilling has other effects on fruit quality, including lack of

ripening and higher incidence of postharvest diseases. According to Shewfelt (1993), tropical and subtropical fruits develop a series of reactions in response to low temperatures leading to an increase of leakage of electrolytes, increase of respiration, ethylene synthesis, accumulation of toxins, and finally cell collapse.

The development of chilling injury occurs when fruits are stored under temperatures above 0 and below 12 °C, for the majority of species. In most cases, temperatures closer to 5 °C are more effective in inducing symptoms of injury (Wills et al. 1998). Nevertheless, in order to develop permanent symptoms, the fruits must remain for a period under inducing chilling temperatures: the longer the periods under the stressing temperatures, the more intense are the symptoms. In general, fruits show symptoms under low temperature, but in some species, visible symptoms will develop only when they are moved to higher temperatures.

Reactive oxygen species (ROS) composed of superoxide, hydroxyls, peroxides, and oxygen singlet may contribute to the resistance to chilling injury development in pepper fruits. The resistance to chilling is related to the activation of the antioxidant system involved in the ROS metabolism. Peroxidases and catalase eliminate the accumulation of hydrogen peroxide delaying the appearance of chilling. In addition, there is an increase in the expression of the alternative oxidase pathway (AOX), associated with the reduction of ROS, detected in fruits of *C. annuum* stored under chilling inducing temperatures (Fung et al. 2004; Purvis 2002).

The fruit maturity stage at harvest is another important factor of the resistance to chilling. As in other fruits, immature and mature green fruits are more sensitive to develop chilling injury than fully ripe fruits. Furthermore, fruits originated from colder regions are less susceptible to chilling than fruits grown in warmer climates. No information is yet available on the degree of sensitivity from the different cultivated *Capsicum* species and cultivars.

The optimum temperature for storage of hot peppers is still to be determined for most of the species and cultivars. The general recommendation is to store between 7 and 10 °C. The hot pepper Pimenta Malagueta had 30 days of shelf life when stored under 12 °C of temperature and relative humidity of 80 %. Nevertheless, the fruit showed some shrinkage at the skin, which can be avoided by wrapping fruits in PVC or a PET plastic box, maintaining elevated humidity inside.

If fruits are stored in PET, it is desired to punch some holes in order to avoid excessive condensation inside the box and the development of fungi and bacterial diseases after 2–3 days if kept at room temperature. The storage of Pimenta Malagueta, De Cheiro, Cumari do Pará, Bode Vermelha, Bode Amarela, and Dedo de Moça wrapped with PVC film had a shelf life of 30 days at 8 °C. These pepper fruits had daily fresh weight of 2.5 % at 24 °C when stored without any PVC film to avoid excessive water loss. However, if wrapped with PVC plastic film, the weight loss was 0.9 % per day and only 0.2 % if stored at 8 °C protected with plastic film (Gravina et al. 2004).

Marques et al. (2005) stored seven *Capsicum* accessions (*C. baccatum*—BGH 1646, 4366, 6029; *C. chinense*—BGH 4213 and 6371; *C. annuum*—cultivars Mirassol and New Mexican) at 5 and 10 °C in PET perforated boxes. Except for

accessions BGH 6029 and Mirassol, all fruits from the others developed symptoms of chilling at both temperatures, with much more intensity at 5 °C. Chilled fruits had dispersed little pale spots at the surface of the pericarp. The first symptoms appeared in those fruits of BGH 4366 stored at 5 °C after 6 days. At 10 °C, the symptoms started to develop after 12 days in BGH 1646, also a *C. baccatum* accession.

Fruits from BGH 6029 (*C. baccatum*) and Mirassol (*C. annuum*) were resistant to chilling injury even after 1 month of storage at 5 or 10 °C. As a general recommendation, temperatures between 7 and 10 °C are more suitable and the relative humidity should stay between 90 and 95 %. Nevertheless, this humidity is hard to get in most refrigerated storage facilities when there is no humidity control. In this situation, it is recommended to use plastic films to avoid dehydration (Finger and Vieira 1997). Several works show that excessive postharvest water loss increases the susceptibility of fruits to chilling, which can be diminished by wrapping with any plastic film.

2.5 Conclusions

Fruits from the cultivated species *Capsicum annuum* C. *frutescens*, *C. baccatum*, *C. pubescens*, and *C. chinense* behave as nonclimacteric fruits. All fruits are susceptible to intense water loss after harvest requiring immediate reduction of temperature and wrapping with plastic film to avoid dehydration and shrinkage.

Regardless of the domesticated species of hot peppers, the majority of the fruits are susceptible to develop chilling symptoms when stored below 10 °C of temperature, which can be reduced by wrapping the fruits with plastic film. For most cultivars, however, there is no information about the best postharvest temperature and humidity of storage.

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Chapter 3
Cytogenetics in *Capsicum* L.

Fabiane Rabelo da Costa Batista

Abstract *Capsicum* is native to the New World and comprises 33–34 species, five of them domesticated. Studies with domesticated and wild species using cytogenetic methodologies have helped to understand the genetic relationships among them. In general, interspecific crosses are associated with genomic homologies between species and genetic gains could be obtained by desirable gene introgressions. In this sense, extending the knowledge about intra- and interspecific genetic variability, the genomic organization, and evolution in *Capsicum* could be very useful in plant breeding programs. Many chromosome markers have been developed, generating more refined and detailed *Capsicum* karyotypes. Association of classical staining, densitometry, CMA/DAPI banding, flow cytometry, and fluorescent in situ hybridization have allowed confirming or reviewing previous taxa, sometimes delicately constructed. All *Capsicum* species are diploid but two groups with distinct chromosome numbers are formed: with $2n=24$ and with $2n=26$. Two possible genome evolution lines are supposed to the genus, but the origin of the thirteenth chromosome is not well-defined. This chapter is a review of classical and modern cytogenetic methodologies applied in *Capsicum* since 1930. The main results and inferences about phylogenetic relationships in the genus are presented, although many questions are still unanswered.

Keywords Chromosomes • Pepper • CMA/DAPI and FISH

The largest part of phenotypic diversity could be attributed to genetic variability, including both nuclear and cytoplasmic genes. Considering new technologies, different methods have been applied in plant science for a better understanding of the organization and functioning of these genomes, generating knowledge to support basic and applied research in this area.

Cytogenetic and molecular analyses have helping to elucidate the evolution genetics and karyotypic stability of many species. New chromosome markers were developed, generating more refined and detailed karyotypes of the *Capsicum*

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species. Association of classical staining and C-banding, densitometry, flow cytometry, and fluorescent in situ hybridization (FISH) have allowed confirming or reviewing previous taxa, sometimes delicately constructed.

Capsicum cytogenetics, including domesticated species and their putative wild ancestors has been studied by plant breeders. Successful interspecific crosses are strongly associated with genomic homologies among selected species. Genetic gains could be obtained by desirable gene introgressions such as genes of disease resistance that are found in wild species. In this sense, extending knowledge about intra- and interspecific genetic variability, the genomic organization, and evolution in *Capsicum* could be helpful to define specific hybridizations in both conventional plant breeding and transgenic breeding programs.

Capsicum is native to the New World and comprises 33–34 species, five of them domesticated (Barboza and Bianchetti 2005; Moscone et al. 2007; Barboza et al. 2011). The first karyological studies in *Capsicum* used conventional staining and demonstrated relative chromosome uniformity among and within the studied species. The chromosome number and ploidy level were described, and all analyzed species were diploid, with $2n = 2x = 24$ chromosomes. Satellites were observed in some species (Huskins and La-Cour 1930; Sinha 1950).

Shopova (1966a), studying metaphase chromosomes of *C. annuum*, *C. frutescens*, and *C. pubescens*, observed that almost all of them were metacentric (m). Although the three species had $2n = 2x = 24$ chromosomes and big heterochromatin blocks close to centromeric regions, they differed about NOR distribution, secondary constrictions, quantity and distribution of heterochromatin along their chromosomes, and frequency of chiasm formation. Long chromocenters and the presence of heterochromatin in telomeric regions allowed distinguishing *C. pubescens* from the other cultivated species. Some irregularities were observed during meiosis (Shopova 1966b), including pyknotic degeneration in or after pachytene, sticky and laggard chromosomes, micronuclei formation and errors during cytokinesis, resulting in anthers with normal, but also with unbalanced and binucleate pollen grains, in significant proportions. The author suggested these anomalies could be consequences of the inbreeding process.

Studying wild accessions of *C. annuum*, Pickersgill (1971) described their karyotypes as 11 meta- or submetacentric (sm) chromosomes and one acrocentric (a) chromosome. Mexican accessions were exceptions and presented two acrocentric pairs. Later, Pickersgill (1977) found a natural tetraploid accession of *C. annuum* ($2n = 4x = 48$ chromosomes). Recently, *C. annuum* (Kumar and Raja Rao 2003) and *C. baccatum* var. *pendulum* (Scaldaferro et al. 2013a) polyploids were obtained by gamma-ray and X-ray induction, respectively, and had their cytogenetic and somatic effects evaluated.

With the aim of localizing some enzyme-coding genes and their linkage relationships in pepper, Tanksley (1984) analyzed cultivars NM6-4 (*C. annuum*), CA4 (*C. chinense*), and their hybrids during mitosis and meiosis and also verified the pollen grain viability of both parents and progenies. In addition, the author utilized primary trisomics from progenies of *C. annuum* cv. Doux des Landes (Pochard 1970, apud Tanksley 1984) to help the identification. The *C. annuum* and *C. chinense*

karyotypes were similar to a previous report by Pickersgill (1971). Pollen viabilities were 84.9, 87.1, and 11.7 %, in *C. annuum*, *C. chinense*, and F_1 hybrids, respectively. During meiotic analyses, 50 % of the hybrids presented normal pairing (bivalents) and 22 % of the cells showed at least one unpaired chromosome. Tri- and quadrivalents were also observed, indicating that both parental species differ by one reciprocal translocation (Tanksley 1984), corroborating the results of Pickersgill et al. (1979). According to these authors, the domesticated forms of *C. annuum* could be distinguished from *C. chinense*, *C. frutescens*, and from semidomesticated forms of *C. annuum* by only one reciprocal translocation. The acrocentric chromosome could be originated by unequal reciprocal translocation between two metacentric chromosomes (Pickersgill et al. 1979). Tanksley (1984) neither observed bridges and fragments during anaphases I and II, nor any evidence that hybrids could be heterozygotic to paracentric inversion. A laggard was visualized in anaphase I, but only in a few cells. The author suggested it could be the chromosome 12, the acrocentric one, or a metacentric chromosome apparently related to the reciprocal translocation that distinguishes *C. annuum* and *C. frutescens*. Lanteri and Pickersgill (1993), with more detailed meiotic analyzes of *C. annuum* × *C. chinense* hybrids, suggested that the cytological difference between these species could be a result of not only one but two reciprocal translocations among three chromosome pairs.

The first version of the linkage map of chromosome 12 in *C. annuum* was based on genetic linkage, trisomic dosage, and translocation data (Tanksley 1984). The *6Pgdh-1* (*6-Phosphogluconate dehydrogenase*) is on the metacentric chromosome, corresponding to the *Noir* trisomic; *Est-3* (*Esterase*) and *Idh-1* (*Isocitrate dehydrogenase*) are on chromosome 12, an acrocentric chromosome that corresponds to *Pourple* trisomic. The *Idh-1* is near the centromere and the *Est-3* is distal on the long arm of that chromosome, presuming a distance of 44 cM between loci. This proposal is supported by the fact that *Est-3* showed a less significant effect on pollen stainability than *Idh-1* suggesting it is farther from the translocation breakpoint. A comparison of the linkage relationships of enzyme-coding genes in pepper with those of putative orthologous loci in tomato revealed that *Est-1-Est-7* and *Pgi-1*(*Phosphoglucoisomerase*)*-Est-4* linkage blocks may have remained intact since the divergence of *Capsicum* and *Lycopersicon*.

Later, Tanksley et al. (1988) studied the genomic homology between pepper and tomato by construction and comparison of their genetic linkage maps, using cDNA clones and in situ hybridization with ribosomal genes. Results of cDNA clones suggest that the two species mostly share the same gene repertoire and the nucleotide sequences of these homologous genes are fairly conserved. Based on orthologous loci, linkage maps were constructed, more specifically 14 linkage groups were obtained, for 80 of the 85 loci detected in pepper. The remaining 5 loci showed no linkage to other markers. By comparing the tomato and pepper maps, the authors suggested a minimum of 32 breakages to transform the order and position of genes in the tomato map to that observed in pepper.

Tomato and pepper not only differ in regard to the number and position of loci containing ribosomal genes, but the two pepper species also differ relative to each other. Two 45S rDNA sites of hybridization in two different chromosomes of *C. chinense*

were observed, but only one in *C. annuum* and tomato. In both pepper species, chromosome 5, a submetacentric chromosome, showed a strong hybridization signal at or near the end of the short arm. The second hybridization site, seen only in *C. chinense*, was located at the end of the long arm of another submetacentric chromosome, the chromosome 6, however, its hybridization signal was smaller and weaker (Tanksley et al. 1988). The authors believe that at least one of the duplications occurred since the divergence of tomato and pepper from their last common ancestor.

Analyses of other *Capsicum* species defined the karyotypic profile of the genus: 12 chromosome pairs ($n=x=12$), from which one or two were acrocentric or telocentric (t) and the others were meta- or submetacentric (Pickersgill 1971, 1977, 1991; Tanksley 1984; Moscone 1992; Park et al. 1999), although recently Souza et al. (2011) had found one *C. chinense* accession with 11 metacentric and one submetacentric pair. The discovery of *Capsicum* wild species with $n=x=13$ chromosomes and their inclusion in cytogenetic and molecular marker studies gave new direction to phylogenetic relationships in the genus.

At the beginning of 1990, *Capsicum* species were grouped according to their similarities in terms of morphology, cytogenetics, and also in their crossabilities (Pickersgill 1991; Zijlstra et al. 1991). Three complexes were defined: *C. annuum* Complex—includes *C. annuum* var. *annuum*, *C. annuum* var. *glabriusculum*, *C. frutescens*, *C. chinense*, *C. chacoense*, and *C. galapagoense*; *C. baccatum* Complex—with *C. baccatum* var. *pendulum*, *C. baccatum* var. *baccatum*, and *C. praetermissum*; and *C. pubescens* Complex—encompasses *C. pubescens*, *C. cardenasii*, and *C. eximium*. Each complex can be considered as a primary gene pool (GP1). In general, successful interspecific crosses occur inside GP1. In the secondary gene pool (GP2), gene transfers are possible and crosses produce at least partially fertile hybrids. In the tertiary gene pool (GP3), pre- or postzygotic barriers exist among groups inhibiting gene introgressions and causing hybridization failures. Species from each complex probably have a common ancestor (Pickersgill et al. 1979; Onus and Pickersgill 2004; Perry et al. 2007; Moscone et al. 2007). Much research has been done since then, involving origin and domestication, dispersion, crossabilities, and cytogenetic studies that corroborate this proposal and allow new additions inside the groups.

Although self-compatibility is the rule in *Capsicum*, unilateral incompatibility was found (Zijlstra et al. 1991) and confirmed in the *C. pubescens* Complex (Onus and Pickersgill 2004). Pistils of species in the *C. pubescens* Complex inhibit pollen tubes of species outside the *C. pubescens* Complex, and the reciprocal crosses are compatible. *C. eximium* and *C. pubescens* are assumed to be derived from self-incompatible ancestors and to have developed self-compatibility relatively recently (Onus and Pickersgill 2004). Self-incompatibility is a particular characteristic of *C. cardenasii* (Pickersgill 1997). It is possible that in *Capsicum*, unilateral incompatibility had arisen by genetic divergence between the *C. pubescens* Complex and the other peppers, not as a product of natural selection (Onus and Pickersgill 2004).

The first research using the FISH in *Capsicum* was conducted by Park et al. (1999). Probes of 5S and 18S–26S rDNA were used to compare and distinguish karyotypes of the domesticated species and also support genetic and evolutionary relationships

of the genus. For all five species, the karyotypes were similar, with 10 or 11 meta- or submetacentric chromosome pairs and one or two (only *C. annuum*) acrocentric pairs, corroborating previous studies. Only one 5S site was located close to the telomeric region of chromosome 1 in all species. This chromosome was identified as chromosome 1 based on genomic synteny between *Capsicum* and tomato. Satellites were observed in one or two chromosome pairs: in *C. annuum* and *C. chinense* it was located in the acrocentric chromosome 12; in all *C. frutescens*, *C. baccatum*, and *C. pubescens*, satellites were found in chromosomes 3 and 12.

On the other hand, number, position, and signal intensity of 18–26S were highly variable among species. *C. annuum* presented two 18S sites, in both chromosomes 4 and 12 (Park et al. 1999). Four 18S sites were observed in *C. chinense*, on chromosomes 4, 7, 11, and 12. C. *frutescens* presented 18S sites distributed in 7 chromosomes: 3, 4, 7, 8, 9, 11, and 12, but with more intense signals in the third, the twelfth, and in both arms of chromosomes 9 and 11. In *C. baccatum* and *C. pubescens*, only two chromosomes did not present 18S sites: the fifth and the sixth, and the fifth and tenth, respectively (Park et al. 1999).

Based on Southern hybridization and rDNA loci, the closest species were *C. annuum* and *C. chinense*. They share several common bands and their chromosomes 1, 4, and 12 presented the same rDNA sites. *C. frutescens* was closely related to *C. chinense*; their chromosomes 1, 4, 7, and 12 were similar. *C. baccatum* was considered an intermediate between *C. frutescens* and *C. pubescens*. The chromosome 3 of these three latter species presented 18S site in its short arm, which did not happen either in *C. annuum* or in *C. chinense*. Thus, in *C. baccatum* and *C. pubescens*, the 18S–26S site was linked to 5S in the short arm of chromosome 1, suggesting they are closely related to each other (Park et al. 1999).

Later, Park et al. (2000) using FISH, confirmed all domesticated species of Capsicum presented 5S rDNA site in the short arm of chromosome 1, however, no secondary constrictions were observed in this chromosome. On the other hand, the 5S sequence sizes varied between 278 and 300 bp among species. Although the coding region was highly conserved in length (120 bp to all species, except for *C. frutescens*, with 119 bp), the spacer sequences varied between 158 and 180 bp. Based on 5S rDNA sequence homologies, it was suggested *C. annuum*, *C. chinense*, and *C. frutescens* formed a separate lineage with a common ancestor. A higher similarity was found between *C. frutescens* and *C. chinense* (89.2 %). *C. baccatum* might be intermediate between this group and *C. pubescens*, the last the most divergent species among them (Park et al. 2000), corroborating the species-complex proposal (Pickersgill 1991; Zijlstra et al. 1991).

Scaldaferro et al. (2006) and Kwon and Kim (2009) also working with FISH and rDNA probes in *Capsicum* confirmed the presence of only one 5S site in all studied species. In contrast, the number and position of 45S sites were variable among and within them. The number of 45S loci was three or four in *C. annuum*, four in *C. chacoense*, 14 in *C. pubescens*, 15 in *C. baccatum* var. *umbilicatum*, and 18 in *C. cardenasii* (Scaldaferro et al. 2006). Kwon and Kim (2009) found one or three 45S signals in *C. annuum*, one or two in *C. frutescens*, two in both *C. chinense* and *C. chacoense*, and four in *C. baccatum*, and suggested these differences in

distribution patterns could be the result of inversions or translocations, satellite DNA amplifications, or dispersion of rDNA sequences.

Based on cytological mapping of rRNA loci, Scaldaferro et al. (2006) suggested many possible chromosome homologies and reinforced relationships among species (Pickersgill 1991; Zijlstra et al. 1991; Moscone et al. 1993). *C. chacoense* and *C. annuum* had rather similar number, position, and size of rDNA gene clusters, with 45S sites being few. On the other hand, *C. cardenasii* and *C. pubescens*, both members of *C. pubescens* Complex, exhibit a 5S site syntenic to a 45S one and a large number of 45S clusters, which are of conspicuous size in the latter species. The eight chromosome homologies between both species should be indicative of their close relationship. In this sense, *C. cardenasii* could be a member of the probable ancestral gene pool from which *C. pubescens* had been originated. Finally, *C. baccatum*, with an increased number of 45S loci and a syntenic 5S site to a 45S one, should be a link between the *C. annuum* and *C. pubescens* Complexes.

Pachytene chromosomes and DNA fibers of *C. annuum*, cultivar CM344, were analyzed. Almost 80 % of the DNA were heterochromatic regions and were intensely DAPI stained whereas the euchromatic regions were localized not only in the chromosome ends but also dispersed in heterochromatic regions, and were lightly stained. The size of the linear 5S rDNA sequence was estimated at 439 kb, which represents 0.016 % of 2,702 Mbp of the pepper haploid genome (Moscone et al. 2003). Considering the used 5S probe had 302 bp, around 1453 5S repeat units tandem arranged were estimated in the pepper haploid genome (Kwon and Kim 2009).

Moscone et al. (1993) used Giemsa C-banding in *Capsicum* to improve the karyological characterization of the genus and the relationships among wild and cultivated species. Centromeric bands were observed in each chromosome of all species, although their number was variable. They were weakly stained in *C. chacoense, C. parvifolium*, and *C. annuum* var. *annuum* but strongly represented in *C. baccatum* var. *pendulum*, *C. pubescens*, and *C. campylopodium*. Smaller to larger distal bands were frequently observed in all species but *C. annuum*, *C. pubescens*, and *C. campylopodium* also presented some intercalary bands. Satellites were always C-positive and the adjacent secondary constrictions were negatively C-banded. The C-banded and classical karyotypes were in agreement. Besides, C-banding allowed chromosome differentiation in all species, considering the small differences in length and arm ratio among them.

Considering both the amount and the distribution of constitutive heterochromatin, species were subdivided in two groups, one with low and the other with high C-heterochromatin content. The first group encompassed *C. chacoense* (with the lowest proportion of C-bands), *C. parvifolium, C. annuum* var. *annuum* (with near similar contents), and *C. baccatum* var. *pendulum.* This latter species presented the highest C-band content within the group and was easily distinguished from the other three species. The other group was composed of *C. campylopodium* and *C. pubescens*, both species with high C-heterochromatin contents (33 % and 28 % of the total karyotype length, respectively) (Moscone et al. 1993).

Moscone et al. (1996), using fluorescent chromosome banding in domesticated species of *Capsicum*, observed dissimilarities of heterochromatic band patterns among and within species. Small differences in heterochromatin content were veri-

fied among *C. annuum*, *C. chinense*, *C. frutescens*, and *C. baccatum*, however, a significant difference was observed in relation to this group and *C. pubescens*. The most common heterochromatic bands were terminal (rarely intercalary and indistinct centromeric). Similar results were obtained by Scaldaferro et al. (2013b) that found a positive correlation between heterochromatin amount and karyotype length, and also constitutive heterochromatin at terminal positions of the chromosomes, except in C. *flexuosum*. The number of NOR-bearing satellited chromosome pairs varied from 1 to 4 (Moscone et al. 1996). Many fluorescent bands obtained in the present work corresponded to C-bands obtained by Moscone et al. (1993). Results from this research support the species-complex proposal (Pickersgill 1991; Zijlstra et al. 1991) and suggest three distinct evolutionary lines to *Capsicum*, in which *C. baccatum* complex could be an intermediate between *C. annuum* and *C. pubescens* complexes.

