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Thesis

GAMIC PROPAGATION OF GLOBE ARTICHOKE (*Cynara cardunculus* subsp. *scolymus*) FOR THE PRODUCTION OF F_1 HYBRIDS

PhD student

Chiara Lo Bianco

Coordinator

Prof. Alberto GRAIFENBERG

Tutors

Prof. Francesco Saccardo
Università degli Studi della Tuscia, Dipartimento GEMINI

Dr. Paola CRINO'
ENEA C.R. Casaccia, Roma

Dr. John Rusty Jordan
BIG HEARTH Seed Company, Ca, USA

This work is dedicated to the biggest love of my life: my father.

Este trabajo esta dedicado al amor más grande de mi vida: mi padre.

Dedico questo lavoro al piú grande amore della mia vita: mio padre.

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SUMMARY

Globe artichoke gives an important contribution to the Mediterranean agricultural economy. However, in the Mediterranean countries, where this crop was first grown and its cultivation developed and increased over the centuries, production has recently remained stagnant since the end of last century.

The principal problems related to the development of globe artichoke as a modern cultivation consist in the irregularity both of production and commercialization calendar as well as in the traditionally adopted vegetative propagation system, which requires expensive agronomical practices and does not guarantee well-being propagation material.

No intensive breeding programs have been carried out. The only real novelty in this sense is represented by the development of few seed propagated hybrids. Male sterility was used to allow production of many experimental F_1 hybrids eliminating the long and expensive operation of floral emasculation.

Seed-planted cultivars have the following major advantages:

1) labor saving and a cheap operation of the mechanical seed sowing;
2) conversion of globe artichoke into annually grown crops and introduction into crop rotation;
3) respect to vegetatively propagated plants, more efficient use of both moisture and fertilizers due to the long vertical tap roots penetrating into the soil deeper than the adventitious roots produced by planted suckers;
4) protection from pathogens and pests by annual cultivation coupled with crop rotation;
5) more vigorous and healthier growth of the plants with a low input of pesticides, fungicides and fertilizers, so making globe artichoke as a valid rotation choice in an organic cultivation system.

For the development of globe artichoke as a modern crop, it is important to produce new stable seed propagated hybrids, but also to set up the techniques necessary to rationalize the cultivation system.

In the process of F_1 hybrid production, it needs to consider some aspects such as (i) the correct management of the cultural cycle, (ii) the adaptability of the new F_1 hybrids to the different cultivation environments as well as to the different production and commercialization calendars, (iii) the economical production of F_1 hybrid seed because the adequate pollination of male-sterile plants still remains difficult to achieve.

During the three years of PhD program, a strategy of development of F_1 hybrids was carried out in order to reach some objectives. The first one was to develop stable male and female parental lines, selecting the germplasm already available and trying to improve their stability. Some male sterile clones were selected and individuated as good female parents; some male fertile genotypes were selected as stable. Moreover, in order to deep the knowledge on the morphological and functional differences between male sterile and male fertile globe artichoke flowers, a study of the floral biology was carried out. The floral male and female structures were staged and studied in order to highlight the differences. The male sterile flowers showed either a normal development of the male reproductive structures or a normal meiosis in the cytological analyses of pollen formation. The block of pollen viability was post-meiotic and the low viability of the male sterile pollen was connected with a less developed external exine structure. Female organs of the male sterile flowers were more elongated than those of the male fertile flowers at the same stages.

Different cross-combinations were developed either in Italy (2006 and 2007) or in USA 82008) in order to compare the different hybrids and to individuate the most homogeneous ones. A focal point of the project was to find an evaluation system capable of distinguishing which hybrids were more homogeneous than others. For this reason, an evaluation of all hybrids based on the morphological traits was carried out using the ***Protocol for Globe Artichoke*** European Union CPVO (2004) and some other traits chosen by Lopez Anido *et al.* (1998) both in Italy and in USA.

In order to analyze the hybrids also from a molecular point of view, five F_1 offsprings produced during the PhD program along with their parents and a commercial hybrid (Opal F_1) used as a control were analyzed by ISSR markers. Molecular work was realized in the laboratory of the Dpto. Ciencia y Tecnologia Agraria of Universidad Politecnica de Cartagena (UPCT), Spain.

Hybrids were all well differentiated by both the molecular and morphological analyses. The results of the molecular analysis were compared with those of the morphological one. The clustering of clones based on the morphological characters resulted consistent with that derived from the molecular analysis.

The last focal point of the research was the development of an effective pollination technique for the production of F_1 hybrids by comparing different pollination systems studied. Their potentialities in seed productivity of our male sterile lines were evaluated.

In 2006, the field experiment were carried out at the Regional Agency for the Development and Innovation of Agriculture (ARSIAL), in Tarquinia (Viterbo, Italy) and

both manual pollination by brush and pollination by bumblebees (*Bombus terresris*) were compared with an open pollination control. In 2008, open field activities were carried out in the experimental fields of Big Heart Seed Company (BHSC), in Brawley (California, USA) and the effectiveness of honey bees (*Apis mellifera*) as pollinators was evaluated in order to verify if, changing the percentage of both male sterile and male fertile plants, there were some differences in the seed production. The results of the first year experiment should be repeated because highlighted interesting potentialities in the use of the bumblebees as valid alternative to the honey bees. Unfortunately, some environment problems reduced seed production and it was not possible to get some definitive results. The results obtained in the third year confirmed that the honey bees are less attracted by the male sterile flowers.

GENERAL PART

1 Taxonomy and nomenclature

Globe artichoke [*Cynara scolymus* L. = *Cynara cardunculus* L. subsp. *scolymus* (L.) Fiori] is an angiosperm dicotyledonous plant belonging to the *Asterales* order. This order groups 19.000 species with the two families *Asteraceae* and *Cichoriaceae* to the subfamily of *Tubuliflorae* and to the tribe of *Cynareae* (Bianco *et al.*, 1990).

Fig. 1 – Heads of (a) globe artichoke, (b) cultivated cardoon, and (c) wild cardoon.

The members of the genus *Cynara* are as follows: *C. cardunculus* L. species complex, this including the globe artichoke [var. *scolymus* (L.) Fiori], the cultivated cardoon (*C. cardunculs* var. *altilis* DC.= *C. cardunculs* subsp. *cardunculus*) and the wild cardoon [*C. cardunculs* var. *sylvestris* (Lamk) Fiori], and other seven wild species such as *C. syriaca* Boiss, *C. cornigera* (Lindely) (syn. *C. sibthorpiana* Boiss.), *C. algarbiensis* Cosson, *C. baetica* (Sprengel) Pau (syn. *C. alba* Boiss.), *C. humilis* L., *C. cyrenaica* (Maire & Weiller) (Wiklund, 1992; Rottenberg and Zohary, 1996; 2005), and *C. tournefortii* Boiss. Et Reuter.

All wild species are perennial and characterized by large spiny leaves and heads. *Cynara algarbiensis*, *C. baetica*, *C. humilis*, and *C. tournefortii* are principally of Western Mediterranean distribution, while *C. cornigera*, *C. cyrenaica*, and *C. syriaca* come from the Eastern part of the Mediterranean region. *Cynara cardunculus*, present in almost all the Mediterranean basin, is reported to contain the two wild sub-species *cardunculus* and *flavescens* Wiklund, which differ both for the geographical distribution and the bract characters. The former is distributed from Cyprus to Greece, Central and Southern Italy while the latter is diffused in the Iberian Peninsula and Macronesian region (Wiklund 1992). Morphological differences refer especially to bract color, head shape and spines, subsp. *cardunculus* showing longer and more acute spines and subsp. *flavescens* possessing a yellowish margin in the middle bract involucres.

In 1753, Linneo gave the name of *Cynara scolymus* L. to artichoke but, respect to other related *Cynareae*, its taxonomic position has been quite debatable. The controversy whether the three *Cynareae* (globe artichoke, cultivated and wild cardoon) must be classified as different species or as sub-species of *C. cardunculus* L. has not been for a long time resolved.