Interspecific crosses among *C. tovarii* and other *Capsicum* species were performed and their F_1 hybrids were cytologically analyzed to better understand its genetic relationship to the three species-complex (Tong and Bosland 1999). Chromosome pairing and behavior were observed at diakinesis, metaphase-I and anaphase-I of pollen mother cells (PMCs). Both meiosis and pollen viability slides were stained with aceto-carmine. From all crosses, only *C. baccatum* var. *pendulum* × *C. tovarii* and *C. praetermissum* × *C. tovarii* produced seeds with embryo and endosperm when *C. tovarii* was used as male parent. Considering the first cross, more than 100 fruits were obtained from each F_1 plant. The 24 chromosomes of the two parental species paired normally as 12 bivalents, but a few cells with rings were visualized in both species. No chromosome bridges or chromosome lagging was observed. On the other hand, complex chromosome associations were found in F_1 cells. Most chromosomes paired as bivalents but irregular meiotic chromosomal pairing as univalents, quadrivalents, and a quinquevalent, along with the chromosome bridges and lagging were also observed. The F_1 pollen viability was 32.2 %. It is possible that both species could differ by, at least, a reciprocal translocation even though they have basically homologous genomes. Therefore, based on all results, the authors concluded *C. tovarii* belongs to *C. baccatum* Complex (Tong and Bosland 1999). Its heterochromatin content and distribution supported this inclusion (Scaldaferro et al. 2013b).

A similar investigation was developed with wild species *C. lanceolatum* ($n = x = 13$) and *C. buforum* ($n = x = 12$) (Tong and Bosland 2003). *C. buforum* was identified as self-incompatible, similarly to *C. cardenasii*, the only self-incompatible species reported since then. *C. buforum* was more closely related to *C. praetermissum*, *C. pubescens, C. cardenasii*, and *C. eximium* than to other *Capsicum* species and different compatibility levels were observed. As no viable seeds were obtained from interspecific crosses, some genetic isolation was attributed to this species. The analyses of PMCs of *C. buforum* showed 12 bivalents. Two to four lagging chromosomes were visualized and this could be the reason for its pollen stainability reduction (74 %). Later, Pozzobon et al. (2006) found $n = 13$ as the basic number of *C. buforum*. According to them, morphological characteristics described by Tong and Bosland (2003) to the putative *C. buforum* are found only in wild species with 26 chromosomes and maybe some mistake had occurred.

In relation to *C. lanceolatum*, none of the hybridizations were successful and no viable seeds were produced. A possible explanation should be the F_1s chromosome pairing in crosses between species with different chromosome numbers ($n=13 \times n=12$). The aneuploidy condition should cause embryo abortion. However, *C. lanceolatum* × *C. ciliatum*, both species with $n=13$, was also a failure. Meiotic analyses showed quadrivalents in 33 % of *C. lanceolatum* cells, suggesting chromosome interchanges. The irregular meiosis may explain the incompatibility in crosses involving this species. Bridges or sticky chromosomes were not found (Tong and Bosland 2003).

The observation of a small chromosome pair in *C. lanceolatum*, similarly to other species with 26 chromosomes (Pickersgill 1991; Moscone et al. 1993) corroborates Moscone et al. (1993) who suggest the 13th chromosome could be originated by centric fission of a long metacentric chromosome. Nevertheless, the origin of this "extra" chromosome was not elucidated. Considering all results in this study and the evolutionary lines suggested by Moscone et al. (1996), *C. lanceolatum* was not in any described species-complex (Tong and Bosland 2003).

Pozzobon and Schifino-Wittmann (2006) analyzed $2n=24$ and $2n=26$ Brazilian species during meiosis and verified their meiotic behavior was generally regular although some irregularities were also found, such as chromosome elimination, nonorientation of chromosomes at metaphase I, irregular disjunction, desynapsis, laggards, and cytomixis, among others. The meiosis was reported for the first time for *C. buforum, C. campylopodium, C. cornutum, C. pereirae, C. friburguense, C. schottianum*, and *C. villosum* var. *villosum*. In general, meiotic indexes were over 90 % in $2n=24$ species. On the other hand, they were lower in $2n=26$ species, ranged from 0.00 to 99.27 %. Pollen viability ranged from 73.96 to 95.98 % for $2n=24$ species and from 58.89 to 96.92 % for $2n=26$ species. Martins et al. (2010) also found values of pollen viability higher than 96 % in accessions of *C. annuum* var. *annuum*, *C. annuum* var. *glabriusculum*, and *C. baccatum* var. *baccatum* despite some irregularities observed during meiosis in both varieties of *C. annuum*. The cytokinesis was "simultaneous type" and tetrads had tetragonal shape. All accessions were considered meiotically stable and suitable for plant breeding programs.

Palynological characterization of Capsicum species were conducted by Martins et al. (2013). Pollen grains of *C. annuum* var. *annuum*, *C. annuum* var. *glabriusculum*, *C. chinense, C. frutescens*, *C. baccatum* var. *pedulum*, *C. baccatum* var. *baccatum*, and *C. parvifolium* were morphologically analyzed in relation to 13 characteristics and were grouped based on them. The first group was composed of the last three species and the second group by the others, corroborating the proposal of species-complexes (Pickersgill 1991; Zijlstra et al. 1991). However, the inclusion of *C. parvifolium* in *C. baccatum* complex should be cautiously considered, inasmuch as no members of the third complex (*C. pubescens*) or other wild species were included in the present study.

Nuclear DNA content can be estimated by flow cytometry using a standard of known DNA content or cells of species with previously determined DNA content. Galbraith et al. (1983) found 2C DNA content of 5.52 pg in *Capsicum annuum*, and Arumuganathan and Earle (1991) reported a range of 5.6–7.51 pg in seven cultivars, using chicken red blood cells (2C genome size = 2.33 pg) as the DNA standard.

Belletti et al. (1998) analyzed 10 *Capsicum* species by flow cytometry using pea (*Pisum sativum*—2C = 9.07 pg) as the internal standard for estimation of *C. annuum* genome size. Pea has 2C genome size closer to *Capsicum* than other standards previously used. The *C. annuum* 2C DNA was calculated as 7.65 pg. The genome sizes were calculated and vary between 3.691 Mbp (*C. annuum*) and 4.690 Mbp (*C. pubescens*). Based on DNA contents, species were grouped as follows: *C. annuum* Complex—*C. annuum*, *C. frutescens*, *C. chinense*, *C. chacoense*, *C. galapagoense*, and *C. tovarii*; *C. baccatum* Complex—*C. baccatum* var. *baccatum*, *C. baccatum* var. *pendulum*, and *C. eximium*; *C. pubescens* Complex—*C. cardenasii*, *C. praetermissum*, and *C. pubescens*.

Moscone et al. (2003) evaluated variation of genome sizes in nine *Capsicum* species by flow cytometry using *Hordeum vulgare* 1C = 5.06 pg as the internal standard. In addition, they analyzed samples of *C. annuum* and *C. pubescens* using Feulgen densitometry and compared results. Both methods resulted in similar relative values. The 1C DNA values varied almost 75 % among species. The smallest and the largest genome sizes were estimated at 3.283 Mbp (1C = 3.35 pg) and 5.655 Mbp (1C = 5.77 pg), corresponding to *C. chacoense* and *C. parvifolium,* respectively. *C. campylopodium* was the only species with significant intraspecific variation (cytotype 1—1C = 5.74 pg and cytotype 2—1C = 4.53 pg). Five groups were formed. The first group was composed of *C. chacoense* (3.35 pg), *C. annuum* var. *annuum* (3.38 pg), *C. frutescens* (3.40 pg), and *C. chinense* (3.42 pg); the second group encompassed *C. baccatum* var. *pendulum* (3.68 pg), *C. baccatum* var. *baccatum* (3.71 pg), and *C. baccatum* var. *umbilicatum* (3.76 pg); the third had only *C. eximium* (4.06 pg); the fourth, *C. pubescens* (4.47 pg) and *C. campylopodium*, cytotype 2 (4.53 pg); and the fifth, *C. campylopodium*, cytotype 1 (5.74 pg) and *C. parvifolium* (5.77 pg) (Moscone et al. 2003). In general, these DNA values were smaller than those found by Belletti et al. (1998). These variances should be attributed to different stains and internal standards used by both groups.

As a general rule, the white-flowered species (*C. annuum* Complex) presented lower DNA contents (Belletti et al. 1998; Moscone et al. 2003), small amounts of GC-rich heterochromatin, simple heterochromatic banding patterns (Moscone et al. 1993), and one or two Ag-NORs per basic chromosome set (Moscone et al. 1995). Species of *C. baccatum* Complex presented higher DNA contents, more GC-enriched heterochromatin, and a higher degree of complexity in heterochromatic banding pattern than the first group. Besides, it has three or four NORs in the basic complement. These characteristics classified it as an intermediate group, although it is close to that previously mentioned (Moscone et al. 2003).

The species *C. eximium* was isolated from the others, although it was an intermediate between *C. baccatum* and *C. pubescens* Complexes (Moscone et al. 2003). Considering its crossability (Zijlstra et al. 1991; Onus and Pickersgill 2004), it is closer to *C. pubescens* Complex than to *C. baccatum* Complex.

C. pubescens and *C. campylopodium* (cytotype 2) were grouped together, even with different basic numbers ($x = 12$ and $x = 13$, respectively), heterochromatin contents, and number of NORs. On the other hand, *C. campylopodium* cytotype 1 and *C. parvifolium* were grouped together, both with large genomes and only one NOR

per haploid complement. Differences between *C. campylopodium* cytotypes should be a starting point of diverging evolutive paths (Moscone et al. 2003).

Considering the positive correlation among DNA content, karyotype length, and heterochromatin amounts (Moscone et al. 1993, 1995, 1996, 2003, 2007), a proposal of genome evolution in *Capsicum* was generated. Species such as *C. chacoense*, with $x=12$, small genome, simpler GC-rich heterochromatic regions, higher number of NORs per haploid complement, and more symmetrical karyotype should be more primitive. On the other hand, species such as *C. campylopodium,* with 13 chromosome pairs, larger genomes, only one NOR per haploid complement, AT-rich heterochromatic regions, more complex heterochromatic band pattern, and more asymmetrical karyotypes should be more advanced (Moscone et al. 2003).

Many species with $x=13$ chromosomes are found in Brazil. However, only recently new approaches with these wild species have been intensified. Considering both geographic distribution and botanical descriptors, Barboza and Bianchetti (2005) identified three Brazilian species, *C. pereirae*, *C. friburgense*, and *C. hunzikerianum* and suggested the first one was closely related to *C. flexuosum* and *C. schottianum*; the second one was closer to *C. scolnikianum*, *C. cardenasii*, and *C. mirabile*, and the last one, close to *C. cornutum*. Later, 14 species were cytologically investigated by Pozzobon et al. (2006) and information about chromosome sizes and presence of satellites was generated. Eight of them had their chromosomes identified for the first time: *C. cornutum*, *C. schottianum*, *C. villosum* var. *villosum*, *C. friburgense*, *C. pereirae*, and three new species that were not botanically identified since then, with 26 chromosomes. As a whole, the 13th chromosome pair was much smaller than the others and was classified as subtelocentric (st) (Pozzobon et al. 2006), whereas Moscone et al. (2007) classified it as telocentric. This difference should be due to the cytological preparations, including pretreatment solutions, stains, or the method that defines the chromosome classification.

Cytogenetic methodologies evidenced the presence of diploid karyotypes based on $x=12$ or $x=13$, the latter being more asymmetrical and derived than the former (Moscone et al. 1993, 1995, 1996, 2007; Pickersgill 1971, 1991; Tong and Bosland 2003; Pozzobon et al. 2006). The occurrence of both basic chromosome numbers in *Capsicum* generated the hypothesis of two distinct evolution lines in the genus but the origin of the 13th chromosome is not well-defined. Moscone et al. (1993) suggested it should have appeared by centric fission. However, Pozzobon et al. (2006) argue that if it was correct, one of the other 12 might present a very short arm or, maybe, only one arm and there is no mention of this "ancient chromosome" in *Capsicum* literature. Pozzobon et al. (2006) suggested $x=13$ as the ancestral basic number of the genus due to the loss of the small chromosome pair typical for several $2n=26$ species. This hypothesis is supported by a morphoecological study with Brazilian species that propose $x=13$ should be more primitive and $x=12$ had arisen by natural selection (Bianchetti 1996).

On the other hand, Moscone et al. (2007), in a relatively recent review on *Capsicum*, suggested a different evolutionary lineage, based on many cytogenetic methodologies such as classical staining (chromosome number, size, and morphology), silver impregnation (number and position of NORs), fluorescent chromosome

banding (amount, type, and distribution of constitutive heterochromatin), flow cytometry (DNA content and genome size), and FISH (localization of telomeric sequences at the chromosome level). In fact, this work reinforces the hypothesis of Moscone et al. (2003) that species with 26 chromosomes could be evolved from species with 24 chromosomes and reinforces the idea of three independent lines leading to the domesticated peppers: the *C. annuum*, *C. baccatum*, and *C. pubescens* Complexes (Moscone et al. 2007).

Thirty-four accessions, representing 20 *Capsicum* species, were characterized by classical and molecular cytogenetics (Moscone et al. 2007). New data were generated for 17 taxa, including the first chromosome description for *C. recurvatum*. Intraspecific karyotype variation was observed in 10 taxa; cytotypes differed with respect to karyotype formula and length, number, and position of NORs, karyotype asymmetry, heterochromatin amount, and banding pattern. On the other hand, high karyotype constancy was found in the cultivated taxa in comparison to the wild ones. In these cases, differences occur only in number of NORs, size of the NOR-associated satellites, and small variations in heterochromatin amounts. Based on the present work and previous contributions, the authors proposed a model of possible chromosome evolution in *Capsicum*. The $2n=24$ and $2n=26$ species should have originated from a common ancestor. They postulated a diploid ancestor with $x=12$, small genome size, 11m + 1 st chromosomes, two active NORs in the basic complement (in chromosomes 1 and 12), little GC-rich heterochromatin, and simple banding pattern of a symmetrical karyotype. All these karyological features regarded as primitive should be present in C. *chacoense*, a species supposed to be involved in the early evolution of the genus.

According to Moscone et al. (2007), $x=12$ should correspond to the ancestral condition in *Capsicum*, whereas $x=13$ should be a phylogenetic progression, probably due to centric fission of one long metacentric chromosome. Considering this assumption, an asymmetrical karyotype in species with $2n=26$ chromosomes should be expected, with two small chromosomes of the t, st, or sm type generally present in the basic set, as well a st (sm) typical of the $2n=24$ species, as found in the present work. Furthermore, none of the $2n=24$ species analyzed show ectopic localizations of the telomeric sequence at an intercalary position as expected in centric fusions, as proposed by Pozzobon et al. (2006). For more details, see Moscone et al. (2007).

In 2011, two new Brazilian species were described: *C. caatingae* and *C. longidentatum*, both endemic of the northeast region (Barboza et al. 2011). Therefore, Brazil can be considered the diversity center of the genus, with 12 endemic species: *C. caatingae, C. campylopodium*, *C. cornutum*, *C. friburgense*, *C. hunzikerianum*, *C. longidentatum, C. mirabile, C. parvifolium, C. pereirae, C. recurvatum*, *C. schottianum*, and *C. villosum* (Barboza et al. 2011; Stehmann et al. 2012).

A list of the 34 recognized *Capsicum* species and some additional information are presented in Table 3.1.

According to the hypothesis of *Capsicum* evolution by Moscone et al. (2007), the *C. annuum* complex had been differentiated comparatively early, just after *C. chacoense*, by a moderate increase in heterochromatin amount and banding

Table 3.1 The *Capsicum* species, domestication status, chromosome numbers, and geographic distribution

	Species	Domestication status[a]	Basic number[b]	Geographic distribution[c]
1a	*C. annuum* var. *annuum*	D	12	Whole world
1b	*C. annuum* var. *glabriusculum*	SD	12	South USA to North/Northeast Brazil
2a	*C. baccatum* var. *baccatum*	S	12	Colombia to North Argentina; South-Southeast Brazil
2b	*C. baccatum* var. *pendulum*	D	12	USA, Central and South America and India
2c	*C. baccatum* var. *praetermissum*	SD	12	Paraguay, Southeast and Central Brazil
2d	*C. baccatum* var. *umbilicatum*	D	12	USA, Central and South America
3	*C. buforum*	W	13	Brazil: SP and RJ
4	*C. caatingae*	W	12	Brazil: BA, PE, and North MG (endemic)
5	*C. caballeroi*	W	–	Bolivia (endemic)
6	*C. campylopodium*	W	13	Brazil: ES, MG, and RJ (endemic)
7	*C. cardenasii*	W	12	Bolivia (endemic)
8	*C. ceratocalyx*	W	–	Bolivia (endemic)
9	*C. chacoense*	W	12	South Bolivia, Paraguay, North and Central Argentina
10	*C. chinense*	D/SD	12	EUA, Central and South America, China, and Japan
11	*C. coccineum*	W	–	Peru and Bolivia
12	*C. cornutum*	W	13	Brazil: RJ and SP (endemic)
13	*C. dimorphum*	W	–	Colombia and Ecuador
14	*C. eshbaughii*	W	12	South-Central Bolivia
15	*C. eximium*	W	12	South Bolivia and North Argentina
16	*C. flexuosum*	W	12	Paraguay, South and Southeast Brazil, and Northeast Argentina
17	*C. friburgense*	W	13	Brazil: RJ (endemic)
18	*C. frutescens*	D	12	EUA, México, Central and South America Africa, India, China, and Japan
19	*C. galapagoense*	W	12	Galapagos Islands (endemic)
20	*C. geminifolium*	W	–	Colombia, Ecuador, and Peru
21	*C. hookerianum*	W	–	South Ecuador and North Peru (endemic)

22	*C. hunzikerianum*	W	13	Brazil: SP (endemic)
23	*C. lanceolatum*	W	13	México and Guatemala
24	*C. longidentatum*	W	12	Brazil: Central BA and PE (endemic)
25	*C. mirabile*	W	13	Brazil: MG, RJ, and SP (endemic)
26	*C. parvifolium*	W	12	Colombia, Venezuela, and Northeast Brazil
27	*C. pereirae*	W	13	Brazil: ES, RJ, and SP (endemic)
28	*C. pubescens*	D	12	México, Central and South America
29	*C. recurvatum*	W	13	Brazil: RJ, SP, PR, and SC (endemic)
30	*C. rhomboideum*	W	13	Brazil: MG, RJ, and SP (endemic)
31	*C. schottianum*	W	13	Brazil: MG, RJ, and SP (endemic)
32	*C. scolnikianum*	W	—	Peru and Ecuador (endemic)
33	*C. tovarii*	W	12	Peru (endemic)
34a	*C. villosum* var. *villosum*	W	13	Brazil: MG, RJ, and SP (endemic)
34b	*Capsicum villosum* var. *muticum*	W	13	Southeast Brazil

Adapted from Bianchetti (1996), Bianchetti et al. (1999), Barboza and Bianchetti (2005), Pozzobon et al. (2006), Nee et al. (2006), Moscone et al. (2007), Barboza (2011), Barboza et al. (2011), Stehmann et al. (2012)

[a]*D* domesticated, *SD* semi-domesticated, *W* wild

[b]Chromosome numbers of seven species were not found in the literature

[c]*RJ* Rio de Janeiro, *SP* São Paulo, *MG* Minas Gerais, *ES* Espirito Santo, *BA* Bahia, *PE* Pernambuco

pattern complexity. They appeared as relatively primitive. A reduction of genome size and a loss of one active NOR evidently had accompanied the differentiation of *C. galapagoense* and *C. rhomboideum*. In the latter, a centric fission had changed chromosome number ($2n=26$) and increased the karyotype asymmetry. Next, *C. parvifolium* had originated after a striking increase in genome size linked to a small increase of heterochromatin content and banding pattern complexity, a decrease in karyotype asymmetry, and the loss of one active NOR. Subsequently, *C. baccatum* and *C. praetermissum* should evolve together, with a moderate increase in genome size, heterochromatin amount, and banding pattern complexity. The acquisition of one or two additional active NORs by *C. baccatum* and the moderately GC-rich banding pattern by *C. praetermissum* should be the basis of both species differentiation.

In the divergence of the three most advanced branches according to Moscone et al. (2007), increases in genome size, heterochromatin amounts, and banding pattern complexities initiated this process. The first branch gave rise to the purple-flowered group, composed of *C. cardenasii*, *C. eximium*, *C. pubescens*, and *C. tovarii*, with a change of one st to a sm chromosome. The second branch had produced *C. flexuosum*, and finally, the third branch, the most evolved group, composed of *C. mirabile* as the core species, *C. schottianum*, *C. pereirae*, *C. campylopodium, C. recurvatum*, and the majority of other $2n=26$ species. The occurrence of two independent centric fission events during *Capsicum* evolution should have formed two $2n=26$ species subgroups. The first subgroup had been composed of *C. lanceolatum* and *C. rhomboideum* and the other by *C. campylopodium, C. cornutum, C. friburgense, C. mirabile*, *C. pereirae*, *C. recurvatum, C. schottianum*, and *C. villosum*. This assumption is supported by morphological features and geographical distribution of these species (Bianchetti 1996; Bianchetti et al. 1999; Barboza and Bianchetti 2005).

In relation to cultivated taxa, Moscone et al. (2007) corroborated the proposal of three independent ancestral lines (Moscone et al. 1993, 1995, 1996, 2003), the first including *C. annuum, C. chinense*, and *C. frutescens* but clearly distinguishing each species; the second, an intermediate line, composed of *C. baccatum* varieties and in which the wild form, *C. baccatum* var. *baccatum*, should have originated the others; and the last line, encompassing purple-flowered species and suggesting the participation of *C. cardenasii* in the origin of *C. pubescens.*

Although a huge step had been made to elucidate phylogenetic relationships in *Capsicum,* many questions are not answered yet. The use of cytogenetic tools associated with other methods could help to find them. All information generated could be used as models in other research with *Capsicum*-related taxa.

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Chapter 4
Genetics and Breeding of Chili Pepper *Capsicum* spp.

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Abstract A breeding program involves several activities such as germplasm bank maintenance, evaluation of genetic diversity, selection of superior genotypes, progenitor's selection, hybridization, and evaluation of segregating populations. These activities are necessary, in general, to develop new cultivars. A considerable number of researchers around the world are dedicated to *Capsicum* breeding programs. Their great challenge is to select high-yield cultivars resistant to pests and diseases, protect them against biotic and abiotic stresses, and improve their fruit quality, and ornamental potential, according to the purpose for use in industry or for fresh consumption. Market type, fruit or plant, has a number of traits that makes it commercially acceptable. Continuous breeding aimed at production and quality depends on the incorporation of new allelic forms into the new cultivars. To achieve their goals, breeders adopt the available breeding methods. In this chapter we further detail aspects of genetic variability, hybridization, genetic of quantitative traits, breeding methods, and postproduction of ornamental peppers showing the main results found by different groups of chili pepper breeders.