On the basis of morphological data, in 1848, De Candolle for the first time suggested that globe artichoke would derive from the species *C. cardunculus* L. var. *sylvestris* (Lamk) Fiori. Studies based both on hybridization programs and use of enzyme markers have demonstrated that these two *C. cardunculus* forms are fully cross-compatible, and give fertile inter-varietal hybrids (Basnizki and Zohary, 1994). The wild cardoon (*C. cardunculus* L. var. *sylvestris*) must be considered as the wild progenitor of the cultivated one (Bianco *et al*., 1990; Basnizki & Zohary, 1994; Rottenberg & Zohary, 1996a e b; Rottenberg *et al.*, 1996) and comprises the primary wild gene pool (GP1) of the cultivated artichoke (Rottenberg and Zohary, 1996a). Globe artichoke and its wild progenitor also possess ribosomal genes of the same length (Maggini *et al.*, 1988).

In contrast, reproductive barriers separate the *C. cardunculus* species complex from the other *Cynara* species (Rottenberg *et al.*, 1996): the crosses between *C. cardunculus* and any of the other species *C. syriaca*, *C. algarbiensis*, *C. baetica* or *C. humilis* produce few seeds, although the hybrids are generally sterile. Moreover, wild *Cynara* species diverge isozymically from *C. cardunculus*; they are, therefore, regarded as comprising the secondary wild gene pool (GP2) of the artichoke crop (Rottenberg and Zohary, 1996a).

2 Origin and domestication

Judging from the distribution of the wild progenitor, the cultivated globe artichoke should have been cultivated in the central and western parts of Mediterranean basin. In particular, it should be native from Southern Europe around the Mediterranean basin and North Western Africa.

Whether artichoke was known to the ancient classical world is still an open question. De Candolle (1890) referred that cultivated artichoke was unknown in classical times but both Greek and Roman writers reported on the consumption of this species and, according to Theophrastus, in 371–287 BC, its cultivation was already present in Sicily but not in Greece

(Montelucci, 1962). On the contrary, artichoke was already well known at the beginning of the Christian era (Marzi, 1967) and an unidentified species of *Cynara* is shown in a mosaic back dating to the Imperial period (3rd century AD) in the Bardo Museum of Tunis. Columella (1st century AD), in *De re rustica*, referred on 'Cynara' cultivation in Italy and defined the plant as 'hispida' (= spiny) stating that '*pinea vertice pungit*' (= its head apex pierces).

Based on Plinius and Columella, Foury (1989) deduced that cultivation of artichoke started around in the 1st century AD; however, it is likely that, in the first century of the modern era, the domestication of artichoke was not yet accomplished (Sonnante *et al.,* 2007). The first records on artichoke diffusion are related to Filippo Strozzi who brought this plant possibly from Sicily to Florence (Bianco, 1990). These reports further support the idea that globe artichoke was originally domesticated in Sicily, as also recently reported by Pignone and Sonnante (2004), and then spread by Arabs, who dominated Sicily for over 200 years beginning from the 9th century, to the regions where nowadays it is a crop of primary importance (Pignone and Sonnante, 2004). The role played by Arabs in spreading the globe artichoke cultivation is confirmed by the Italian (carciofo), Spanish (alcachofa), and Portuguese (alcachofra) current name of the plant that comes from the Arabic word 'al harshuff'. Interestingly, in English, French, and German languages, as well as in north European and in Russian languages, the name of this plant comes from the late Latin/old Italian 'Alcocalum', 'Articocalus', 'Articiocco' or 'Articoca' of uncertain origin, but possibly related to the Latin 'coculum' (= cardoon; Lonitzer, 1551–1555), while in Greek the plant is known as 'Agginara' (= Αγγιναρα), which relates to old Greek 'Kyon' (= Κυον, dog), possibly for the spines remembering the dog teeth. This strongly suggests one more that Italy was the bridge for the diffusion of artichoke into Europe.

3 Geographic distribution

Almost 90% of the overall world acreage of globe artichoke is distributed in Italy (50,120 ha), Spain (21,000 ha), France (10,500 ha), and Greece (2,500 ha), while only 10% involves South America (mainly Argentina with 4,700 ha and Chile with 4,300 ha), California (3,320 ha), North Africa (Egypt with 3,800 ha, Algeria with 2,800 ha, Morocco with 3,300 ha, Tunisia with 2,100 ha), Middle East (Turkey with 2500 ha, Israel with 670 ha, Syria with 510 ha), and China (10,000 ha) (FAOSTAT, 2007).