Keywords Mass selection • Hybridization • News cultivars • Improvement • Ornamental pepper

4.1 Introduction

The first breeders of the genus *Capsicum* were the indigenous peoples of the Americas, who domesticated the *Capsicum* species (Heiser 1979) through selection, developing many types of the fruits that exist today, such as the peppers called jalapeño, serrano, and ancho.

In Brazil, *C. baccatum*, *C. chinense*, *C. annuum*, and *C. frutescens* are the species most commonly sold (Lannes et al. 2007; Rêgo et al. 2012a). The botanical varieties in Brazil are well known and receive different names from those of the

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extern international market, for example, Malaguetas, Malaguetinha, Malaguetão, or Malagueta-amarela (*C. frutescens*); pimenta-de-cheiro, pimenta-bode, Cumari-do-pará, Biquinho, or Murupi (*C. chinense*); Doce, Bola, or Cereja (*C. annuum*); Dedo-de-moça, Cambuci, Chapéu-de frade, or Chapéu-de-bispo (*C. baccatum* cv. *pendulum*); and Cumari (*C. baccatum* cv. *bacctatum* and *C. baccatum* cv. *praetermissum*; Casali and Couto 1984; Rêgo et al. 2012a), which are the types most commonly found in open markets to be consumed as fresh as dried spice. The most common cultivar of Pimenta-doce is Agronômico 11, with nonpungent, elongated fruits 18 cm in length (Casali and Couto 1984; Rêgo et al. 2012a). Cereja's fruits are round and small and may or may not be pungent. Pimenta-de-mesa is the common name for dwarf colored plants for ornamental uses (Rêgo et al. 2009a).

The breeding of peppers has been performed via mass selection in African species, and, recently, some breeders have given emphasis to the use of hybridization in breeding programs (Tavares 1993; Geleta and Labuschagne 2004a; Patil and Salimath 2008; Rêgo et al. 2009b, 2012b, c, 2015a; Nascimento et al. 2014; Ferreira et al. 2015; Fortunato et al. 2015).

The great challenge today is to select high-yield cultivars resistant to pests and diseases, protect them against biotic and abiotic stresses, and improve their fruit quality, according to the purpose for use in industry or for fresh consumption. Peppers have a great breeding potential in terms of nutrition, because of their high content of vitamins A and C, carotenoids, and capsaicin. In recent years, peppers have stood out in the market of ornamental plants (IBPGRI 1983; Poulos 1994; Bosland and Votava 2003; Bontempo 2007; Rêgo et al. 2009a, b, 2011a; Barroso et al. 2012).

Each type of pepper, depending on market type, fruit or plant, has a number of traits that makes it commercially acceptable (Bosland and Votava 2003; Poulos 1994; Rêgo et al. 2009a, b; Table 4.1). Some traits are more difficult to manipulate than others, as is the case of pungency content (Zewdie and Bosland 2000, 2001).

To achieve their goals, breeders adopt available breeding methods. The breeding methods employed on autogamous plants, such as pepper, usually involve hybridization in the production of new sources of variability. In populations with high variability, on the other hand, selection-based methods may be utilized. For a breeding program to be successful, however, the breeders must know the genetics of the traits of interest and the compatibility within and between species (Allard 1971; Fehr 1987; Rêgo et al. 2009a, b, 2011a, 2012b, c, 2015b; Nascimento et al. 2014; Ferreira et al. 2015; Fortunato et al. 2015).

Continuous breeding aimed at production and quality depends on the incorporation of new allelic forms into the new cultivars. It is not yet known, however, which alleles will be useful in future commercial varieties until the need arises (Hancock 1992; Allard 1971; Fehr 1987; Nascimento et al. 2014). In the hybridization-based breeding methods, the selection of parents is a critical step. In general, parents are chosen based on their performance and on the complementarities among them (Allard 1971).

Another important factor to be taken into account in a breeding program is the available germplasm. Several countries of Latin America, among them Brazil,

Table 4.1 Objectives of breeding for fruit and plant quality according to the type of consumption

Market type	Important fruit and plant quality traits
Fresh fruit	Visual appearance, color, pungency, shape, size, number, flavor, pericarp thickness, endocarp/seeds ratio, provitamins A, vitamins C, E, B1, B2, and B3, yield
Processed fruit (sauces, pastes, pickles)	Color, pungency, flavor, pericarp thickness, soluble solids content, endocarp/seeds ratio, yield
Dried fruit (whole or powder)	Color, pungency, flavor, dry weight, endocarp/seeds ratio, low fiber content, soluble solids content, productivity
Oleoresin extraction	Essential oils (color and pungency), oil production
Ornamentation (plant)	Visual appearance (color, shape, and size of flowers, leaves, and fruits), plant height, canopy width, ratio between plant height and canopy width with pot size, fruit yield/plant.
Ornamentation (fruit)	Color, pungency, shape, number of fruit ripening stages (at least two), position of the fruit on the plant (standing out in the foliage)
Postharvest (fruit)	Firmness, water loss (wrinkling), color
Postproduction (plant)	Fruit firmness, persistence of leaves and fruits on the plant, color of fruits and leaves, longevity in the pot (in days), resistance to ethylene

Source: Bosland (1993), Yuen and Hoffman (1993), Poulos (1994), Rêgo et al. (2009a, b)

are considered to give top priority to the *Capsicum* germplasm collection (IBPGRI 1983).

The few Active Germplasm Banks of *Capsicum* in Brazil usually contain more varieties of domesticated species, although wild species are a source of resistance genes (Bianchetti and Carvalho 2005). The Active Germplasm Banks of pepper in Brazil belong to the Federal University of Paraíba (BAG-UFPB, Areia-PB), Federal University of Viçosa (BAG-UFV, Viçosa-MG), State University of Norte Fluminense (BAG-UENF, Campos dos Goytacazes-RJ), Instituto Agronômico de Campinas (BAG-IAC, Campinas-SP), and Embrapa Vegetables (Brasília-DF). In the following sections we further detail these aspects, showing the main results found by different research groups in Brazil and in the world.

4.2 Genetics

4.2.1 Genetic Variability

The first list of genes of the genus *Capsicum* contained 50 genes, and the rules of nomenclature and standardization of genes was determined by Lippert et al. (1965). Daskalov and Poulos (1994) and the *Committee of Capsicum and Eggplant Newsletter* expanded this list and described protocols for names and symbols. Recently, Wang and Bosland (2006) have conducted a review describing 292 genes for the genus.

Fig. 4.1 Controlled self-pollination of the *Capsicum* species (*Source*: Rêgo 2001)

Cytogenetic studies on the structure and morphology of chromosomes have been conducted by several authors since 1940, as seen in a previous chapter. The DNA content of the different species, however, was determined by Belletti et al. (1998).

The genetic variability of morphoagronomic traits, within and between accessions from the germplasm bank and of commercial varieties, has been the focus of many studies, for example, Inoue and Reifschneider (1989), Rêgo (2001), Rêgo et al. (2003), Sudré et al. (2005), Rêgo et al. (2011b, c), Nascimento et al. (2014), Silva Neto et al. 2014; Pessoa et al. 2015; and Nascimento et al. 2015; Rêgo et al. 2015a, b.

The phenotypic variability within the line, as a consequence of natural hybridization, is often found in elite lines in a breeding program or in released cultivars. The cross-pollination rate in the *Capsicum* species is not always known. In practice, it is easy to find contamination of sweet-pepper fields from the crossing with pungent peppers over generations of uncontrolled pollination. A way to prevent cross-pollination is to cover the plants individually with organza (Fig. 4.1) (Rêgo 2001), with fabric cages for more than one plant (Bosland 1993), or even glue the flower bud when it is in preanthesis (Fig. 4.2; Rêgo et al. 2012d).

4.2.2 Hybridization and Compatibility

Hybridization is an important factor in the evolution of plants as a source of new genetic combinations and as a mechanism of speciation. This procedure is also utilized to insert genes that provide desirable traits to cultivated plants (Cruz and

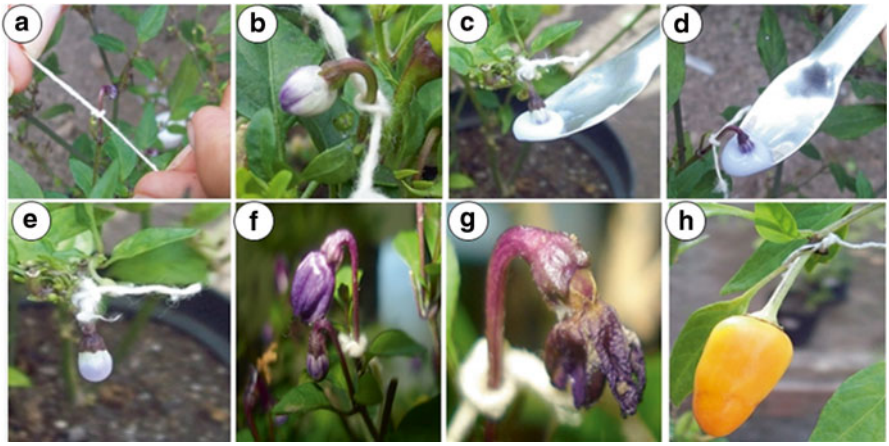

Fig. 4.2 Stages of self-pollination: (**a**, **b**) identification of the bud; (**c**, **d**) bud gluing; (**e**) bud with glue; (**f**) bud after 3 days; (**g**) bud after 5 days; (**h**) fruit in intermediate stage (Rêgo et al. 2012d)

Regazzi 1994; Gonçalves et al. 2011). According to Nascimento et al. (2012b), hybrids are, in general, more stable, uniform, and productive than cultivars from open pollination, for most traits.

Hybridization within pepper species, involving different types or cultivars, has not been explored much (Legg and Lippert 1966; Rêgo et al. 2009b). According to Rêgo et al. (2012d), among the factors contributing to the restriction of the use of hybridization in the breeding of *Capsicum* are the difficulty to handle the flowers and the low production of seeds per fruits. The steps for the manual crosses are shown in Fig. 4.3.

The hybridization between varieties of a same species, in general, produces the sufficient amount of seeds. Although some intraspecific crosses show a low percentage of fruit set, around 20 % (Nascimento et al. 2015a, b, c, d). Contrastingly, seeds originating from interspecific crosses are harder to obtain due to the incompatibility and/or incongruity of crosses (Bosland and Votava 2003; Costa et al. 2009; Rêgo et al. 2011d; Nascimento et al. 2012).

Nascimento et al. (2012) demonstrated that the cross between *C. annuum* and *C. chinense* has a varied fruit set rate (0–29 %). Costa et al. (2009) obtained crossing rates varying from 8.88 to 40 % between these two species. Nascimento et al. (2012) and Nascimento et al. (2015b) also demonstrated reciprocal effects on the fruit set rate of crosses and on the number of seeds formed, in intra- and interspecific crosses. Barroso et al. (2015) showed the importance of seed quality in the establishment and development of *Capsicum* plants. These authors highlighted low heritability values and epistatics effects for germination at 14 days. On the other hand Medeiros et al. (2015) found high heritability values of traits related to germination and only additive effects for seed germination in vitro.

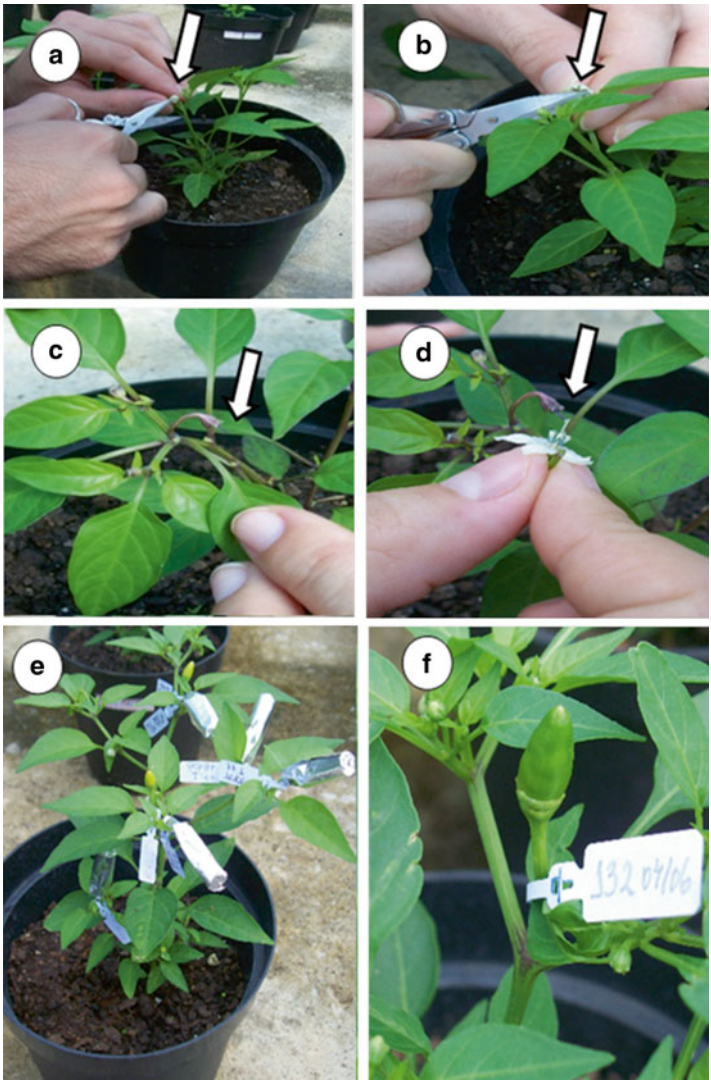

Fig. 4.3 Manual crossing in *Capsicum*. Stages: (**a**, **b**) emasculation; (**c**) emasculated bud; (**d**) pollination, exhibiting the full flower; (**e**) covered bud; (**f**) labeled fruit (Rêgo et al. 2011d)

4.2.3 Male Sterility

Male sterility (MS) was first described by Martin and Crawford (1951) and soon after by Peterson (1958), working with *C. annuum*. Male sterility is a trait of interest in the breeding of *Capsicum*, as it is easier to obtain hybrids due to the absence of

viable pollen in the flower (Shifriss and Frankel 1969; Corrêa et al. 2007; Monteiro et al. 2011). Genetic male sterility (GMS) and cytoplasmic male sterility (CMS) were described by Shifriss (1997). The former is determined by a series of recessive alleles (ms), which can interact with a plasma gene S (Shifriss 1973; Shifriss and Frankel 1969). More than a dozen MS alleles have been described. These are natural mutants, or obtained by mutagenesis (Shifriss 1997). Producing and maintaining a male-sterile line is a hard task and, thus, its use is limited (Daskalov and Mihailov 1988). These same authors studied the CMS. However, this system is unstable and can generate fertile pollen in some conditions (Shifriss and Frankel 1971). Fertility can be easily restored through backcrosses with one or both parents. Details on how to keep males sterile and restore fertility can be viewed in Shifriss (1997).

4.2.4 Maternal Effects, Heritability, and Combining Ability of Quantitative Traits in Capsicum

4.2.4.1 Maternal Effects

Rêgo (2001) performed analyses of reciprocal effects in *Capsicum baccatum* utilizing 28 hybrids and their reciprocals. These authors evaluated 14 fruit-quality and morphoagronomic traits and, based on the tests utilized, reciprocal effects were detected in all fruit and morphoagronomic traits. In contrast, no parent showed significant differences in more than 50 % of the crosses, which showed the importance of these maternal effects on these traits, except for pericarp thickness. According to Rêgo et al. (2009b), despite the existence of reciprocal effects, they may be considered irrelevant, in this species, especially in programs aimed at the generation of lines, because no reciprocal general combining ability (GCA) effect was detected. However, if the objective of the program is to obtain hybrids, these effects should be considered. Nascimento et al. (2015b) stated that the intraspecific compatibility also varied with the directions of crosses. These authors showed the importance of the knowledge of the directions of crosses for the success in a hybrid breeding program.

4.2.4.2 Heritability and Combining Ability of Quantitative Traits

Some authors report the scarcity of research studies on narrow-sense heritability, in peppers, although estimates of broad-sense heritability have been well-studied for several traits (Poulos 1994; Sreelathakumary and Rajamony 2004; Hasanuzzaman et al. 2012; Silva et al. 2013).

Rêgo (2001) determined the presence of an epistatic effect on the following traits: total soluble solids, fruit dry matter, pericarp thickness, plant height, first bifurcation height, canopy diameter between plants, and yield. The additive-dominant model

could be applied to the traits of major and minor fruit diameter, fruit length, fresh matter, and fruit fresh matter content, canopy width between rows, and fruit yield per plant. Sousa and Maluf (2003), however, detected epistatic effects also in the determination of seed yield per fruit. Anandhi and Khader (2011) found epistatic effects for the trait's plant height, number of branches, fruit yield per plant, fruit length and diameter, seed yield per fruit, and green fruit yield per plant.

The knowledge of the combining ability of parents is a prerequisite in the direction of crosses aimed at production of good hybrids and lineages. The GCA is related to the additive genetic effects, whereas the specific combining ability (SCA) is related to the nonadditive genetic effects. Hybrid combinations with favorable SCA, good performance per se in the traits of interest, and which involve at least one parent with good GCA, are of interest in the plant breeding program (Kirsch and Miller 1991; Rêgo et al. 2009b; Nascimento et al. 2014; Ferreira et al. 2015).

The effects of GCA and SCA referring to 14 traits in the *C. baccatum* species were evaluated by Rêgo et al. (2009b), who demonstrated the importance of the additive and nonadditive effects on the expression of several quantitative traits. The traits of minor fruit width, soluble solids, pericarp thickness, first bifurcation height, plant height, canopy diameter between rows and between plants, and yield showed a prevalence of nonadditive genetic effects, which can be better explored in specific programs for hybrid production. A similar study was conducted by Nascimento et al. (2014) and Ferreira et al. (2015) in *Capsicum annuum*, in which the authors observed the significance of the CGA and SCA effects on all traits analyzed. Reciprocal effects were also observed by these authors, except for the traits of fruit length, pericarp thickness, placental length, and seed yield per fruit. Similar data were found by other authors with other species of the genus (Zambrano et al. 2005; Ahmed et al. 1999; Geleta and Labuschagne 2004a; Schuelter et al. 2010).

On the other hand, Sousa and Maluf (2003) determined that the nonadditive effects predominate in the production traits of fruit length/width ratio, fruit dry matter, production of capsaicin, and seed yield per fruit. For these same traits and also precocity traits (days to flowering and fructification), pericarp thickness, fruit yield, total soluble solids, and ascorbic acid content, Geleta and Labuschagne (2004b) determined the existence of dominance effects, which was also reported by Geleta et al. (2004). In addition, Rêgo et al. (2012b) determined dominance effects for days to flowering.

Rêgo et al. (2012b) determined that both the additive and the nonadditive genetic effects influence the plantlet and flower traits, except anther length. Ferreira et al. (2015) found predominant additive effects determining corolla length and number of stamens. Fortunato et al. (2015) also showed the predominance of additive effects for corolla length, petal width, and anther and style length.

For the traits in which the genetic additive effects predominate, it is suggested to utilize backcrossing or selection-based methods. For variables with predominance of nonadditive genetic effects, however, exploring the hybrid vigor may be a good strategy.

4.3 Breeding Methods

Several methods can be utilized in the development of a new cultivar. These should be determined by the breeder according to the objectives of the program and the existence of genetic variability in the basic population. The most widely used methods in the development of new *Capsicum* varieties are mentioned below.

4.3.1 Mass Selection

This method should be used for populations with genetic variability and selected in environments where the traits express themselves and for those of high heritability, inasmuch as selection is based on the phenotype.

In Brazil, this method has been used efficiently by the breeding groups of the Federal University of Paraíba (UFPB), State University of Norte Fluminense (UENF), and Embrapa Vegetables.

4.3.2 Pedigree

This method is based on hybridization and involves the ancestry record of each plant selected within and between lines (Fehr 1987). The peppers BRS Sarakura and BRS Garça, adapted to Central Brazil, were developed by Embrapa Vegetables employing this method (Carvalho et al. 2009). Segregating generations F_3, F_4, and F_5 are being evaluated and selected at UFPB for ornamental purposes by the genealogical method. Cultivar Ouro Negro, or UFPB2, was selected using this method.

4.3.3 Backcross

This method is effective when one aims at transferring one or a few genes. A successful case of its use was the transfer of virus resistance from the species *C. chinense* to *C. frutescens* (Greenleaf 1986). This method has been utilized efficiently to introduce genes of resistance to diseases.

4.3.3.1 Recurring Selection

This method involves interpopulation crossing for the formation of a new population base. It is used for the selection of quantitative traits of low heritability. Palloix et al. (1990a, b) utilized this method in the development of two lines of pepper (*C. annuum*) resistant to *Verticillium dahliae* and *Phytophtora capsici*.

4.3.4 SSD (Single Seed Descent)

This method involves the advance of generations without selection (Fehr 1987). Generation advance can be performed in greenhouses. Villalon (1986) utilized this method to fix recessive genes of resistance to potyvirus. Moreira et al. (2009) utilized this method to obtain lines resistant to bacterial spot and with high yield.

4.3.5 Mutation Breeding

This is not exactly a breeding method, but a way to generate new mutant alleles of interest. Mutants for pericarp color in pepper were successfully introduced chemically and by ionizing radiation, generating stable individuals through selection in subsequent generations (Bhargava and Umalkar 1989). Venkataiah et al. (2005) obtained, by chemical induction, mutants of *C. praetermissum* resistant to streptomycin. Chemical and physical mutagens have been utilized successfully in the generation of genetic variability for fruit and plant traits by the research group of the Federal University of Paraíba. Nascimento et al. (2015a) found different forms of fruit in mutated plants and determined the ideal ethyl methanesulphonate (EMS) and exposure time to obtain pepper mutants.

4.4 Correlations Among Traits

The knowledge of the association among traits is of great importance in breeding works, especially when the selection of one of them is difficult due to low heritability, or problems of measurement and identification. The simple correlation coefficients may not be completely informative as to the relationship between two variables, because the effects caused by other variables may be confusing these values. The partial correlation coefficient, which removes the effects of other traits on the studied association, and the path analysis, which deploys the correlation coefficient to direct and indirect effects on the basic variable, are auxiliary measures in the study of correlations. Rêgo et al. (2001) employed path analysis and partial correlations in the choice of selection strategies for 10 important traits in the breeding of pepper. For the path analysis, the trait yield was considered the basic variable. The variables of pericarp thickness, fruit length, plant height, and fruit yield per plant showed the highest partial correlation coefficients with yield (0.67, 0.77, 0.63, and 0.88, respectively), despite the low simple correlation coefficients (0.28, 0.14, 0.51, and 0.36, respectively). They also displayed the highest direct effects on the principal variable, indicating that pleiotropy and/or epistasis with genes, which control the other morphological variables, mask the effects of these traits on yield. Despite the low correlations, the direct effect is high; thus, the traits can be utilized

in a selection index. The simple and partial correlation coefficients for the variables of major and minor fruit diameter, canopy width, first bifurcation height, and dry matter yield were low, and their effects on the principal variable have an indirect origin, via other variables, mainly pericarp thickness and plant height.