In the last 15 years, some world areas, in particular South America and China, increased significantly the artichoke growing areas (*Fig.2*) and are emerging as globe artichoke producers (FAOSTAT, 2007).

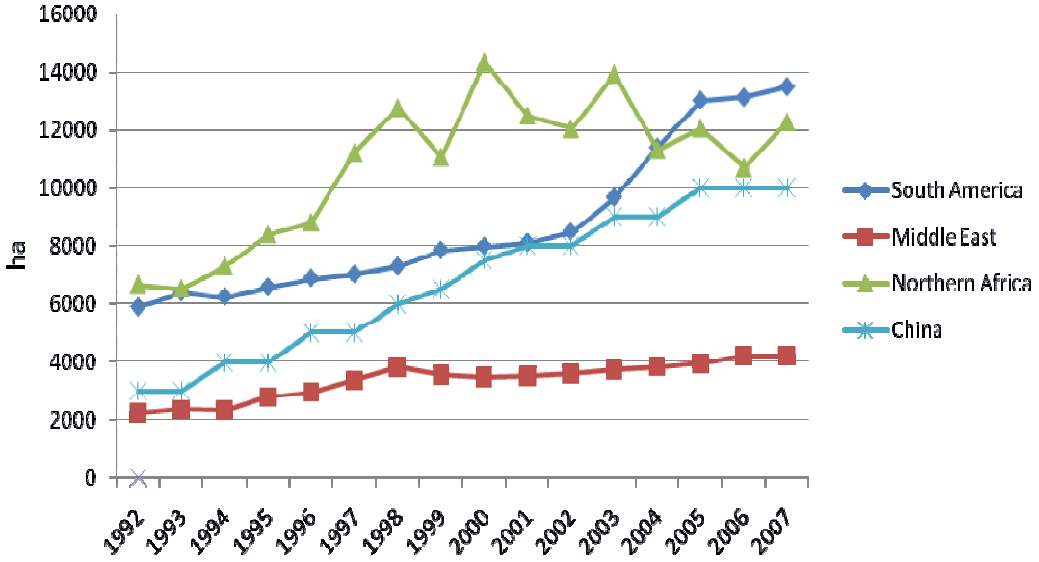

Fig. 2 - Increase of some world areas in the artichoke acreage (FAOSTAT, 2007)

South American countries were the first ones to start growing artichokes in a great amount: Peru has now over 4,000 hectares, most of which are covered with plants grown from seeds; Chile has 2,500-3,000 hectares of plants grown from offshoots of Blanca de Tudela variety, and Ecuador has 500 hectares of seed planted varieties. In China, however, cultivation of this crop is much more recent, only starting in 1993 with new plantations consisting entirely of seed propagated plants covering approximately 1,000 hectares in Kunming area and 200 hectares in and around Wuhan territory. The rapid expansion of this crop in completely new areas is due to the use of seeds. This allows a large number of plants to be grown in a short period of time, in contrast to the use of traditional vegetative propagation methods limiting the number of new plants. The varieties being grown in almost all these new countries are Imperial Star, Lorca or A-106 (Macua, 2007). The fact that artichokes are now being grown in countries where they were previously unknown means that domestic demand is nil. All the production is canned, bottled or frozen, or in the case of China, is preserved in barrels for later re-packing, and destined for export. The zones chosen for production have

mild weather conditions and a long growing season, this in turn leading to a high degree of productivity (20-30 t/ha). With regard to the Mediterranean basin, the situation has improved in some of the southern countries such as Egypt and Morocco, or to a lesser extent Turkey.

Globe artichoke cultivation is of great importance for Italian horticultural economy and its cultivation area is exceeded only both by tomato and potato. The major Italian production areas are located in southern (Apulia, Sicily) and central regions (Latium, Sardinia, Campania, Tuscany), where globe artichokes are generally planted from July through September and year-round grown generally as perennials (3 to 4 years). In southern Italy, globe artichoke (e.g. 'Violetto di Sicilia' (b), 'Spinoso Sardo', 'Brindisino') is grown for autumn, winter, and early spring harvesting while, in central Italy, it is cultivated only for spring production (e.g. 'Campagnano', 'Castellamare', 'C3', 'Terom', 'Violetto di Toscana').