Utilizing path analysis in fruit traits, Silva et al. (2013) determined that the fruit dry matter is negatively correlated with pedicel and fruit lengths, fruit width, pericarp thickness, and average fruit weight.

Gains in yield can be achieved by selecting tall plants, with a higher fruit yield per plant, and longer fruits with a thicker pericarp. In this context, the use of selection indexes would be the most recommended strategy for the generation of new improved genotypes. If the objective is to select plants with fruits that have a greater dry matter content, plants bearing fruits with a smaller width should be selected.

4.5 Ornamental Peppers

The sale of ornamental plants in pots is becoming increasingly widespread; in general, more than that of cut flowers. In the ornamental pepper industry, the diversity of supply of new types opens new markets (Casali and Couto 1984; Rêgo et al. 2009b, 2011d). Among the ornamental plants grown in pots, the cultivation and search for peppers have increased, because they have a double purpose, especially when grown in pots or in gardens. The use of ornamental peppers for decoration and for consumption adds value to this product, increasing the financial return to the producer (Finger et al. 2012).

Ornamental peppers have had great prominence and good acceptance by the consumer market; they are popular in Europe and are gaining popularity in the United States. In Brazil, the sale of ornamental pepper is still restricted to street markets and some supermarkets, but the scenario has been changing, and consumers with higher purchasing power are already acquiring peppers at flower shops. This business is an important source of income to agricultural populations (Bosland et al. 1994). Family farming has been primarily responsible, in Brazil, for the expansion of the pepper-growing area in several states (Rêgo et al. 2011d).

Not every pepper cultivar adapts to cultivation in a pot, with variations present even within the same species (Fig. 4.4). Only those which show reduced plant size and harmony in the pot can be grown and marketed as ornamental plants. The traits of plant height, total height (heights of plant and pot), canopy width, and color and position of the fruit and flower are criteria utilized by consumers at the moment of purchase (Table 4.1) (Barroso et al. 2012; Nascimento et al. 2013).

To obtain good harmony, it is advisable that the ratio between plant height and canopy diameter, be 1.5–2 times the pot height or width, respectively (Barbosa 2003; Barroso et al. 2012). Pots with 900 mL capacity are often used successfully in the production of ornamental pepper (Fig. 4.5). Further research on the best containers and their dimensions is important, as they will influence the final production costs of ornamental peppers, so unnecessary expenses can be reduced.

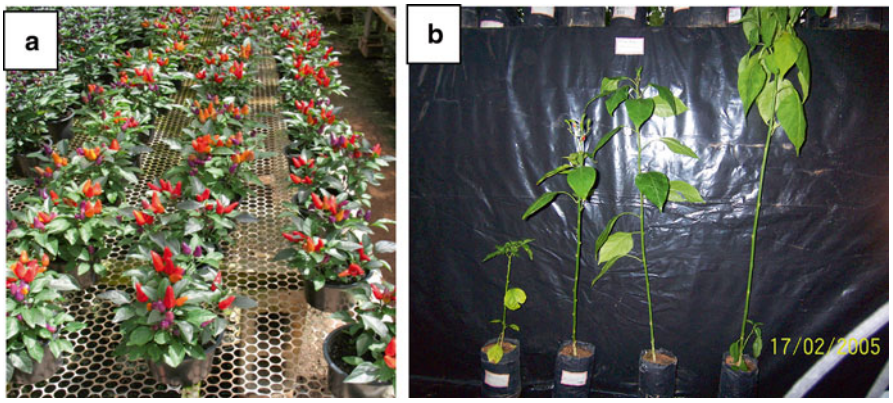

Fig. 4.4 (**a**) Cultivars of pepper adapted to pots; (**b**) pepper cultivars not adapted to pots. Different cultivars of the *Capsicum frutescens* species with different plant heights (**a**: Finger FL and **b** Rêgo et al. 2011d)

Fig. 4.5 Ornamental pepper plants grown in a greenhouse in Areia-PB, Brazil, in pots with 900 mL capacity

4.6 Postproduction

Peppers are, in general, demanding plants in terms of temperature and, for this reason, in most pepper-growing regions of Brazil they are planted in the early spring. In regions of low altitude and mild winters, they can be cultivated all year round (Filgueira 2003). However, few studies have been carried out with ornamental peppers on production factors such as size, precocity, aging capacity in the pot, and postproduction factors such as sensitivity to ethylene, capacity to maintain

Table 4.2 Broad-sense heritability (H^2_b), narrow-sense heritability (H^2_n), allelic interaction, and gene interaction for leaf and fruit abscission in ornamental pepper

Trait	H^2_b	H^2_n	Allelic interaction	Gene interaction
Leaf abscission	98.57	0.01	Overdominance	**
Fruit abscission	99.62	95.00	Overdominance	ns

** significant at 1% of probability by t test.
ns = not significant

Table 4.3 Correlation between leaf abscission and flower and fruit traits after exposure to ethylene (10 μL L^{-1})

Trait	Leaf abscission	H^2_b	H^2_n
Anther length	0.95*	0.36	0.13
Major fruit diameter	0.98*	0.84	0.75
Pedicel length	0.95*	0.80	0.62
Pericarp thickness	0.95*	0.86	0.66
Dry matter content	0.96*	0.71	0.71

* significant at 5% of probability by t test

photosynthesis under low- and high-luminosity conditions, and the use of inhibitors of the ethylene action to increase postproduction longevity in pots.

If there is too much ethylene in the circulating air (exhaust gases or ripe fruits), ethylene-sensitive flowers and plants will suffer wilting, bud drying, and abscission of leaf and fruits, among other problems (Woltering et al. 1996). However, the concentration of ethylene required to cause these effects depends on factors including time of exposure, temperature, developmental stage, and sensitivity of the species or variety (Hoyer 1996; Segatto et al. 2013).

The response of ornamental peppers to ethylene was studied by Segatto et al. (2013), who determined that after 48 h in the presence of 10 μL L^{-1} of ethylene, there was a significant difference in the chlorophyll contents in the different genotypes of *C. annuum* tested.

Rêgo (2015) (data not published) worked with five generations (parents, F1, F2, BC1, and BC2) treated for 6 h with 10 μL L^{-1} ethylene for 48 h and determined heritability for leaf and fruit abscission, allelic and gene effects, and correlation with morphoagronomic traits (Table 4.2). These authors determined the presence of overdominance and gene interaction for the trait leaf abscission in treated peppers (Table 4.2).

Nascimento et al. (2015c) determined the existence of a high positive correlation between flower and fruit traits and leaf abscission caused by ethylene (Table 4.3). Plants more resistant to ethylene can be selected by selecting plants with smaller fruits with a thinner pericarp and lower dry matter content. These data were confirmed in the study of these authors who also determined that there is no correlation between fruit drop and leaf senescence after exposure to ethylene.

Santos et al. (2013) studied the sensitivity to ethylene in seven F2 populations of ornamental pepper and observed significant differences between the evaluated populations in leaf and fruit abscission (Table 4.4 and Fig. 4.6).

Table 4.4 Percentage of leaf and fruit abscission within seven F_2 families (A–G) of ornamental peppers treated with ethylene

Family	Leaf abscission (%)	Fruit abscission (%)
A	66.81–100	0.0–92.62
B	68.36–100	0.0–100
C	72.26–100	0.0–48.70
D	73.46–100	7.5–92.02
E	72.40–100	3.30–100
F	100	6.97–100
G	86.55–100	0.0–100

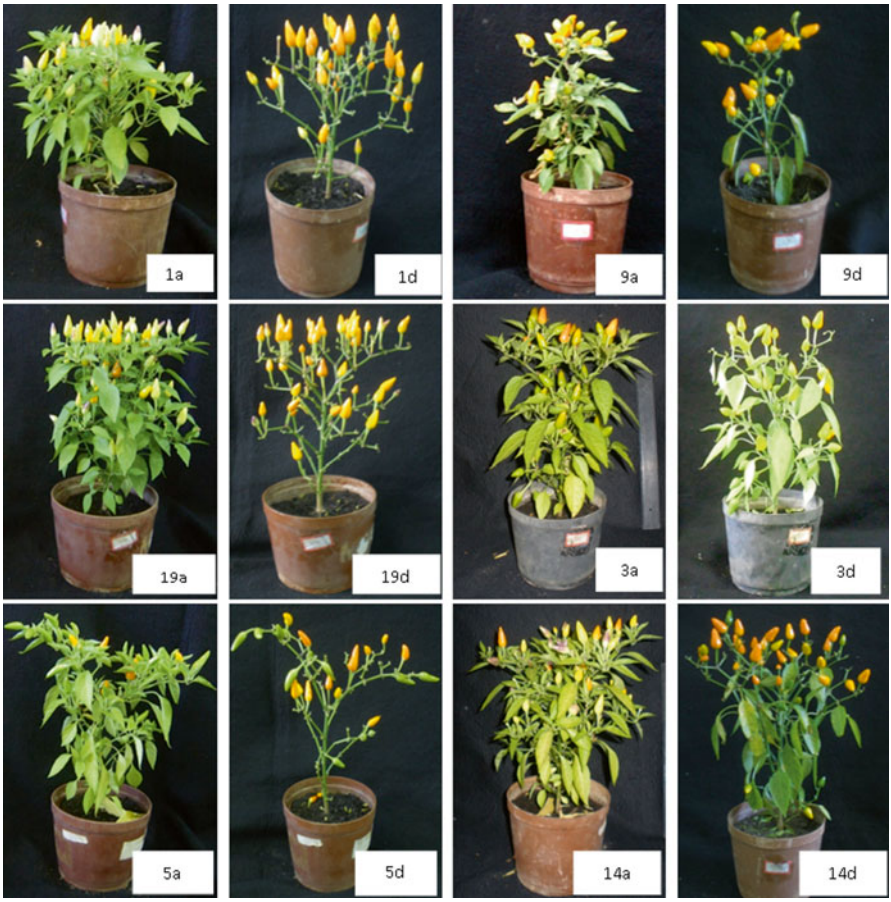

Fig. 4.6 Effect of ethylene in segregating populations of ornamental pepper. (*a*) before application of ethylene; (*d*) after application of ethylene (Santos et al. 2013)

The resistant populations were selected and are being evaluated in the breeding program of the Federal University of Paraíba, in Areia-PB, Brazil, for ornamental purposes. In contrast, susceptible populations were selected to be utilized for the production of cut stem bouquets (Fig. 4.7).

Fig. 4.7 Plant with leaf abscission and fruit persistence after ethylene treatment

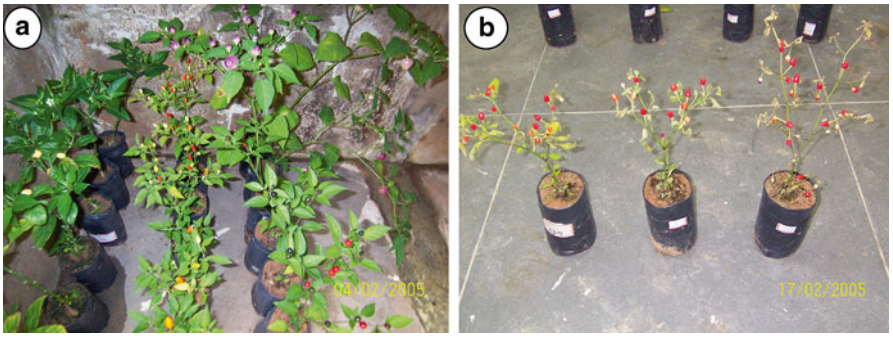

Fig. 4.8 Cultivars of ornamental pepper subjected to transport simulation (**a**) and a more susceptible variety after 13 days at room temperature (**b**) (Rêgo et al. 2011d)

Rêgo et al. (2009a) and Silva et al. (2009) demonstrated that the longevity of ornamental pepper in pots can vary from 13 to 72 days, after being subjected to simulated transport for 48 h, depending on the cultivar (Fig. 4.8).

4.7 Breeding for Ornamental Purposes

In general, the seeds from the ornamental varieties available in the Brazilian market are hybrids and the available cultivars are Gion red, pirâmide, espaguetinho ornamental, and grisu f-1 (Fabri 2008). There is a growing demand for new cultivars with colorful, attention-getting fruits and flowers that stand out in the foliage, with a small size and with postproduction quality.

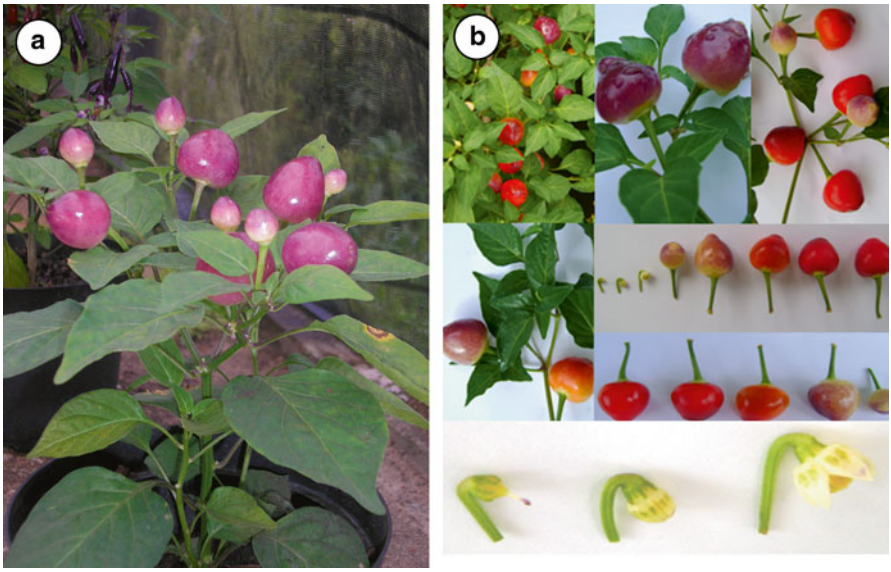

Fig. 4.9 Plant (**a**) and fruit and flower (**b**) aspects of cultivar Eliza's rainbow (Rêgo et al. 2011d)

In this regard, the Federal University of Paraíba (UFPB) has been developing, together with the Federal University of Viçosa (UFV), a breeding program of peppers for ornamental purposes with the following objectives: (1) to select pepper lines for family farmers; (2) to promote intra- and interspecific hybridization among the selected lines; (3) to advance generations through segregating populations; (4) to perform molecular analyses; and (5) to conduct studies of postproduction longevity. Many results have been obtained, such as selection of lines with a longer postproduction time (Rêgo et al. 2010), selection of ethylene-resistant lines (Santos et al. 2013), development of 290 hybrids of the *Capsicum annuum* species, and maintenance of segregating populations in a greenhouse (Rêgo et al. 2015a, b).

Eliza's rainbow (UFPB 1) and Ouro Negro (UFPB 2): new cultivars of potgrown ornamental pepper.

Two new cultivars were obtained. Eliza's rainbow (UFPB 1) was obtained through five cycles of mass selection with progeny testing, for three consecutive years, in a basic population of a cherry-like fruit of *Capsicum baccatum* chili pepper. Cultivar Ouro Negro (UFPB 2) was obtained by the genealogical method from the advancement of generations obtained by diallel crosses (Nascimento et al. 2012, 2014; Rêgo et al. 2012b, c). These segregating populations were evaluated for six consecutive cycles.

Cultivar Eliza's rainbow presents anthocyanin in the stem, densely branched plants with medium density of green leaves, and green, erect fruits with anthocyanin spots, and four fruit-ripening stages with the colors beige, purple, orange, and red (Figs. 4.9 and 4.10). The flower is erect, white, and has green-yellowish spots in the

Fig. 4.10 New cultivar, Eliza's rainbow (*purple* fruits), subjected to the cultivar registration, compared with commercial cultivar Calypso (*yellow* fruits) (Rêgo et al. 2011d)

corolla (Fig. 4.9b). The plant height values (49.24 cm) confirm its ornamental use as compared with the control cultivar (Calypso; Fig. 4.10). Cultivar Eliza's rainbow (UFPB 1) is recommended both for use in gardens and in pots. All characterizations was done following the Capsicum descriptors (IPGRI, 1995).

Cultivar Ouro Negro has sparse, green foliage and erect fruits that are black when not ripe and yellow when ripe (Fig. 4.11). The values obtained for plant height (39 cm) confirmed its ornamental use. Cultivar Ouro Negro is only recommended for use in pots, because of its very small size (Rêgo et al. 2015a, b).

Both cultivars are in final evaluation trials for registration in the National Cultivar Registration Systems.

4.7.1 Ornamental Hybrids

Parallel to the development of new cultivars by mass selection and by the genealogical method, intraspecific hybrids of the *Capsicum annuum* species have been produced, and they are currently being evaluated in comparison with commercial cultivars (Fig. 4.12a, b). At present, 53 hybrids are being evaluated (Fig. 4.12b), aiming at use as ornamental pepper in pots, resistant to the action of ethylene, and for production of different populations (Figs. 4.13 and 4.14), which will be used as a basic population in the breeding of *Capsicum* with ornamental purposes.

Fig. 4.11 Plant and fruit aspects of cultivar Ouro Negro, developed at CCA-UFPB (Rêgo et al. 2015a, b)

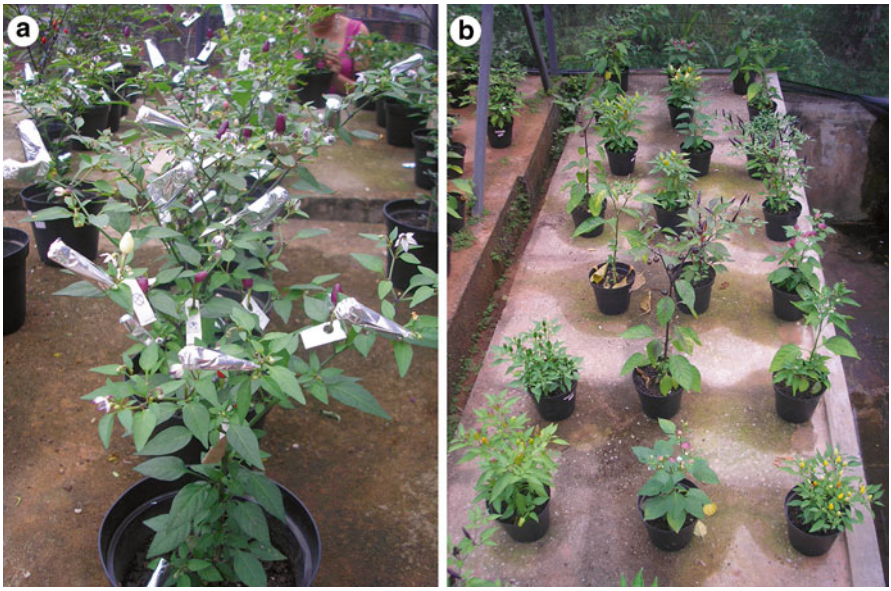

Fig. 4.12 Controlled hybridization of ornamental peppers (**a**) and new hybrids (**b**) under testing at CCA-UFPB, Areia-PB, Brazil (Rêgo et al. 2011d)

Fig. 4.13 F2 generation of *C. annuum* ornamental peppers (Rêgo et al. 2011d)

Fig. 4.14 Genotypes representing the different phenotypic classes observed in six generations of pepper (*C. annuum*) (Nascimento et al. 2015d)

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Chapter 5
Molecular Markers in *Capsicum* spp. Breeding

Rosana Rodrigues, Fabiane Rabelo da Costa Batista, and Monique Moreira Moulin

Abstract The genetic diversity among and within *Capsicum* species can be estimated by different methods and their choice is dependent on the available resources and the desired precision of the researcher. As well as in some species of economic interest, there are several studies using molecular markers such as RAPD, AFLP, SSR or microsatellites, and ISSR to support breeding in *Capsicum*. Although there are other molecular markers used for *Capsicum*, this chapter only emphasizes results of these markers, due to their large usage. They have been used aiming to estimate genetic diversity and to identify duplicates in germplasm collections; to develop DNA fingerprinting; to evaluate seed genetic purity; to assisted selection in breeding programs; for genetic mapping, and gene isolation research. These markers differ with each other on the ability to detect polymorphisms, at application cost, ease of use, and result consistency.

Keywords DNA • *Pepper* • Microsatellites • RAPD and ISSR

As well as in some species of economic interest, there is a certain amount of research using molecular markers such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), SSR (simple sequence repeat) or microsatellites, and ISSR (inter single sequence repeats) to support breeding in *Capsicum*. These markers have been used aiming to estimate genetic diversity in germplasm collection; to develop DNA fingerprinting; to evaluate seed genetic purity; to assist selection in breeding programs; and for genetic mapping and gene isolation research. These markers differ with each other on the ability to detect polymorphisms, at application cost, ease of use, and result consistency. Although

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there are other molecular markers used for *Capsicum*, we only emphasize results of the four above-mentioned markers, due to their large usage within the genus.

Williams et al. (1990) developed RAPD markers. They are based on PCR and promote arbitrary DNA segment amplification throughout the genome, from short and unique primers (10 bp) (Jiang 2013). RAPD markers have been widely used for variability analysis and genotyping, however, they have low repeatability. This technique has also been used for other purposes, ranging from studies at the individual level (e.g., genetic identity), for studies involving closely related species and studies of population gene mapping to aid in saturation of areas not covered by other markers (Kumar et al. 2009). The RAPD tool is more efficient in studies of genetic divergence, in which the analyzed individuals are genetically close or belong to the same species (Collard and Mackill 2008).

As advantages, the RAPD technique is distinguished by its simplicity, ease to be performed; fast data attainment, relatively reduced cost when compared to other molecular techniques, and immediate applicability to any organism. Moreover, it does not require a previous specific probe library of the target organism, and a minimum DNA amount is necessary for genomic analysis enabling results with great practical validity (Caixeta et al. 2006). Contrarily, a main disadvantage of RAPD markers is their dominant inheritance. Thus, it is not possible to identify if a band of RAPD gel comes from amplification of one or two DNA segments and dominant homozygote individuals are not differentiated from heterozygotes (Williams et al. 1990). Furthermore, these markers have low repeatability at laboratory scale, being considered, underthe genetic point of view, as less efficient than RFLP and SSR ones (Alzate-Marin et al. 2005). RAPD allows analyzing a larger loci number for trial and does not need previous selection, which makes significant progress in genetic mapping speed possible, and RAPDs have been widely used for this end. According to the literature, these markers have been used to map *C. annuum* species (Ben-Chaim et al. 2001; Sugita et al. 2005; Minamiyama et al. 2006; Barchi et al. 2007).