In Italy, Spain and California, approximately 80-75% of the harvested globe artichokes are sold to the fresh market; the remaining 30-25% are processed as canned hearts and crowns or as frozen, quartered artichokes. In California, a limited market has started for fresh, trimmed artichoke hearts, mostly for the upscale restaurant trade.

California produces all of the commercially grown artichokes in the United States; in particular, it occurs in four central-coast areas (Monterey, San Mateo, Santa Cruz, Santa Barbara), where the climate for artichoke production is especially favourable. The Central Coastal Region of California represents the location of 84% of USA artichoke acreage. The remaining part is grown in the South Eastern Region, mainly in the area of Riverside, and in the South Coastal region of Santa Barbara, San Diego, Ventura, Imperial, and Orange as well as in the San Joaquin Valley region counties of Fresno and San Joaquin.

4 Production and international trade

In the Mediterranean region, European countries such as Italy (470,213 Mt), Spain (188,900 Mt), France (52,500 Mt) and Greece (35,000 Mt) are world leaders accounting almost 80% of the overall world production of globe artichoke. In this area, in the last 45 years, a decreasing trend of total productions has been recorded for France (- 67%) and Greece (- 41%), while increased values of 13 and 52% were observed in Italy and Spain.

At present, globe artichoke is an important vegetable also in Argentina (88,000 Mt), Egypt (70,000 Mt), Morocco (53,000 Mt), Algeria (35,000 Mt), Chile (32,000 Mt), Turkey f(30,000 Mt), and California where, since 1961 to now, its total production showed increases

of 62%. Recently the production remained stagnant in the countries were globe artichoke cultivation developed and increased. However, two new horizons have opened up for this vegetable in opposite sides of the world: one in the east (China), and the other in the west (South America). China represents an emerging country because globe artichoke, which was not cultivated 50 years ago, is now producing 55,000 Mt. Only small amounts of globe artichoke are produced in other countries of North Africa, Middle East, and Latin America.
Over 99% of the commercial artichoke production of the United States is located in California, where the vegetatively propagated variety Green Globe currently accounts for nearly 90% of artichoke production, while seeded artichoke varieties include Imperial Star and Emerald. California is exporting artichokes to Canada, Japan, Mexico, and Europe.

5 Biology and ecology

The globe artichoke is a perennial plant that grows to a height of 90-180 cm. During each growing season, offshoots or suckers emerge from a permanent crown, at the base of the stem. The number of offshoots varies with the age of the plant; young plants produce a single offshoot, while 3-4 year old plants may produce a dozen or more offshoots. Each offshoot forms a cluster of large basal leaves, from the center of which the flower-bearing stalks grow. Edible buds are produced both at the tips of these elongated stalks and their branches.

Normally, a new cropping cycle begins when the plant is cut back slightly below the soil surface to stimulate the development of new offshoots (De Vos, 1992). If the buds are not removed, they develop into purple-centered, thistle-like flowers with heads 10-15 cm in diameter. The timing of this ‘cut-back’ operation influences when the artichokes are ready for harvest. Perennial production is essentially continuous throughout the year, although about 70% of the crop is harvested in a specific part of the year. Fields with winter-spring cycles produce the highest yields, and are continuously harvested from September through May. Earliness of the re-flowering types is frequently lost because of a phenomenon commonly considered as ‘degeneration’ of the crop.

Proper climatic conditions are extremely important in globe artichoke production. This is a cool-season crop well adapted to a wide temperature range of growth; temperatures of 20-22ºC in the day and 12-14ºC in the night represent the optimal values to obtain compact and tender heads for an extended period. The minimal biological temperature ranges between 7-9

ºC, while the lethal one is lesser than –10ºC (Bianco *et al.*, 1990). Plants are tolerant to high temperatures (> 30°C) that, however, tend to decrease the quality of edible heads.