On the other hand, AFLP are known as the most powerful markers to detect genetic variability, once they explore restrict and amplified polymorphisms, and are considered a valuable tool as they generate a great number of polymorphic loci and with great repeatability. As well as in other species, it has often been applied in studies with *Capsicum* (Ben-Chaim et al. 2001; Lanteri et al. 2003; Toquica et al. 2003; Paran et al. 2004; Geleta et al. 2005; Minamiyama et al. 2006).

The microsatellite markers or SSR were developed by Hamada et al. (1982). Meanwhile, the "microsatellite" term was built up, for the first time, by Litt and Luty (1989), being afterwards called SSR by Jacob et al. (1991) or STR (short tandem repeats by Edwards et al. 1991).

Microsatellite loci are sequences containing one to six nucleotides that might be repeated in tandem, and differing in nucleotide composition. In general, repeated di-nucleotide, tri-nucleotide, and tetra-nucleotide sequences are widely distributed in the plant genome. The repetition number is variable for each individual and is responsible for polymorphism. Although repetition is variable, sequences flanking the microsatellites are preserved, being used for primer anchoring and locus

amplification (Jiang 2013). These markers have been used at various genetic research fields either for domesticated or wild species, as well as for parental identification, lineage assignment, genetic mapping, improved population germplasm evaluation, and genetic diversity or similarity studies (Lacape et al. 2007). In addition, they are much used for QTL (quantitative trait loci) mapping in animal and vegetal species.

Microsatellite markers have innumerable advantages: codominant inheritance and multiallelic, they are abundant in genomes, have high repeatability and are informative, besides possessing loci that are frequently conserved among related species, which facilitates comparative mapping (Simko 2009). The comparative mapping reveals the degree of collinearity between closely related species, which allows the exchange of markers between them (Zhu et al. 2012). Nevertheless, a great limitation of using SSR markers on a large scale is to attain primers that will be used in PCR to amplify alleles in each locus. Thus, it is a high cost and intensive labor technique, considering the entire process (Buso et al. 2000). Conventionally, SSR marker development requires selection and scanning of genomic libraries to select repetitive sequences (Portis et al. 2007).

Microsatellite marker identification and development have allowed a significant increase in the number of linking maps obtained by other markers, aside from enabling studies on phylogeny and species evolution. According to Novelli et al. (2000), microsatellite markers can also serve as anchors with genitor maps, building consensus or comparative maps, giving great robustness among maps that share these markers at same positions.

A large number of microsatellite markers are available for *C. annuum.* According to Sugita et al. (2013), this marker type is considered useful and very trustworthy for genetic map and QTL mapping in *Capsicum* populations. Huang et al. (2001) have published the first microsatellite markers used for the species. In this work, 58 pairs of primers of an enriched genomic library with ten different replications were drawn, from which only five were considered polymorphic. Afterwards, Lee et al. (2004) developed 40 pairs of reliable primer microsatellites of an enriched genomic library for GT, GA, TTG, and ATT repetitions.

The number of available markers for *C. annuum* increased considerably since the research of Minamiyama et al. (2006). These markers were developed by enriched genomic libraries for repetitions GA, GT, AAG, and AAT. It appears to be that of 626 drawn primer pairs, 153 had revealed polymorphism, and 106 followed Mendelian segregation and were used to build a linkage map for *C. annuum*.

Sugita et al. (2013) developed 265 pairs of SSR primers that were used to build a precise genetic map. Hence, a number greater than 500 microsatellites were developed for *C. annuum*, based on DNA genomic libraries. *Capsicum* genus has an oversized genome and a narrow genetic basis; therefore, more SSR markers are to be developed to construct high-density genetic maps for peppers (Kong et al. 2012).

SSR are characterized by possessing a high transference rate among related species and even among genera in the same family (Gutierrez et al. 2005). Although it is true that for some species, the polymorphism rate generated by microsatellites is very low, there is a need for interspecific crossings, allowing greater primer transferability.

Regarding the ISSR technique, it is characterized by 16–25 bp primers that amplify microsatellites at different sizes. This technique was first reported by Zietkiewicz et al. (1994) and devised based on microsatellite primers (Kumar et al. 2009). Exhibiting polymorphism resulted from individual microsatellite variation, in which presence or absence differs by genome (dominant loci) and from conserved microsatellite region variations (codominant loci).

ISSRs enable species inter- and intraspecific diversity identification, genetic identity analysis, genetic map construction, phylogenetic studies, and germplasm characterization and evaluation (Zietkiewicz et al. 1994; Lijun and Xuexiao 2012). In this technique, various loci are simultaneously generated, detecting fragment presence or absence of certain sizes, having high polymorphism. This method has high repeatability, great flexibility, does not require any sequence information to build primers, uses small amounts of DNA, and results in a high level of polymorphism (Kumar et al. 2001). As a limitation, the segregation consists of a dominant Mendelian inheritance and might have repeatability problems (Kumar et al. 2009). Reports on genetic maps based on ISSR markers do not exist for the *Capsicum* genus. Studies using this marker are concentrated in genus diversity analyses (Patel et al. 2011; Lijun and Xuexiao 2012; Ahmed 2013; Dias et al. 2013). Ahmed (2013) described that although these markers are widely used for characterization and discrimination of plant samples, they are excellent tools for genetic mapping. According to Patel et al. (2011), these markers have great potential for genetic mapping and gene location, having as example other species that had their genetic maps made by ISSR markers (Chen et al. 2011; Gupta et al. 2012; Priyamedha et al. 2012).

5.1 Marker-Assisted Selection in *Capsicum* Breeding for Disease Resistance

Disease resistance is one of the main goals in plant breeding programs. Development of new disease-resistant genotypes has allowed species cultivation in places where it was not possible before, due to pathogen presence or its severity. Furthermore, resistant cultivars reduce yield losses and pesticide use, producing low-cost food with greater quality and environmental friendliness.

Once certain disease resistance is performed by a gene (dominant or recessive), one of the improvement methods most used to attain a resistant cultivar is backcrossing (BC). Briefly, this procedure consists of crossing an elite, but disease susceptible, cultivar, which is called recurrent, and a resistant cultivar, however, deficient in good agricultural traits (donor), followed by successive backcrossings and selections based on inoculations, until the improved material is achieved.

The use of molecular marker plant breeding programs aim at disease resistance and have received the greatest attention by breeders, because in programs based on backcrossings, markers can maximize selection efficiency, increasing the probability of conversion and reducing the time of attainment, by monitoring the genitor–genome ratio at different RC generations. Additionally, it is possible to identify

individuals in which the transferred genes are linked to a lower number of residual genes of the donor (linkage drag).

Other aspects refer to identification of individuals that possess two or more resistance genes to a certain pathogen. Selection becomes more difficult when there is a gene pyramid; once produced phenotypes (resistant) are similar among individuals with one or more distinct genes. Thus, by molecular analysis, genes will be able to be identified regarding resistance gene presence, without a lineage test need, which also implies time reduction, less inoculation labor, and cultivation of a large number of plants.

In simple inheritance mapping, as in the majority of disease resistance cases, bulk segregation technique can be used (bulk segregation analysis, BSA). The technique is based on the construction of two contrasting DNA bulks for a target trait, among segregating population individuals. In this way, these bulks are tested with molecular markers, and polymorphisms have great chances of being connected to the contrasted trait in bulk (resistance × susceptibility), because others will segregate randomly. The connection between obtained markers and desired phenotype is tested by cosegregation analysis. The identification of these markers connected to the resistance gene allows monitoring and speeding up these gene introgressions into commercial varieties, and enabling pyramidation of two or more genes in this variety.

PVY (Potato virus *Y*) is a virus that infects *Capsicum* plants among others. Various markers related to different potyvirus resistance genes are described for *Capsicum*. The loci *pvr1* (Murphy et al. 1998), *pvr2* (Caranta et al. 1997), *pvr6* (Caranta et al. 1996), and *Pvr7* (Grube et al. 2000) confer resistance through a wide range of potyvirus, which were located by different markers following varied location strategies. In the case of *Pvr4*, two distinct strategies with AFLPs and RAPDs were applied to get useful marks for marker-assisted selection. Caranta et al. (1999) used BSA technique combined with AFLP, and Arnedo-Andrés et al. (2002) employed BSA associated with RAPD markers. The latter authors identified a RAPD marker linked in repulsion-phase to *Pvr4* gene. Therefore, this marker was converted into a SCAR marker and located upon a *Capsicum* genetic map at linkage group 10 (Livingstone et al. 1999). Moreover, it can be useful to identify a PVY resistant genotype,by means of marker-assisted selection (MAS) in segregating pepper populations.

Other diseases that have brought losses in production for this crop are *Pepper Yellow Mosaic Virus* (PepYMV) and *Phytophthora wilt*, caused by *Phytophthora capsici* fungus. In this sense, Izioka et al. (1997), through RAPD markers, studied *C. annuum* variability for some cultivars, seeking to evaluate their genetic basis in relation to *P. capsici* resistance. Three groups were formed by the UPGMA method, and in accordance with known resistance levels, the evaluated genotypes were distributed into susceptible, intermediate, and resistant.

It is important to mention that molecular markers still have proportionate increments in genetic gains and time reduction for attainment of superior genotypes. Most probably, plant breeding program trends are to integrate classic or conventional techniques with DNA marker use. After all, the association of both tech-

niques has allowed the knowledge magnification about agricultural crops on genome terms. Subsequent to a new genotype development, markers can also be used for varietal identification and genetic purity of *C. annuum* hybrids as described for Ilbi (2003). The author used RAPD markers successfully aiming to improve seed quality control and material protection. Based on results, it is possible to foresee that molecular marker use has increasingly become an important and decisive tool for *Capsicum* breeding programs, a plant genus that still has a wide variability to be explored for both domesticated and wild species.

5.2 DNA Markers and Gene Mapping for *Capsicum* spp.

In plant breeding, genetic maps are important tools that have allowed genome analysis, complex genetic trait breakup into Mendelian components, location of genomic regions that control relevant traits, and their effect quantification for a characteristic under study. The construction of a genetic map encourages the acquisition of important information for the breeding of a species, such as the association of molecular markers and qualitative characters of the same location on linkage groups, and the identification of genomic regions associated with quantitative traits among others.

Several characteristics have been used in genetic map construction. The first developed genetic maps used morphological markers: qualitative traits easily visualized, such as seed and flower colors, hypocotyl, and floral and foliar morphology, among others. Nevertheless, due to the limited number, a possibility of significant associations between these markers and traits was reduced. Following, the use of isoenzymatic markers led to an increase of available genetic markers, and by the aid of molecular biology techniques, new markers capable of directly detecting genetic polymorphism at the DNA level have appeared, resulting in a large number of markers and complementation and saturation of genetic maps for several vegetal species.

Research was carried out seeking to identify genes with great agricultural traits and a diverse number of genetic maps were constructed for *Capsicum* with the aid of molecular markers (Prince et al. 1993; Lefebvre et al. 1995; Livingstone et al. 1999; Ben-Chaim et al. 2001 and others). In that respect, these genetic maps for *Capsicum* were constructed using plant populations from intra- and interspecific hybridizations.

The first linkage map for *Capsicum* was built based on few markers, using an interspecific crossing of *C. annuum* cv. NuMex RNaky and *C. chinense* PI 159234 populations (Tanksley 1984) in order to map enzyme codification genes. However, the first genetic map with the widest genome cover, which had 85 RAPD markers, was developed by Tanksley et al. (1988) with *C. annuum* and *C. chinense* PI 159234 crossing. Another aspect is that this population was mapped with markers derived from tomato mapping studies (*Solanum lycopersicon*), which had high conservation

of gene repertory but the gene order was greatly modified. Lately, a study using the same crossing generated a more precise map with 192 markers (Prince et al. 1993).

In a study carried out by Inai et al. (1993), a molecular marker was found connected to a single nuclear gene in *C. annuum* responsible for plant dwarfism at recombination rate of 6 % when interacting with plant cytoplasm in *C. chinense*. In consequence, several maps derived from intraspecific crossing populations of *C. annuum* were made available (Lefebvre et al. 1995, 2002; Paran et al. 2004; Minamiyama et al. 2006; Sugita et al. 2006; Barchi et al. 2007; Mimura et al. 2012).

Lefebvre et al. (1995) developed the first integrated map for *C. annuum*, using RAPD and RFLP markers and di-haploid lineages, getting 85 marks, covering throughout 820 cM, and arranged in 14 linkage groups. The authors estimated that this map represented from 36 to 59 % of the genome and found three genes of agricultural interest: *L*, TMV hypersensitivity controller; *up*, fruit erect habit controller; and *C*, pungency controller, which was the most precisely located. Also using RAPD and RFLP markers, Massoudi and Cantrell (1995) constructed a linkage map for *C. annuum* and identified resistance marks related to *Phytophthora capsici*.

Livingstone et al. (1999), using different molecular techniques such as RAPD, isoenzymes, AFLP, and RFLP, built a genetic map for *Capsicum* and compared it to other genera from the Solanaceae family, such as tomato and potato, aiming to clarify some evolution aspects of this family.

On other hand, Kang et al. (2001) constructed a linkage map for pepper, evaluating 107 F_2 individuals from interspecific crossing between *C. annuum* and *C. chinense*. For that, a number of 150 RFLP and 430 AFLPs and 16 linkage groups were obtained covering around 1320 cM of the genome. Among the mapped genes are those responsible for carotenoid and capsaicinoid biosynthesis, which can further contribute to studies on secondary metabolites of *Capsicum*. Lee et al. (2009) made an integrated map from the same crossing, in which 805 markers were arranged in 1858 cM. The same research group, using the resultant lineage from this interspecific crossing detected a responsible QTL to explain 27 % of the fruit length trait (Lee et al. 2011).

Kaloo et al. (2002), working with sterile male lineages and a fertility-restoring gene, developed a protocol to validate RAPD marks associated with the *Rf* gene in pepper (*C. annuum*), concluding that a 1400 pb fragment might be connected to the *Rf* gene. In the same year, RFLP markers were used to map the *C* gene that controls pungency in *Capsicum* fruit, which was located in chromosome 2 (Blum et al. 2002).

Lefebvre et al. (2002) constructed a more saturated map for *C. annuum*, in which three maps were integrated, enabling formation of 12 linkage groups, corresponding to the basic number of pepper chromosomes. This first functional consensus map for pepper contains 100 markers of known function genes and five loci of agricultural interest traits were mapped (resistance to nematodes *Me3* and *Xanthomonas Bs3*, determination of capsaicin content, fruit erect habit control, and yellow fruit coloration).

Paran et al. (2004) also constructed an integrated genetic saturated map for *C. annuum*, made from six individual maps, consisting of 2262 markers (1528 AFLPs, 440 RFLPs, 288 RAPDs, and 6 TAGs). The markers were sorted into 13 linkage groups with a total length of 1832 cM. A number of 320 anchor markers were used, that is, markers simultaneously present at least in two individual maps for the accomplishment of this integration. Therefore, in this new map, marker density raised from one per each 2.1 cM up to 0.8 cM, with space reduction (gaps) of more than 10 cM between markers. Even so, several small linkage groups remained, indicating the need of higher saturation for a more effective genome covering.

Lee et al. (2004) built a linkage map named SNU-RFLP, obtained from the interspecific crossing of *C. annuum* TF68 and *C. chinense*, Habanero. Subsequently, the generated map was improved and further called *SNU2*, which contained 333 markers, being 46 SSRs and 287 RFLPs, distributed into 15 linkage groups covering 1761.5 cM with an average distance of 5.3 cM between markers.

Sugita et al. (2005) proposed a linkage map for sweet pepper (*C. annuum*), where 518 molecular marks were mapped (328 AFLPs, 122 RAPDs, 3 RFLPs, 7 SCARs, and 4 CAPSs) into 11 groups of larger linking and five smaller, covering 1043 cM. After that, Sugita et al. (2013) developed a map with the largest genome covering for *C. annuum* that was based in 265 SSR markers distributed into 12 groups covering a total genetic distance of 2028 cM.

Marques et al. (2006) developed a genetic map for *Capsicum annuum* using microsatellites. For that, they used 186 F_2 individuals obtained from crosses of *C. annuum* cultivars, contrasting for the characteristic of *Phytophthora wilt* and Pep-YMV resistance. Then, 50 polymorphic SSR markers were obtained for the F_2 genotype, 40 of them being used for genetic map formulation. In addition, these markers were mapped over eight linkage groups, totaling 523.88 cM.

Minamiyama et al. (2006), using 106 SSR markers to map a lineage composed by 117 F_2 individuals, have elaborated another map. The generated map had 13 linkage groups with 1042 cM. F_1 di-haploid population segregated from *C. annuum* was used to generate a linkage map. Lately, the map was saturated and contained 253 markers (151 SSRs, 90 AFLPs, 10 CAPSs, and 2 TAGs) covering 1336 cM of the genome. This was the first map of *C. annuum* based in SSR markers where marks were placed into 12 linkage groups (Mimura et al. 2012).

Genetic maps derived from interspecific crossings between *C. frutescens* and *C. annuum* were also generated and Rao et al. (2003) used a population derived from the crossing of *C. frutescens* "BG2816" with *C. annuum* cv. Maor to study production traits. The mapping was performed based on 248 plants arising from backcrossings; and 92 RFLP marks were distributed throughout genome, 10 production traits were mapped, and 58 QTLs detected. Wu et al. (2009), using an F_2 population of *C. frutescens* and *C. annuum* crossing, mapped 299 orthologue markers comparing pepper and tomato, which is the first map generated from interspecific crossing where the linkage group number corresponded to the chromosome number. Moulin et al. (2015) published a reference map for *C. baccatum* using an F_2 population consisting of 203 individuals, based on 42 SSR, 85 ISSR and 56 RAPD markers. The genetic map consisted of 12 major and four minor linkage groups covering a total genome distance of 2547.5 cM.

5.3 QTL Mapping in *Capsicum*

It is believed that the development of genetic maps has great utility for plant breeding programs. In this line, molecular markers have facilitated genetic map building that has great application in the study of loci that control quantitative traits and with complex inheritance, which are called QTLs (Tanksley 1993). One major application of linkage maps for genetic breeding of several crops has been QTL mapping in intraspecific crossings (Chutimanitsakun et al. 2011).

Therefore, QTL mapping can lead to a better understanding of genotype and phenotype interaction. According to Bernardo (2008), QTL mapping has as its objective to increase knowledge of genetic trait inheritance and identify molecular markers that are able to aid in assisted selection for relevant phenotypical traits such as production and disease resistance.

Although this technique makes it possible to estimate the position and number of loci that control the studied quantitative trait, as well as the gene action manner (additive, dominant, and epistatic), the disintegration of genotype × environment interaction at each QTL level likewise allows synteny studies or comparative mapping (Ferreira and Grattapaglia 1998). QTLs necessarily do not take the identification of a specific gene, being able to correspond to region identification that may possess dozens of genes. However, one of the great difficulties faced by researchers is QTL position identification and its effect size (Broman and Sen 2009).

Therefore, to perform mapping, information is needed such as identification of the target quantitative trait, molecular marker data, which are associated with QTLs, and an adjusted method to associate them (Toledo et al. 2008). Since the end of the 1980s, several methods have been proposed to identify QTLs. Among them, simple mark, interval, and composite interval mapping methods are highlighted. The simple mark mapping is based on comparison among marker genotypical class averages, carried through linear regression, "*t*" test, variance analysis, and likelihood ratio. Yet in the interval method, the association between the marker and QTL can be estimated by maximum likelihood, which is based on joint frequencies of a pair of adjacent marks and an occasional QTL. Regarding the composite interval mapping, it is used as a combination of simple interval mapping and multiple linear regression (Schuster and Cruz 2004).

Nevertheless, in order to estimate the magnitude of the QTL effect, genetic maps must have a good saturation. As stated by Wu et al. (2007), the longer the distance between markers, the lower the QTL estimate precision is between these markers. Moreover, three factors have to be considered in a coherent association of molecular markers and QTLs: (1) information repeatability between marker linkage and respective QTLs at different populations; (2) QTLs × environment interaction, and (3) simultaneous selection for various traits (Ferreira and Grattapaglia 1998).

A QTL evaluation related to *Capsicum* fruit was conducted by Ben-Chaim et al. (2001), in which the studied F_3 generation was derived from two *Capsicum annuum* genotype crossings, one owning fruit in bell form and another with small fruit. RFLP, AFLP, and RAPD markers and morphological markers were applied in the

research. Accordingly, a total of 55 QTLs were identified and several of them have demonstrated high genetic correlation, such as fruit diameter and weight, pericarp thickness, and pedicel diameter; thus, they were placed at similar regions of the linkage groups. A large number of QTLs in the *Capsicum* genus seemed to correspond to similar positions in tomato for loci that control the same traits, in this way suggesting the hypothesis of being orthologue QTLs when comparing the two species.

In another QTL mapping, Ben-Chaim et al. (2006) analyzed F_3 families using microsatellite markers to detect the spot responsible for capsaicinoid content control and found six QTLs in three chromosomes. In addition, two QTLs controlling fruit mass were identified without verifying any relation between capsaicinoid content and fruit mass.

Some studies with pepper involving genetic parameters of a trait series and the respective QTL mapping have been reported (Alimi et al. 2013). Most of them have focused on commercial characteristics such as fruit quality and production (Rao et al. 2003; Wang et al. 2004; Zygier et al. 2005; Lee et al. 2008, 2011; Barchi et al. 2009; Alimi et al. 2013), in addition to some research for QTL associated with disease resistance (Lefebvre et al. 2003; Thabuis et al. 2003; Voorrips et al. 2004; Sugita et al. 2006; Minamiyama et al. 2007; Mimura et al. 2009; Kim et al. 2011). Conversely, a limited amount of research involves the QTL identification associated with vegetative parts (leaves, stem, internode, and petioles) (Barchi et al. 2009; Mimura et al. 2010).

Meanwhile, Lee et al. (2011) have located a QTL related to pepper fruit length, which was placed at linkage group 3, explaining 27 % of phenotypical trait variation. Additionally, Lu et al. (2012) identified 23 QTLs of great effect on 12 morphological traits. Hence, a very dense map consisting of 458 molecular markers was used, this linkage map being derived from integration of some maps generated by other studies.

Finally, Alimi et al. (2013) evaluated 16 physiological characteristics at four different environments and identified 24 QTLs. The average number of QTLs for characteristics was two, varying between zero and six. The total explained phenotypical variance for the characteristics varied between 9 and 61 %. In the high density map built by Yarnes et al. (2013), 96 QTLs were estimated for 38 agricultural traits, including 12 QTLs associated with capsaicinoid contents in the *Capsicum* population under study.

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Chapter 6
Tissue Culture of *Capsicum* spp.

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Abstract Plant tissue culture comprises a set of in vitro techniques, methods, and strategies that are part of plant biotechnology. In this chapter, we discuss some difficulties and recent advances in the in vitro culture of peppers, aiming at the breeding of cultured species belonging to the genus. It also reports our experience and contribution to the induction of androgenic haploid embryos, sensitivity and resistance to ethylene, flowering, fruiting and seed production in vitro, as well as embryo rescue and their utilization in the breeding program of ornamental pepper from Brazil.

Keywords In vitro culture • Breeding • Ornamentals • Haploids and ethylene resistance

6.1 Introduction

Tissue culture in vitro is one of the plant biotechnological tools that explores the natural totipotency of plant cells (Haberlandt 1902), demonstrated unmistakably by Steward et al. (1958).

Ever since the first report on the use of tissue culture in *Capsicum* by Smith and Heiser (1957), who obtained F1 plants from the germination of well-developed zygotic embryos of the *C. pendulum* × *C. annuum* hybrid, tissue culture has achieved extraordinary advances in the genus. This technology has enabled the creation and preservation of genetic variability, selection and cloning of superior genotypes, elimination of bacterial diseases and viruses, rescue of embryos resulting from intra- and interspecific crosses, preselection of materials tolerant to abiotic stress, and acceleration of breeding programs for the genus *Capsicum*.

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When associated with molecular biology, tissue culture has allowed for the incorporation of genes and expression of new traits through genetic engineering. Overall, this technology has contributed markedly to the breeding of peppers (Kothari et al. 2010).

In this chapter, we discuss some difficulties and recent advances in the in vitro culture of peppers aimed at the breeding of cultured species belonging to the genus. Among the main difficulties we mention morphogenetic recalcitrance, formation of rosette shoots, genotypic dependence, and sensitivity to ethylene. And the main advances have been achieved in the regeneration system, direct and indirect organogenesis, somatic embryogenesis, culture of protoplasts and anthers for the production of haploids and double haploids (DH), and acceleration of breeding programs and genetic transformation. Finally, we have reported our experience and contribution to the induction of androgenic haploid embryos, sensitivity and resistance to ethylene, flowering and fruiting in vitro, and response to ethylene suppression.

6.2 Some Difficulties Inherent to Tissue Culture in Peppers

Of the many problems inherent to the tissue growth of peppers, those of greatest importance are: natural morphogenetic recalcitrance, formation of rosette shoots, and genotypic dependence, which have limited some research studies and ought to be better evaluated and understood in order to optimize results and application in plant breeding.

6.2.1 Natural Morphogenetic Recalcitrance

Recalcitrance is defined as the inability of cells, tissues, and organs to respond to an in vitro culture. Tissue culture's responses are greatly influenced by three primary factors: the physiology of the explant donor plant, in vitro manipulation, and the stress physiology in vitro (Benson 2000).

Working with tissue culture of the genus *Capsicum* has been difficult compared with other species belonging to the Solanaceae family, some of which are used as model systems (potato, tobacco, and tomato), due to its great ability to regenerate plants. Although several studies report the relative success of morphogenesis in the genus *Capsicum* (for review see Fari 1988; Kothari et al. 2010; Steinitz et al. 1999), genetic engineering is still restricted to the low morphogenetic potential of these species (Steinitz et al. 1999; Ochoa-Alejo and Ramírez-Malagón 2001). The selection of the explant itself in a specific responsive stage, and the modification of different components in the nutrient medium and other additives should minimize the effects of recalcitrance. Valera-Montero and Phillips (2005) suggested that one possible reason for organogenic recalcitrance in pepper might be a narrow time window in the regenerative process.

6.2.2 Formation of Rosette Shoots

One of the most important factors limiting the regeneration of the tissue culture in the genus *Capsicum* is the formation of rosette shoots (rosettes), which do not elongate. Several attempts to overcome shoot-elongation difficulties were made by Arroyo and Revilla (1991), who rooted rosettes and elongation occurred in the field itself. Another methodology employed was the addition of different supplements to the medium, such as silver nitrate ($AgNO_3$; Hyde and Phillips 1996), phenylacetic acid (PAA; Husain et al. 1999), and a lactone, 24-epibrassinolide (Franck-Duchenne et al. 1998) to overcome the bud- and shoot-elongation problem. However, the problem yet persists in many species of peppers.

6.2.3 Genotypic Dependence

The dependence on genotype is the main factor influencing organogenesis in species of the genus *Capsicum* when subjected to tissue culture. The existence of a strong specificity of the genotypes in the regenerative capacity of the different cultivars represents an important limiting factor that makes the development of a new regeneration pattern protocol for the genus infeasible, and there is, in fact, a different need for each cultivar (Fari 1988; Steinitz et al. 1999; Kothari et al. 2010).

6.2.4 Sensitivity to Ethylene and Gas Exchanges

Ethylene is a simple gaseous plant hormone that triggers a wide array of physiological and morphological responses in plants such as inhibition of cell expression, promotion of senescence in leaves and flowers, and induction to fruit ripening and abscission (Bleecker and Kende 2000; An et al. 2010).

Ethylene production in plants is regulated by signaling cascades of the mitogen-activated protein kinases (MAPKs). This activation occurs in response to several external stimuli, in which stress conditions usually cause an increase in the endogenous levels of ethylene (Mayak et al. 2004; Siddikee et al. 2011).

Little is known about the effects of ethylene in vitro, together with the growth regulators in the growth medium, and their influences on the growth of explants (Lin et al. 2009). Peppers are highly sensitive to ethylene during their development in in vitro conditions (Hyde and Phillips 1996; Santana-Buzzy et al. 2005, 2006; Núñez-Pastrana et al. 2011). These authors observed symptoms such as chlorosis and abscission of the leaf primordia during their development, followed by vigor loss. We have observed that when these explants are kept in containers with higher exchange rates, the regeneration and development of plantlets of ornamental pepper occur normally.

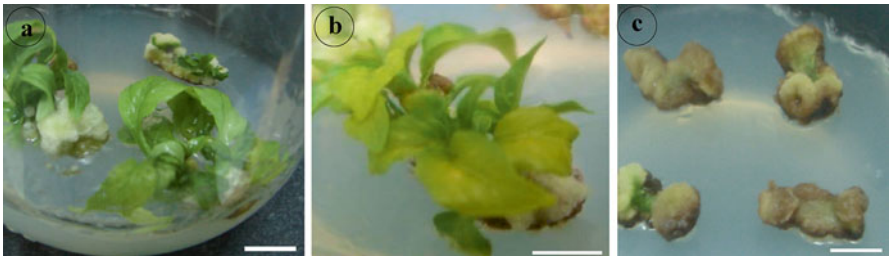


Fig. 6.1 Organogenesis of *C. annuum* L. in vitro 30 days after inoculation: (**a**) expanded leaves; (**b**) presence of adventitious buds and calluses on hypocotyledonary explants; (**c**) callogenesis in hypocotyledonary explants (Barr = 1.0 cm). (Batista et al., 2013)

In addition to the gas exchanges, another alternative to reduce the effects of ethylene is to use inhibitors of its biosynthesis. Some substances used in the inhibition are aminoethoxyvinyl glycine (AVG), silver ions, for example, silver nitrate ($AgNO_3$), carbon dioxide, and polyamines, which are compounds of aliphatic nature that play various functions in the growth and development of plants (Bregoli et al. 2006), also acting in the plant morphogenesis (Bais and Ravishankar 2002), promoting cell elongation and division by inhibiting ethylene production (Hu et al. 2006).

In our research group, we analyzed ethylene suppression to promote the morphogenesis of *C. annuum* in vitro, using AVG inhibitors, silver thiosulfate (STS), and the polyamines (putrescine, spermidine, and spermine), as well as the polyamine-inhibitor methylglyoxal-bis(guanylhydrazone; MGBG) and mercury perchlorate (Batista et al. 2013).

There were three different types of morphogenetic responses in the treatments: (1) direct organogenesis with emission of adventitious buds (Fig. 6.1a), (2) treatments that showed callogenesis and direct organogenesis (Fig. 6.1b), and (3) only formation of calluses (Fig. 6.1c).

Greater formation of calluses occurred in the treatments with the highest levels of ethylene [control and methylglyoxal bis(guanylhydrazone; MGBG)]. On the other hand, when the polyamines (ethylene scavenger) were added to the growth medium, ethylene levels were lower than the control, and the frequency of regeneration and emission of adventitious buds was higher (Fig. 6.2) (Batista et al. 2013).

We observed in this experiment that the ethylene levels during the 30-day growth period were highest for ACC, followed by Put, MGBG, and control, which were detected at approximately day 15, and for the control, at approximately day 12 (Fig. 6.2). For flasks supplemented with AVG, STS, MP*, Spd, and Spm, no ethylene was detected on or before day 9, but ethylene was detected from day 12 onward. No ethylene was detected in flasks treated with Spd and Spm on day 30 of cultivation. Mercury perchlorate (MP) was the only treatment in which ethylene was not detected at any time. Flasks that had the highest levels of ethylene accumulation (control, MGBG, ACC, and Put) showed more callusing (Fig. 6.1b, c). On the other

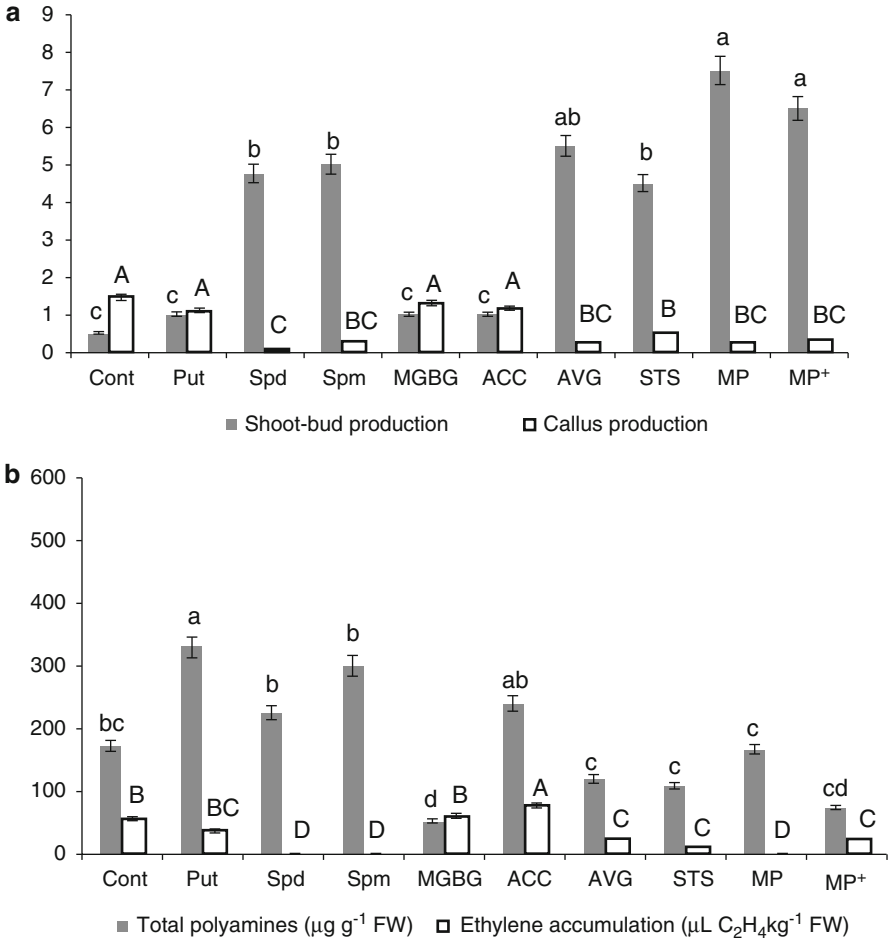


Fig. 6.2 Characteristics evaluated 30 days after induction of organogenesis in *C. annuum. Cont* control, *Put* putrescine, *Spd* spermidine, *Spm* spermidine, *MGBG* methylglyoxal bis(guanylhydrazone), *ACC* 1-Aminocyclopro-pane-1-carboxylic acid, *AVG* aminoetho-xyvinylglycine, *STS* silver thiosulfate, *PM* mercury perchlorate, *PM** mercury perchlorate removed on the tenth day of growth: (**a**) numbers of shoots and calli; (**b**) levels of ethylene and polyamines. Means indicated by the same letters (lowercase above the *gray bars* and uppercase above the *white bars*) are not significantly different as assessed by Tukey's test at the 5 % probability level. Error bars indicate standard error. (Batista et al., 2013)

hand, treatments with the highest regeneration frequencies (MP, MP*, AVG, Spm, Spd, and STS) had the lowest levels of ethylene (Fig. 6.1a, b).

Our results suggest a coordinated regulation of ethylene and polyamines, because when we suppressed the ethylene levels using its inhibitors, polyamines or mercury perchlorate, the regeneration frequency and morphogenetic response of *C. annuum* L. increased. Therefore, the use of ethylene inhibitors and the addition of increasing concentrations of PAs may be important tools for reducing ethylene levels and reducing recalcitrance of peppers cultured in vitro.

6.3 Plant Regeneration Systems

The pepper regeneration systems in vitro serve three main purposes: (1) the micropropagation of elite or special genotypes [e.g., male-sterile plants (Steinitz et al. 1999), or F1 plants showing heterosis (Gupta et al. 1998)]; (2) generation of double-haploid plants originating from a culture of microspores, anthers, ovule or ovary unfertilized (Regner 1996); and (3) production of transgenic plants.

The regeneration of peppers has been achieved mainly via organogenesis, but other regeneration modes such as somatic embryogenesis or regeneration from protoplasts and anther culture for production of androgenic haploids have been explored (Table 6.1).

6.3.1 Organogenesis

Organogenesis is a complex phenomenon involving the de novo formation of organs (shoots and roots). There are two types of organogenesis: direct and indirect. In the direct organogenesis, the shoots-bud can be derived by differentiation of nonmeristematic tissue, known as formation of adventitious shoots, or by a pre-existing meristematic tissue, known as formation of axillary shoots. Both processes require synergistic interactions between chemical and physical factors.

A good plant regeneration protocol requires the appropriate choice of explant, age of the explant-donor plant, well-defined medium formulation, specific growth regulators, responsive genotypes, carbohydrate source, gelling agent, physical factors including light regime, temperature, and moisture, and other factors.

In indirect organogenesis, the differentiation of shoots and roots occurs on the surface of a mass of cells called the callus. The callus is a mass of undifferentiated cells that multiply rapidly and are little organized; thus, the possibility of appearance of somaclonal variants is increased, which is an undesirable process when aiming at in vitro cloning. The indirect organogenesis is usually a result of the hormonal balance between the medium and the endogenous contents of plant hormones.

6.3.1.1 Genotype Effect

Among the different factors affecting regeneration in the genus *Capsicum*, the dependence on genotype is ranked first. According to Ochoa-Alejo and Ireta-Moreno (1990), pepper cultivars have a very clear influence on the capacity of formation of adventitious shoots from hypocotyl tissues. Explants from 16 cultivars including the types Pimento, Ancho, Serrano, Jalapeño, Pasilla, Caloro, and Anaheim were grown in an MS medium (Murashige and Skoog 1962) supplemented with different concentrations of indole acetic acid (IAA), benzyladenine (BA), and

Table 6.1 Summary of some studies on in vitro regeneration of pepper plants (*Capsicum* spp.)

Species	Type of explant	Regeneration mode	Culture medium + growth regulator	References
Capsicum annuum L.	Cotyledon	Direct organogenesis	MS + BA (8.88 μM) + AIA (2.85–5.71 μM)	Gunay and Rao (1978)
	Zygotic embryo		MS + BA (22.2 μM)	Agrawal and Chandra (1983)
	Plantlets		MS + BA (2.22–44.4 μM)	Phillips and Hubstenberger (1985)
	Hypocotyl, cotyledon, stem, leaf, root, stem apex, and zygotic embryo		MS + BA (22.2 μM) + AIA (2.85–5.71 μM)	Batista et al. (2013)
	Cotyledon, hypocotyl		MS + BA (2.22; 4.40; 8.80 μM) + AIA (0.57; 5.71 μM)	Agrawal et al. (1989)
	Plantlets		MS + BA (22.2 μM) + ANA (0.54 μM)	Arroyo and Revilla (1991)
	Mature seeds		MS without regulators	Ebida and Hu (1993)
	Immature zygotic embryo		MS without regulators	Ezura et al. (1993)
	Immature zygotic embryo	Direct somatic embryogenesis	MS + 2–10 % sucrose, 10 % CW, and 2.4-D (4.53–22.6 μM)	Gatz (2014)
	Mature zygotic embryo	Direct somatic embryogenesis	MS + 6–10 % sucrose + 2.4-D (9.0 μM)	Harini and Sita (1993)
	Cotyledon	Indirect somatic embryogenesis	MS + 3 % sucrose + 2.4-D (452 μM)	Binzel et al. (1996)
	Stem apex	Direct organogenesis	MS + BA (8.8 μM) + AIA (2.85 μM) + $AgNO_3$ (5.8 μM)	Büyükalaca and Mavituna (1996)
	Injured hypocotyl	Axillary meristem	MS + BA (8.8 μM)	Hyde and Phillips (1996)
	Cotyledon	Direct organogenesis	MS + BA (66.6 and 88.8 μM)	Madhuri and Rajam (1993)
			MS	Christopher and Rajam (1994)
	Cotyledon	Direct organogenesis		
	Zygotic embryo	Direct organogenesis	MS + BA (13.35 μM) + AIA (3.4–5.9 μM) + EBr (0.1 μM)	Ramírez-Malagón and Ochoa-Alejo (1996)

(continued)

Table 6.1 (continued)

Species	Type of explant	Regeneration mode	Culture medium + growth regulator	References
	Plantlets, embryonic explant	Direct organogenesis	MS + BA (22.2–31.0 μM) + PAA (14. μM)	Dabauza and Peña (2001)
	Leaf and cotyledon	Direct organogenesis	MS + BA (22.2 μM) + ANA (5.37 μM)	Husain et al. (1999)
	Cotyledon	Direct organogenesis	MS + TDZ (4.5–9.0 μM)	Arous et al. (2001)
	Leaf, cotyledon, hypocotyl	Direct organogenesis	MS + TDZ (4.5–13.5 μM)	Dabauza and Peña (2001)
			MS + BA (22.2 μM) + PAA (14.7 μM)	Venkataiah et al. (2003)
			MS + BA (8.8 μM) + AIA (11.4 μM)	Joshi and Kothari (2007)
			MS + BA (22.2 μM) + Kinetin (4.6 μM)	Sanatombi and Sharma (2008)
				Shreya et al. (2014)
C. frutescens	Cotyledon	Direct organogenesis	MS + BA (8.88 μM) + AIA (2.85–5.71 μM)	Gunay and Rao (1978)
	Stem apex	Axillary proliferation	MS + BA (22.2 μM) + Kinetin (4.6 μM)	Sanatombi and Sharma (2007a, b)
	Leaf, cotyledon, hypocotyl	Direct organogenesis	MS + BA (8.8 μM) + AIA (11.4 μM)	Sanatombi and Sharma (2008)
C. praetermissum	Stem apex			
C. chinense	Leaf, cotyledon, hypocotyl	Direct organogenesis	MS + BA (8.8 μM) + AIA (11.4 μM)	Sanatombi and Sharma (2008)
			MS + TDZ (18.16 μM)	Kehie et al. (2013)
			MS + BA (35 μM) + Kinetin (35 μM)	Gogoi et al. (2014)
				Rêgo et al. (2013)
C. baccatum	Cotyledon	Direct organogenesis	BA (13.3 μM) + IAA (5.71 μM) BA (44.4 μM) BA (22.2 μM)	Christopher and Rajam (1994)

2-isopentenyl adenine (2iP), and the differences between the cultivars regarding the formation of shoots were obvious, determined by the number of shoots formed per explant and the frequency of shoot formation.

Differences in organogenic capacity were observed in different genotypes, cultivars, and species of pepper when different sources of explants and culture media were used (see Fari 1988; Steinitz et al. 1999; Kothari et al. 2010; Rêgo et al. 2013).

More recent studies (Venkataiah et al. 2003) also have supported the influence of genotype on the organogenic response. These authors worked with ten cultivars of peppers in thidiazuron (TDZ)-supplemented medium and found that the response is highly dependent on genotype. Of the ten genotypes of *C. annuum* L. evaluated, only three responded satisfactorily. Our group also performed screening for the regeneration potential of some genotypes of *C. chinense* belonging to our germplasm bank and subjected to different levels of TDZ and observed high genotypic dependence in all five accessions evaluated (Rêgo et al. 2013). The most responsive accessions produced only 3.5 shoots per explant at the best level of TDZ (1.0 mg L^{-1}) (Rêgo et al. 2013).

Comparing the response of cultivars belonging to different species (e.g., *C. annuum*, *C. chinense*, and *C. frutescens*) reported by Sanatombi and Sharma (2008), we observe that, in general, the cultivar of the species *C. chinense* showed the best response in terms of number of shoots produced per explant.

On the other hand, there are reports in which the environmental effect is higher than the genotype effect, concerning the germination of completely developed or immature zygotic embryo (Manzur et al. 2013).

6.3.1.2 Selection of the Explant

The morphogenesis and growth of the plant tissue under in vitro conditions are highly influenced by the appropriate choice of the explant. Thus, the regeneration of peppers also depends on the age and type of explant involved. Different explants, including cotyledons, hypocotyls, leaves, stem apexes, zygotic embryos, leaf primordia, stems, internodes, mature seeds, and roots have been employed in the regeneration of plants of the genus *Capsicum* (Table 6.1). However, to the present moment, no reports of plants, buds, or shoots regenerated from the explant from root have been published in the genus.

Ever since the pioneering study on the regeneration of pepper plants conducted by Gunay and Rao (1978), in which the cotyledons showed to be more responsive than the hypocotyl, several others have been conducted (read Fari 1988; Kothari et al. 2010).

Golegaonkar and Kantharajah (2006) investigated the shoot-formation capacity of leaf and cotyledon explants of five cultivars of peppers in India and reported that the regeneration frequency is highly influenced by the explant (49.7 % of the total variation found), the growth medium (29.2 %), and lastly, the cultivar (14.2 %). Christopher and Rajam (1996) reported that leaf explants are more efficient than explants derived from hypocotyl or cotyledon in the three genotype groups evaluated.

The organogenic response is also dependent on the position of the explant on the donor plant or plantlet (apical, medium, or basal region, in the case of hypocotyl). For instance, shoot regeneration was only observed in a hypocotyl segment of the apical region of cultivar *C. annuum* cv. T. Havani, whereas sections of the medium and basal region produced only calluses and roots (Fari and Czako 1981; Fari 1988). The age of the explant-donor plantlets also influences the shoot-formation efficiency.

Another factor that may affect the organogenic response efficiency is the treatment of the explant itself. It has been observed that cotyledon ornamented with petiole in *C. annuum* cv. Yatsufusa displayed a significantly higher frequency of shoots than the petiole of the cotyledon (Sripichit et al. 1987).

6.3.1.3 Effect of Growth Regulators on Organogenesis in the Genus *Capsicum*

Plant hormones have a key role in the control of metabolic pathways involved in the growth and development of plants. They regulate the growth speed of individual parts and integrate these parts to produce the whole plant (George et al. 2008). Auxins and cytokinins are required synergistically to induce cell division and growth in the plant tissue culture. Studies on whole plants and excised tissues have demonstrated the existence of antagonistic and additive interactions between these two main types of plant hormones.

The regeneration of shoots in *Capsicum* species is also highly influenced by the formulations of the nutritive media containing the plant hormones and other growth regulators. Overall, a standard response is observed in studies from different laboratories: first, the hormonal composition and the sequence of application of the growth regulators has been adapted to each genotype and type of explant; second, cytokinins are applied alone or in combination with an auxin, which is essential to inducing adventitious shoots (Table 6.1).

The first report on organogenesis in *Capsicum* was by Gunay and Rao (1978), who evaluated the effects of different hormonal regimes on the organogenesis of the genus, evaluating two cultivars of *C. annuum* and one hybrid of *C. frutescens*, cultured in MS medium supplemented with the auxins AIA, ANA, 2,4-D, and the cytokinins BA, kinetin, zeatin, 6-benzyl-9-tetrahydropyran adenine, adenine, and coconut water. After this report, several works have been published (Table 6.1), which we have subdivided to facilitate our understanding, into four categories according to the type of organ regenerated.

Induction of Buds and Shoots

The formation of shoots from explants of cotyledons in the two genotypes of *C. annuum* and of the *C. frutescens* hybrid is dependent on BA. However, superior regeneration of shoots only was achieved in basal medium supplemented with 5.71

μM AIA combined with 8.88 μM BA, after 6 weeks of incubation (Gunay and Rao 1978).

Sripichit et al. (1987) recognize that BA is more efficient than kinetin in shoot induction in *Capsicum*, and Agrawal et al. (1989) report that the combination of BA and AIA is the best for shoot induction when compared with other auxins (AIB, ANA, and 2,4-D) and in combination with kinetin.

Combinations of AIA with 2-iP and zeatin showed to be efficient in inducing bud and shoot formation in different extents in explants from peppers of different cultivars (Alibert 1990; Ochoa-Alejo and García-Bautista 1990; Berljak 1999; Steinitz et al. 1999).

Recently, Venkataiah and Subhash (2001) and Ahmad et al. (2006) reported that TDZ induced the maximum number of adventitious shoots depending on the type of explant and genotype compared with other treatments. Experiments conducted in our laboratories have shown that TDZ at 1.0 mg L^{-1} produced a larger number of adventitious shoots compared with other treatments (Rêgo et al. 2013).

Others substances, unlikely regulators of growth of plants, induced a very high proliferation of axillary buds from the application of a long-chain alcohol found in bee wax—triacontanol (TRIA)—in the amount of 2.0 μg L^{-1} in *C. frutescens* (Reddy et al. 2002). TRIA also improved the growth of shoots and the chlorophyll content of leaves.

Joshi and Kothari (2007) also reported that when the level of $CuSO_4$ was increased 30 times, as compared with the normal concentration of the MS medium, it also increased the number of shoots per explant. Also, according to Hyde and Phillips (1996), treatments with silver nitrate ($AgNO_3$) are necessary for the production of multiple shoots in peppers grown in vitro, perhaps because $AgNO_3$ acts as a potential regulator of ethylene activity and plant growth modulator (Kumar et al. 2009).

Shoot Elongation

The combination of BA with gibberellic acid is commonly used to promote elongation (Table 6.1). The employment of 24-epibrassinolide (EBR), a plant steroid lactone, has been reported as a promoter of shoot elongation in some *Capsicum* cultivars (Franck-Duchenne et al. 1998). These authors observed that EBR acts indirectly on the elongation of stems as an elicitor of elongation in combination or synergistically with regulators of endogenous growth or added exogenously.

The addition of PAA, to the medium improved shoot elongation significantly (Husain et al. 1999), as well as higher levels of copper in the medium to improve differentiation and elongation of buds from explants of cotyledons of *C. annuum* (Joshi and Kothari, 2007).

Lastly, some laboratories have reported the occasional elongation of buds after several subcultures, and the shoots developed into whole plants in growth-regulator-free MS medium (Valera-Montero and Phillips 2005).

Despite some cases of successful elongation, it is known that the main problem during elongation is the severe loss of vigor by the shoot and, in some species, for example, *C. frutescens*, the loss of vigor is more severe than in pimenta-de-cheiro varieties. Defects in differentiation of meristems from shoots or organization of the primordia are responsible for the low recurrent incidence of regeneration of normal plants. Ideal conditions that enable the growth of normal shoots on a large scale are yet to be reported.

Callus Induction

Indirect organogenesis is an important alternative source of genetic variation that generates somaclones with desirable traits of agronomic or industrial interest. Indirect organogenesis in peppers is rarely reported, although the first reports of friable callus were induced in the presence of 2,4-D, reported by Gunay and Rao (1978). Berljak (1999) was the first to report regeneration of shoots from callus tissues induced in MS medium with 2,4-D and then transferred to medium containing BA (17.8 μM) and GA_3 (5.8 μM). The plants regenerated from callus cultures and grown ex vitro showed differences in their morphological and physiological traits.

The establishment of an organogenic culture of calluses from explants from half of a seed of *C. baccatum* in MS medium supplemented with 22.2 μM BA, 5.7 μM AIA, and 6.1 μM gibberellic acid was described by Valera-Montero and Phillips (2005). This organogenic callus culture was able to regenerate whole plants.

Root Induction

Auxins and half-strength MS medium stimulate the rooting of shoots. Excellent rates of rooting of regenerated shoots was obtained in MS medium supplemented with 5.71 μM AIA (Venkataiah et al. 2006). Rooting was observed in 72–94 % of the shoots obtained from TDZ-containing regeneration medium after the elongation treatment; in contrast, only 8–22 % of the shoots rooted without the elongation treatment.

Reddy et al. (2002) also reported a strong positive effect of a long-chain alcohol ($C_{30}H_{62}O$), triacontanol (TRIA), on the rooting of *C. frutescens*, influencing its induction and stimulating its growth.

6.3.1.4 Effect of Carbon Source, Light Regime, and Temperature on Organogenesis in Capsicum

The regeneration of plants in vitro requires a carbon source to succeed and develop axillar buds and shoots. Three percent sucrose is the carbohydrate source commonly used. Addressing tissue growth, Murashige and Skoog (1962) asserted that, in general, the use of 3 % sucrose is better than 2 or 4 %. However, there are many reports

on the use of other carbohydrate sources in the initiation and proliferation of shoots in *Capsicum*. Phillips and Hubstenberger (1985) explored the carbon source and the importance of light regime and growth regulators on organogenesis, and observed that glucose is a superior carbohydrate source compared with sucrose.

Organogenesis of shoots and roots from plantlet explants was restricted to primary cultures or those with less than 3 months and under a 16-h photoperiod at 25 °C and for 8 months under continuous light at 28.5 °C.

A favorable effect of continuous light when compared with a photoperiod of 12 h of light and 12 h of darkness and continuous darkness on organogenesis was reported by Phillips and Hubstenberger (1985); these results were corroborated by Alibert (1990). They also studied the influence of the containers utilized in the in vitro culture (Petri dishes, test tubes, and vials) on organogenesis, although no pronounced effect was observed.

Ramage and Leung (1996) investigated the simultaneous effects of the presence of BA and sucrose in the initial stages of the initiation of shoots and found that these were mandatory in the formation of shoots with a high frequency in *C. annuum* L., cultivar Sweet Banana.

In conclusion, the deprivation of exogenous sucrose from 6 to 20 days of growth has no effects on the shoot-formation response. The effect of sucrose was not due to an osmotic effect, inasmuch as mannitol, with the same osmotic potential, was not able to replace sucrose.

6.3.1.5 Influence of Gas Exchanges

Several studies have demonstrated the influence of gas exchanges on the morphogenesis in vitro of diverse species (reviewed by Kozai 2010), and also in *C. annuum* L. (Mohamed and Alsadon 2011).

Our laboratory has analyzed the problem and we have found that caps with membranes that enable gas exchanges influence the morphogenetic development of ornamental peppers (*C. annuum* L.), favoring the rusticity and maturity traits of the material, demonstrated by the elevation in the fresh and dry matter values. Plantlets grown in pots that provided greater gas exchanges present thicker epidermal cells and vascular tissues are more differentiated, and have greater calibers and lignin content (Fig. 6.3). We have also observed that plantlets grown in conditions of greater gas exchange showed less leaf abscission, slower senescence, and lower incidence of chlorosis and hyperhydricity (data not shown).

The vials without membrane usually retain a relatively higher moisture content than those with membranes. They also form water droplets on their walls (Mohamed and Alsadon 2011). Therefore, the increase in gas exchanges increased significantly the percentage of explants that developed shoots.

On the other hand, plantlets kept in unventilated pots have an early leaf fall and formation of calluses on leaves and stems due to the accumulation of ethylene. These effects caused by the ethylene can be prevented by using ethylene inhibitors, such as mercury perchlorate or high levels of polyamines (Batista et al. 2013).

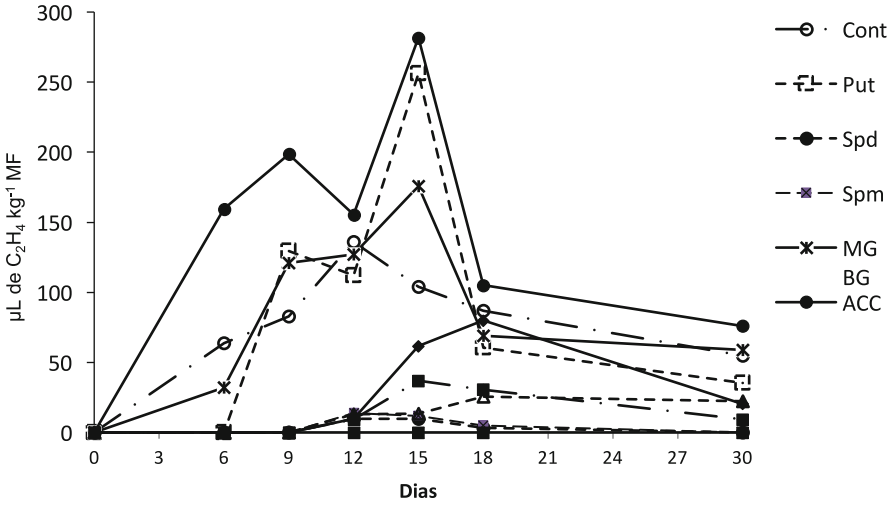


Fig. 6.3 Ethylene accumulation levels (µL of C_2H_4 kg-1 FW) measured throughout the organogenesis-induction period (30 days): *Cont* control, *Put* putrescine, *Spd* spermidine, *Spm* spermidine, *MGBG* methylglyoxal bis(guanylhydra-zone), *ACC* 1-Aminocyclopropane-1-carboxylic acid, *AVG* aminoetho-xyvinylglycine, *STS* silver thiosulfate, *PM* mercury perchlorate, *PM** mercury perchlorate removed on the tenth day of growth. (Batista et al., 2013)

6.4 Micropropagation of Ornamental Peppers

The peppers did not show a natural ability for vegetative propagation; additionally, and despite being considered autogamous, they display a high level of cross-pollination (Tanksley 1984), leading to high heterozygosity, an undesirable trait when producing commercial seeds. Therefore, in vitro propagation methods can be used for their clonal multiplication. Apical and axillary meristems, or apical buds and meristems can be used as explants to generate genetically identical plants on a large scale by micropropagation. Cultures of apical meristems from accessions of *C. annuum*, *C. chinense*, *C. frutescens*, *C.* baccatum, and C. *pubescens* were established in vitro for the first time by Fari (1988), with varying degrees of success. After this report, several protocols for the micropropagation of peppers using stem apex and nodal segments of different species and cultivars of *Capsicum* have been published by many authors (Table 6.1).

In most laboratories, the rate of plant regeneration from explants is low. Steinitz et al. (1999) observed that some genotypes present serious problems in plant regeneration, and these failures are obviously not published.

The most efficient procedure for multiplication of shoots and plant regeneration in *Capsicum* was obtained by Venkataiah et al. (2006), who used TDZ and regenerated an average maximum number of 4.2–22.4 shoots in all tested species of the genus *Capsicum*, surpassing the maximum limit reported by Steinitz et al. (1999). The plantlets obtained from TDZ-containing medium were normal double haploids

($2n = 24$) and were prepared to be established on the soil in greenhouse conditions with a survival frequency of 68–84 %. The regenerated plants were developed morphologically normal and produced viable seeds.

Other authors have also reported efficient protocols for *C. annuum* (Sanatombi and Sharma 2007a), *C. frutescens* (Sanatombi and Sharma 2007b), and *C. chinense* (Santana-Buzzy et al. 2005).

Ezura et al. (1993) were able to induce adventitious buds and whole-plant regeneration in 14 cultivars of *Capsicum* using explants from the proximal part of the hypocotyl and radicle from mature seeds, cultured in MS medium without any exogenous-growth regulator. After 2 weeks of growth, the explants from hypocotyls elongated, and the adventitious buds differentiated around their surface. After an additional period of 2 weeks of growth, the leaves developed from the adventitious buds. When the explants with adventitious buds were subcultured, shoots elongated after 3 weeks of growth in 57 % of the subcultured explants. When the shoots were excised and subcultured in half-strength MS medium, they rooted after 2 weeks of growth.

In practice, we can state that from the standpoint of micropropagation via organogenesis of adventitious shoots, several pepper genotypes are considered recalcitrant (Steinitz et al. 1999; Fari 1988; Ezura 1993; Batista et al. 2013; Rêgo et al. 2013). In spite of the described difficulties and a few cases of success, Ma et al. (1991) reported an encouraging example, producing 4.7 million micropropagated plants based on a multiplication rate of 9:1. Although laborious and done manually, this propagation scenario will be viable economically if the culture-derived plants are cheaper than the seed-derived seedlings. However, today in Brazil, the conventional production of pepper seedlings is done through seeds. This method has some disadvantages such as the short viability period, low germination rate, emergence of plantlets, and high risk of contamination by several diseases and pests.

6.5 Flowering and Fruiting of Peppers In Vitro

The flowering, fruiting, and seed production in vitro allows generation of seeds of high genetic quality and free of pathogens, as well as make selective hybridization, especially in the utilization of pollen stored or preserved in vitro, overcoming some pre- and postfertilization barriers imposed by hybridization. It would also facilitate the understanding of the flowering physiology, which largely depends on the level and interaction of exo- and endogenous phytohormones, sugars, mineral salts, phenolic compounds (Tanimoto and Harada 1981), quality and quantity of light, and the photoperiod length during the in vitro culture (Taha and Hasbullah 2010).

After the production of flowers, fertilization in vitro may also be performed, thus overcoming self-incompatibility barriers (Hogeboom 1972). Seeds from precious interspecific hybrids can also be produced. Thus, the flowering and fruiting in vitro can contribute significantly to genetic breeding programs of ornamental peppers, involving rare actions that are not accessible through conventional plant breeding.

Table 6.2 Distribution of average number of buds, flowers, fruits, and seeds per fruit in five accessions of ornamental pepper from the Germplasm Bank of the Center for Agricultural Sciences at the Federal University of Paraiba (CCA/UFPB) (Data not published)

Accession	Average number of flower buds per plant	Average number of open flowers per plant	Average number of fruits per plant	Average number of seeds per fruit
UFPB-06	–	–	–	–
UFPB-72	–	–	–	–
UFPB-132	3.50	3.00	1.58	5.00
UFPB-137	2.75	0.37	–	–
UFPB-390	2.45	0.27	–	–

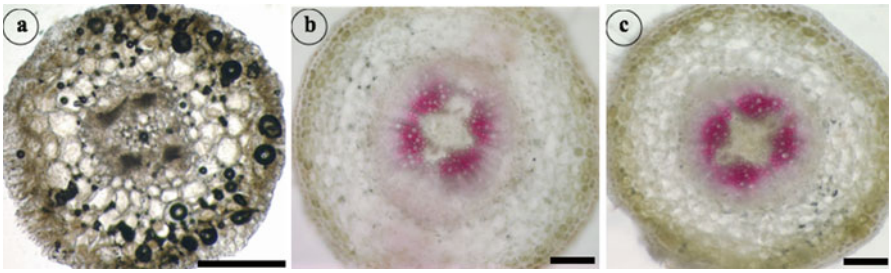


Fig. 6.4 Stem transversal sections of *C. annuum* L. 30 days after germination: (**a**) section of stem from seedlings grown in bottles without membrane; (**b**) stem from seedlings grown in bottles with a membrane; and (**c**) stem from seedlings grown in containers with two membranes. Bar 100 μm (positive reaction for lignin: *red* color). (Batista et al., 2012)

The first reports of flowering, fruiting, and formation of seeds in vitro in *Capsicum* were in *C. frutescens* L. (Tisserat and Galletta 1995), using bioreactors, in which 5–10 % of the flowers formed fruits, and, subsequently, in *C. annuum* L. cv. Sweet Banana (Bodhipadma and Leung 2003), in which success was only achieved utilizing STS combined with 5.3 μM naphthalene acetic acid (NAA); however, in both cases, the obtained seeds did not germinate.

Although flowering and fruiting in vitro in peppers were first cited in the 1990s (Tisserat and Galletta 1995), reports on these occurrences are sparse even today, despite their high scientific value (Bodhipadma and Leung 2003). In this regard, our laboratory has performed investigations aiming to adjust growth media, and screened genotypes of ornamental peppers responsive to in vitro induction of flowers, fruits, and seeds. Our results demonstrate great genotypic dependence regarding the evaluated traits (Table 6.2, unpublished data). Some genotypes simply do not respond (UFPB-06 and UFPB-72); others only produce flower buds and the flowers opened (UFPB-137 and UFPB-390); whereas accession UFPB-132 produced flower, fruits, and seeds, and all its seeds also germinated in vitro and formed whole functional plants (Table 6.2 and Fig. 6.4).

Another factor that contributed to accessions UFPB-06 and UFPB-72's not producing flowers was their sensitivity to the ethylene accumulated in the vial during the in vitro culture, which was also reported by Bodhipadma and Leung (2003); before the flowers reached the balloon stage, they senesced, preventing their formation. Therefore, we suggest the utilization of capped vials that enable the gas exchanges from the internal to the external media in future studies on the flowering and fruiting of ornamental peppers in vitro.

6.6 Somatic Embryogenesis in Peppers

Somatic embryogenesis emerged during the course of evolution, as an alternative way to overcome diverse genetic and environmental factors that prevented fertilization in plants. It is defined as a process through which somatic cells differentiate into somatic embryos. Somatic embryos are morphologically similar to zygotic embryos (George et al. 2008).

Harini and Sita (1993) were the first to report regeneration of peppers via direct somatic embryogenesis from immature zygotic embryos of cultivar California Wonder (*C. annuum*). Later, Binzel et al. (1996) also reported direct somatic embryogenesis in *C. annuum* growing immature somatic embryos of two cultivars from New Mexico (Rajpur Hirapur, pungent; and New Mexico-6, nonpungent). Notably, the percentage of regenerated somatic embryos ranged from 10 to 85 % (Binzel et al. 1996) or in all inoculated explants (100 %) (Harini and Sita 1993). The multiplication rate (somatic embryos/zygotic embryos) was 13 (Harini and Sita 1993) or higher than 8 (Binzel et al. 1996). In the breeding of plants, relatively high rates of somatic embryos (8–13) are considered advantageous when compared with other propagation systems in vitro, as it reduces the multiplication time and provides a more efficient system for the propagation of plants with high genetic uniformity (Stasolla and Yeung 2003).

In general, the MS medium supplemented with 4–18 μM 2,4-D and 3–10 % sucrose has been used to promote the formation of somatic embryos in pepper explants, whereas cytokinins have no significant effect, or have an equally inhibitory effect on somatic embryogenesis in peppers. The germination of somatic embryos has been induced in MS medium supplemented with GA_3 or TDZ alone or combined (Binzel et al. 1996), whereas abscisic acid (ABA) has been utilized to promote the maturation of somatic embryos (Büyükalaca and Mavituna 1996).

Ever since the research conducted by Harini and Sita (1993) and Binzel et al. (1996), several research groups all over the world have reported direct or indirect somatic embryogenesis using explants from mature and immature zygotic embryos, plantlets, leaves, or segments of stem (see Table 6.1).

Indirect somatic embryogenesis was first reported by Büyükalaca and Mavituna (1996), who also developed a protocol to obtain recurrent somatic embryos in liquid medium, from an embryo suspension culture in a bioreactor. The multiplication rate obtained by this process was significantly higher than that obtained by common

multiplication. The production of artificial seeds, consisting of encapsulated somatic embryos of *C. annuum*, was also achieved by Büyükalaca et al. (1995).

Despite the advances in somatic embryogenesis, we should not forget the strong genotypic dependence of peppers, as described above, which require some adaptations of the protocols of large-scale micropropagation of elite material, in an automated computer-controlled system.

Another aspect to be considered is the somaclonal variation between plants derived from the in vitro culture and detected by Shen et al. (1994). Thus, future research and the development of mass propagation protocols should include the genetic stability and fidelity of the produced propagules.

6.6.1 Induction of Androgenic Haploids through Anther Culture

The anther culture is one of the most widely known methods to produce double-haploid lines. The pioneering work of regeneration of haploid plants via anther culture was conducted by Guha and Maheshwari (1964) in *Datura innoxia.* The importance of haploid plants was attributed to their considerable plant-breeding potential (Sarvesh et al. 1993). Haploid plants can be utilized to facilitate the detection of mutations, recovery of single recombinants, and generation of homozygote lines (double haploids) after doubling the number of chromosomes, usually with chemical agents. They can occur spontaneously in the nature, or be induced experimentally. Spontaneous haploids occur in species of the genus *Capsicum* (Pochard and Dumas de Vaulx 1979).

The origin of plants with a single set of chromosomes is ordinarily attributed to the parthenogenetic development of an egg cell or haploid accessory cells (Campos and Morgan 1958). Different authors have reported anther culture and regeneration of haploid species of *Capsicum* species and hybrids (recently reviewed by Irikova et al. 2011).

Wang et al. (1973) were the pioneers in anther culture and regeneration of haploid plants in peppers. Anthers with microspores in the uninucleate stage were grown in MS medium with modifications in some micronutrients and vitamins, supplemented with kinetin, ANA, or 2.4-D (Table 6.1). Green plantlets started to appear from the anthers' sacs after 33 days of growth. Anthers with microspores in the uninucleate stage have been preferred as the material to start in vitro culture (Lantos et al. 2009).

An experiment was developed in our laboratory aiming to relate the flower-bud size to the microspore developmental stage (Fig. 6.5). In the first experiment we utilized accessions 40 (small red fruit with low pungency) and 43 (small, round, yellow fruit with high pungency) of the species *C. annuum* and *C. chinense*, respectively, belonging to the Germplasm Bank of the Federal University of Roraima. In the second experiment we used *C. annuum* accession 390 from the Germplasm Bank of the Federal University of Paraiba.

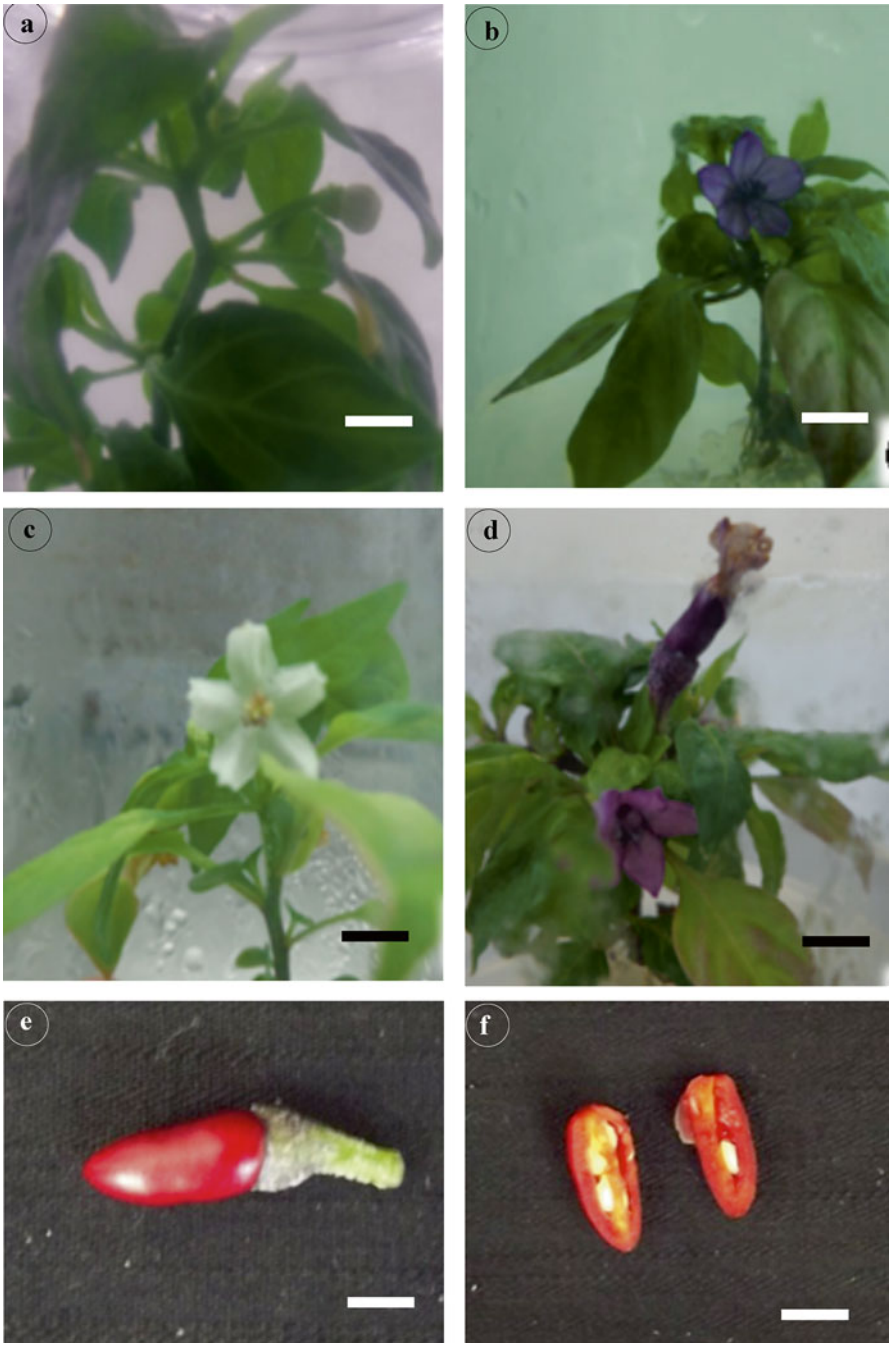


Fig. 6.5 Flowering, fruiting, and seed production in vitro in ornamental peppers (*C. annnuum* L.): (**a**) flower bud; (**b**, **c**) flowers from genotypes UFPB-132 and UFPB-137, respectively; (**d**, **e**) developing and ripe fruit from accession UFPB-132; (**f**) viable seeds from accession UFPB-132. Barr = 1 cm. (Data not published)

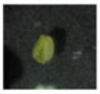
Appearance of the bud	Appearance of the anther	Stage of microsporogenesis	Bud length (mm)	Flower bud diameter (mm)	Characteristic of bud, anther,and microspore
			X ± SD	X ± SD	
			1.76 ± 0.21	1.73 ± 0.25	In this stage, the flower bud shows an anther with a bright green color, containing a microspore mother cell
			2 ± 0.1	1.93±0.11	In this stage, the flower bud shows an anther with a darker green color, and contains microspores in the tetrad stage
			2.4 ± 0.2	2.27 ± 0.15	In this stage, the bud shows a sepal of apporoximatcly the same length as the pctals; the anther has a more whitish color, containing uninucleate microspores in most of it

Fig. 6.6 Different developmental stages of embryos derived from anther culture *C. annuum* L: (**a**) globular; (**b**) cordiforme; (**c**) early cotyledonar; (**d–f**) cotyledonar embryos. Barr = 0.05 mm (Barroso not published)

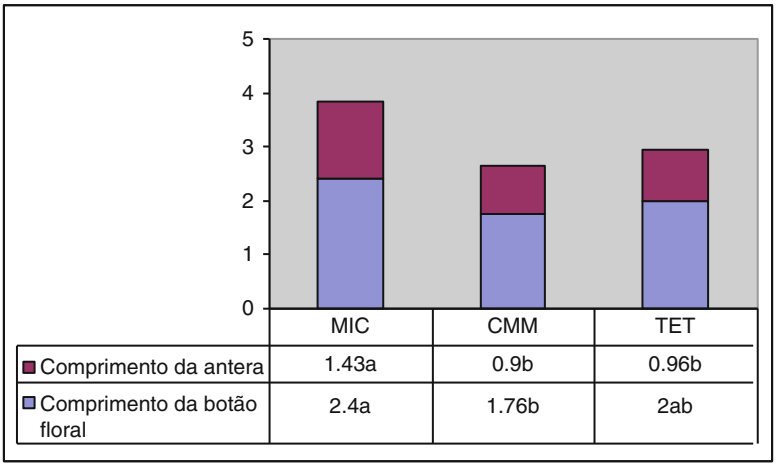


Fig. 6.7 Distribution of the microspore developmental stages in relation to the mean values of bud and anther length. Means followed by equal letters in the row do not differ statistically according to Tukey's test ($P < 0.05$). (Rêgo et al., 2012)

Flower buds in different developmental stages were collected, disinfected, and the anthers were aseptically removed from the flower bud to determine the developmental stage of the microspore, and inoculated in nutrient medium (Rêgo et al. 2012; Barroso et al. 2015). The analysis of the data revealed an overlap between the developmental stages of the microspores and the evaluated traits; that is, there is no synchronism between the cells of a same anther that enter meiosis. The appearance and size of the buds and anthers, the microspore stage, and their characteristics are shown in Fig. 6.5. The microscopic analysis revealed that the highest frequency of uninucleate microspore occurs when the sepal length equals the petal length.

We also have observed significant differences between the microspore developmental stages, the flower bud length (FBL), and the anther length (ANL; Fig. 6.6),

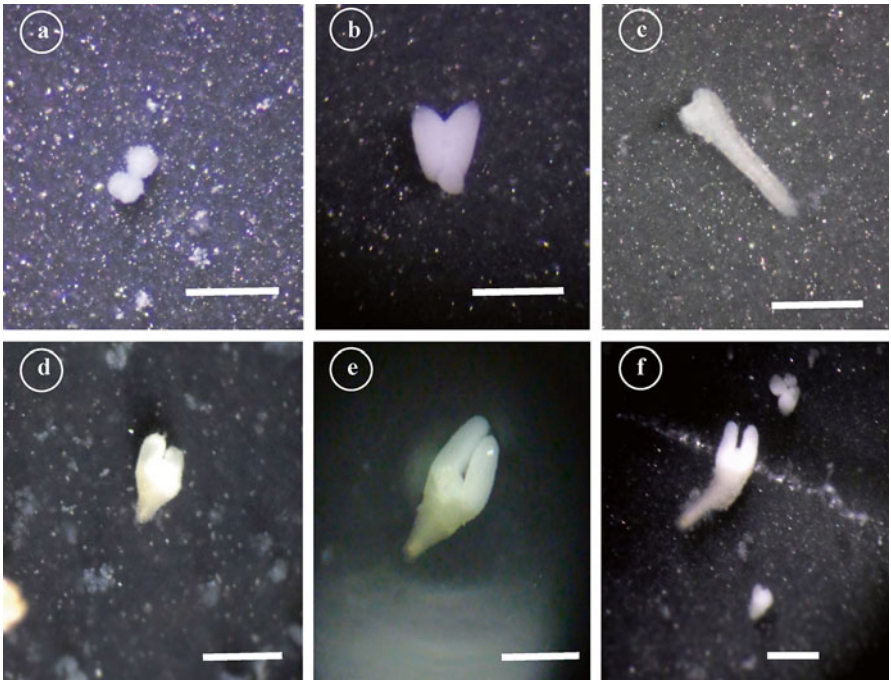


Fig. 6.8 Different developmental stages of embryos derived from anther culture *C. annuum* L: (**a**) globular; (**b**) cordiforme; (**c**) early cotyledonar; (**d**–**f**) cotyledonar embryos. Barr = 0.05 mm. (Barroso, 2016)

and that the ANL is related to the development of most uninucleate microspores (Rêgo et al. 2012; Barroso et al. 2015). The relationship between bud morphology and microspore developmental stage was analyzed by a correlation analysis. It was observed that the number of cells in the uninucleate stage was not correlated with any of the bud morphological traits, indicating a concentration of cells specifically in one of the classes. The test of means confirms that the largest presence of uninucleate microspores is in class 2 (buds with petals and sepals of approximately the same length). They were not found in the first established class (petals shorter than sepals), and occurred in small amounts in class 3 (petals larger than sepals) (Barroso et al. 2015).

Because no correlation was found, and because there is a statistical difference between the averages of uninucleate cells/established size class, it is possible to determine the ideal bud size to find the largest amount of microspores in the uninucleate stage, utilizing petals and sepals with approximately equal lengths to determine the class.

The regeneration of haploid plants from microspore or anther culture is highly dependent on the *Capsicum* species, cultivar, hybrid, or equally on the genotype. The pretreatment of the anther-donor plant or the flower buds before the in vitro culture has been a very common practice to increase the regeneration efficiency of pepper haploids. Low temperature (4 °C) or high temperatures (35 °C) have been applied as pretreatment.

The original protocol for in vitro anther culture developed by Dumas de Vaulx et al. (1981), or some variations of this protocol, has been found in the literature because it is frequently utilized for the regeneration of haploid plants (Irikova et al. 2011) including ours (Barroso et al. 2015).

The protocol of Dumas de Vaulx et al. (1981) involves the simulation of androgenesis in vitro of peppers at a high temperature. Pepper anthers are pretreated at 35 °C for 8 h and then grown at 25 °C in the presence of 0.45 μM 2,4-D and 9.3 μM kinetin.

Morrison et al. (1986) developed the double-layer culture technique, a good alternative for regeneration of haploid *C. annuum* L. plants from anther culture.

More recently, Supena et al. (2006) developed the shed-microspore culture protocol, and then refined it to improve the embryo quality. The medium consists of a double layer: that is, the lower layer is semisolid and the upper layer is liquid. The lower layer consists of the Nitsh medium components (Nitsch and Nitsch 1969), with 2 % maltose, and 0.5 to 1.0 % activated carbon, solidified with 0.6 agar. The upper layer contains only the Nitsh components and 2 % maltose. The pH of the double-layer medium was adjusted to 5.8 and the growth regulators were sterile-filtered and added after autoclaving.

The critical factors of the protocol are: selection of flower buds with more than 50 % of the microspores in the last unicellular stage; pretreatment of the buds for 1 day at 4 °C followed by the anther culture in a double-layer system for 1 week at 9 °C, and subsequently at 28 °C in continuous darkness. The medium contained the Nitsh components (Nitsch and Nitsch 1969) and 2 % maltose, with 1 % activated carbon in the solid sublayer and 2.5 μM zeatin and 5.0 μM AIA in the upper liquid layer. The 10 genotypes evaluated responded to this protocol. The best genotype produced 4–7 plants per flower bud.

Recent investigations on microspore or anther culture in *Capsicum* have been carried out by Supena and Custers (2011), Cheng et al. (2013), Olszewska et al. (2014), and also by our lab (Barroso et al. 2015).

In our laboratory we have applied the protocol developed by Supena et al. (2006, 2011) to screen several accessions from the Germplasm Bank of the Center for Agricultural Sciences of the Federal University of Paraiba, and we have obtained a high frequency of embryos derived from anther culture (unpublished data; Fig. 6.5). We have observed a large genotypic dependence of the androgenic response and clear differences in the effectiveness of androgenesis of ornamental peppers.

In our lab, have found genotypes that respond differently to different protocols; for example, accession UFPB-132 shows a null response to the method described by Dumas de Vaulx (1981), and a positive response to the shed-method described by Supena et al. (2006). In contrast, the accession UFPB-1, shows a null response to the second method and a positive response to the first method; although these genotypes belong to the same type, they have different fruit characters.

Therefore, before starting a breeding program based on double-haploid production it is advisable to assess the response of each variety to the different protocols available.

6.7 Plant Regeneration from Protoplasts

The importance of protoplast culture lies in the possibility of recovering somaclonal variants, somatic hybrids in the case of interspecific sexual incompatibility, and sterility of F1 hybrids. There are few reports on the regeneration of plants via protoplasts. Although pepper protoplasts can be obtained easily, the subsequent cell regeneration and the plant regeneration is a rare event.

Since the groundbreaking work of isolation and culture of protoplasts in *Capsicum* conducted by Saxena et al. (1981) from axenic cultures of shoots of cultivar California Wonder, a few studies have been published on the matter (Diaz et al. 1988; Murphy and Kyle 1994; Prakash et al. 1997; Lim and Lian 2001).

The protocol of Saxena et al. (1981) consisted of enzymatically dissociating the cells of the leaf tissue of the shoot with a mixture of enzymes (2 % cellulase, and 0.4 % Macerozyme) in liquid MS medium supplemented with 0.5 M mannitol as an osmotic regulator. The protoplasts were grown in a medium containing the mineral salts of DPD (Durand 1979) and NT medium (Nagata and Takebe 1971) with 4.5 μM 2,4-D + 5.37 μM ANA + 4.44 μM BA, 2 % sucrose, and 0.5 M mannitol. The chloroplast suspension density was adjusted to 5×10^4 cells per milliliter. The protoplasts proliferated and formed a callus mass in the two growth media under dark conditions. The callus mass differentiated into shoots in the differentiation medium (22.83 μM AIA, 11.90 μM kinetin, and 3 % sucrose) when exposed to light. Shoots were induced to form roots in medium containing 5.71 μM AIA and 0.186 μM kinetin.

Leaf protoplasts were isolated from an axenic culture of shoots established in MS medium without growth regulators of plantlets from four cultivars of *C. annuum* and one genotype of *C. chinense* (Diaz et al. 1988). The shoots were cut and plasmolyzed for 1 h in CPW13M medium (Power and Chapman, 1985), and then treated with an enzyme mixture of 1 % cellulase (Onozuka R-10) and 0.25 % Macerozyme (R-10) in CPW13M medium. After incubation and washing, the protoplasts were transferred to a KM8P/KM8 (2:1) medium (Kao and Michayluk 1975) mixture at a density of 5×10^4 to 1×10^5 protoplasts per milliliter. The protoplast suspension was dispensed as droplets in medium with agarose in Petri dishes and bathed in liquid medium. Incubation was performed under illumination conditions. The colonies were transferred to MS medium supplemented with 4.56 μM zeatin and formed calluses. The calluses were finally cultured in MS medium with 8.88 μM BA and the regenerated branches were rooted in growth-regulator-free MS medium.

Murphy and Kyle (1994) developed a protocol for the isolation of protoplasts from leaf tissue of *C. annuum* cultivars and two genotypes of *C. chinense*. Leaves were sliced and plasmolyzed by submersion in a mannitol solution and agitated at 170 rpm for 10 min at 25 °C. The tissues of *C. chinense* genotypes were incubated in the presence of 0.43–0.45 M mannitol to produce better protoplasts. The mannitol solution was decanted and the plasmolyzed leaves were submerged in a solution of enzymes consisting of 1 % macerase, 0.25 % pectolyase, and 1 % Cellulysin dissolved in mannitol. The leaves in the enzymatic solution were placed in a shaker

at 70 rpm at 25 °C for 4 h. Approximately 650,000 protoplasts were produced per every 0.5 g of leaf tissue. The protoplasts from these preparations are excellent systems for viral RNA infection by electroporation.

Plant regeneration from leaf mesophilic protoplasts of bell pepper (*C. annuum*) cultivar California Wonder was reported by Prakash et al. (1997). Protoplasts isolated from three-week-old, fully expanded leaves from cultures of axenic branches were grown in TM medium (Shahin 1985) supplemented with 5.37 μM ANA + 4.52 μM 2,4-D + 2.22 μM BA. The antioxidants ascorbic acid and polyvinylpyrrolidone in the medium and the incubation in the darkness helped to overcome the protoplasts' necrosis. Micro- and macrocalluses were formed in the TM and MS media, respectively, containing 10.74 μM ANA + 2.22 μM BA. Two-to-five microbranches per callus emerged in gelled MS medium enriched with 2.85 μM AIA + 5.78 μM GA_3 + 44.4 μM BA. The rooting of branches was achieved in a medium containing half the concentration of the salts of MS containing 5.37 μM ANA + 2.22 μM BA.

Factors affecting the isolation and culture of protoplasts in *C. annuum*, *C. baccatum*, and *C. chacoense* have been studied by Lim and Lian (2001). These authors successfully isolated protoplasts from cotyledon, hypocotyl, and mesophilic tissues using a combination of Cellulysin (1 %), Macerozyme (0.25 %), and 0.65 M sorbitol. The antioxidant MES in the enzymatic solution helped to overcome the protoplasts' necrosis.

6.8 Genetic Transformation

The reports describing the generation of transgenic pepper plants is recent, starting with Liu et al. (1990). Communications from laboratories in China have indicated successful transformation of *C. frutescens* (Wang et al. 1991) and *C. annum* (Dong et al. 1992; Zhu et al. 1996), and more recently, in Mexico, *C. chinense* was transformed using leaf explants and mediated *Agrobacterium tumefaciens* (Arcos-Ortega et al. 2010).

The Korean group described the transformation of hot pepper (*C. annuum*) with a CMV satellite construct (Lee et al. 1993; Kim et al. 1997). Partial attenuation of symptoms and a decrease of virus titer were observed. Posteriorly, another chili pepper species (*C. frutescens*) also was transformed by the Japanese group with a phenylalanine ammonia-lyase gene (Yamakawa et al. 1998), and in India Manoharan et al. (1998) described the generation and characterization of transgenic hot chili (*C. annuum*).

In spite of this promising progress and of the efforts invested by researchers in the direction of transgenic pepper breeding, several obstacles to genetic transformation remain, such as: (a) extremely low transformation rates; (b) strong genotypic dependency; (c) the use of similar strategies by the different successful groups: *Agrobacterium*-mediated transformation was exclusively used, cotyledons were generally preferred as the target explants, and NPT II was the selective tool in all cases. More recently, Min et al. (2015) reported a useful protocol with a suitable

selection method. The most important aspect of the pepper transformation protocol is selecting shoots growing from the callus, which is referred to as callus-mediated shoot formation. This protocol is a reproducible and reliable system for pepper transformation.

6.9 Strategy to Accelerate Breeding Programs of Ornamental Peppers

In conventional breeding programs of ornamental peppers the most usual methods are: (1) pedigree, selection of individual plants combined with controlled self-pollination; (2) backcross, particularly for traits controlled by one or few genes, which involves selection of individual plants and successive crosses to a recurrent parent; (3) recurrent selection, which involves selecting individuals from a population followed by intercrossing to form a new population; and (4) single seed descent method, which does not need selection during the breeding process (see Chapter 4 for details).

In any breeding program shortening the selection cycles will be very helpful for two reasons: to accelerate breeding programs and, indirectly, to decrease the costs of growing plant materials. An alternative to overcome this problem is to excise and cultivate in vitro zygotic embryos from immature fruits. This procedure could include marker-assisted selection for specific traits of interest, which might allow breeders to avoid time-consuming evaluations and use the first fruits set in the precocious generation.

The isolation and in vitro germination of immature zygotic embryos might be helpful to shorten breeding cycles and accelerate breeding programs. Manzur et al. (2014) evaluated the efficiency of this strategy in *C. annuum* under different growing conditions [autumn–winter (AW) and spring–summer (SS)]. Five accessions, representing different varietal types, were included in the experiment and immature advanced embryos (torpedo-early cotyledonary) were used because of their high in vitro germination aptitude.

In the conventional breeding program of pepper, no more than two generations per year are possible in peppers. By contrast, the in vitro strategy reduced the cycle length to 70 days in the AW season and to 56 days in the SS season, showing the highest shortenings. These findings show that this strategy will allow Capsicum breeders to obtain three generations per year, and up to four generations in cayenne peppers. Furthermore, compared to controls, in vitro-germinated plantlets showed the same high pollen fertility, and no deleterious effects were observed in their subsequent development (plant height and biomass). Therefore, these plants can be integrated safely in breeding programs.

In our lab, we evaluated the method described by Manzur et al. (2014) in three different species of Capsicum (data not shown) and the preliminary results indicated the possibility of achieving up to four generations per year in our improvement program of ornamental chili pepper.

6.10 Conclusions and Future Prospects

The research developed in the last three decades with tissue culture in the genus *Capsicum* in laboratories around the world has evolved substantially as regards the refinement of the developed protocols of regeneration of functional plants, in spite of the persistent dissatisfaction with the state of the art among scientists, breeders, and producers. We have observed a large genotypic dependence in studies on regeneration from somatic and microspore cells for a given set of growth conditions, making it impossible or perhaps unrealistic to hope to develop valid and repeatable protocols that encompass a broad range of *Capsicum* genotypes. Consequently, regeneration is an obstacle that will have to be overcome through the adjustment of culture conditions for the individual cases that help breeding programs. The proliferation of somatic embryos in computer-controlled bioreactors will probably be the preferred technology for mass micropropagation in the future. Regarding androgenesis, the adjustments in the refinement of protocols help to produce absolutely homozygous double-haploid lines in shorter time periods. Today, anther culture is a successfull part of most *Capsicum* breeding programs, including our laboratory. Yet a lot is still to be done in the next decades, making the *Capsicum* biotechnology an interesting and dynamic field, especially with regard to selection in vitro for resistance to diseases and tolerance to abiotic stress (drought, salinity). Finally, we have understood that, despite the advances, genetic transformation is still in its infancy in ornamental chili pepper.

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