Seed globe artichoke germination generally occurs at temperatures of 15-20 ºC and there is not seed dormancy (Basnizki, 1994). A large variation in emergence rate and germination percentage has been shown. However, germination characteristics are negatively influenced by temperatures > 25°C; this condition is usually recorded during the sowing season, thus temporary thermo-dormancy may be induced in seed (Damato and Calabrese, 2007). Some techniques allows seeds to germinate more rapidly under unfavorable environmental conditions (Damato and Calabrese 2005). An example is represented by the osmoconditioning that, however, influences negatively the germination characteristics of the seeds (Damato and Calabrese, 2007).

Globe artichokes are obligated long day plants with a critical photoperiod of 10,5 hours. In seed planted individuals, the transition from vegetative to reproductive stage depends on the interaction between the following three factors (Basnizki, 1985): 1) attainment of a critical plant size (usually a rosette of seven to eight leaves) by the seedling; 2) low temperatures; 3) photoperiod. Different cultivars vary in their temperature requirements; normally for the floral induction they require 200-250 hours of temperatures in a range of 7-10 °C (Pécaut and Foury, 1992).

The globe artichoke can be grown on a wide range of soils, but it produces better on deep, fertile, well-drained soils with a pH between 6 and 8. The plant is deep rooted and should be planted on soils that afford adequate area for root development. Where coastal climatic and soil conditions are satisfactory, planting can be also made on gently sloping hills. However, hillside soils usually require more fertilizers and a careful management of irrigation water to be as productive as those that are well leveled.

Globe artichoke is sensitive to salinity and intolerant to prolonged waterlogging. It requires frequent irrigations during its growing period. A deficiency of moisture, particularly during the time of head formation, results in an inferior quality. In temperate and cool climates, irrigation is important for the offshoot spouting to anticipate the production while, in warm climates, it is necessary especially for the reawaken of the early cultivars.

Irrigation can be performed by sprinkling with both mobile and fixed installations. However, the use of local low-pressure drips is becoming widespread, especially where the evapo-transpiration demands are high and the water supplies are limited. The seasonal water

requirements of globe artichoke correspond to 4.000-5.000 m^3 per hectare and per year, with the highest crop water requirement during the head formation (Bianco *et al.*, 1990). Finally, irrigation is performed when the soil humidity reaches 25-30% of the field capacity.

Globe artichokes need an adequate amount of plant nutrients for an optimum plant growth and development. However, after removal of the heads during harvest, the bulk of the vegetative parts of the plant can return to the field as result of a nutrient recycling. Supplemental nutrient demands are usually not too high.

6 Botanical description

Globe artichoke is an herbaceous perennial plant with diploid chromosome complement (2n=2x=34). Seed-propagated plants have hypogeous seed germination and produce a conspicuous, thick, fleshy tap-root apparatus.

During the vegetative growth, the plant produces a rosette of large, deeply lobed or divided pubescent green-grayish leaves attached to a compressed stem. Leaves differ sensibly among cultivars for their margin, color, shape, length, presence/absence of spines (*Fig.4*).

Fig..4.: Differences in the color of the leaves (a: light green; b: green-greyish; c: dark green) and for presence of spines

The base of the stem produces axillary buds from which offshoots (suckers) can grow in a variable number (*Fig. 5*), depending both on the variety and on its attitude to the vegetative propagation. Each offshoot produces adventitious roots that initially are mostly fibrous and thickened and, during the first year of growth, differentiate fleshy storage organs (rhizomes).

Fig. 5: Attitude of the plant to produce offshoots.

In the spring, above the rosette of leaves, an apical or primary bud appears and a floral stem can elongate above 1 m of height. Floral stem induction is influenced both by both temperature and photoperiod and the cultivars differ in their requirements of low-temperature and day length. Secondary, tertiary, and higher-order buds develop on branching stems from leaf axis of the primary stem; the primary terminal bud achieves the largest size, this decreasing sequentially for secondary, tertiary and higher-order flower buds. Each vegetative offshoot produces an erect flower-bearing stem.

The head or *capitulum* is composed by many florets crowded onto a fleshy receptacle and surrounded by a whorl of multiple rows of bracts, thick and fleshy in basal parts and progressively thinner in upper portions; the outermost bracts are large and fibrous while the inner ones are progressively smaller and tenderer. The inner tender portions of the receptacle form the ‘heart’ of the globe artichoke.

A well-developed head can contain some 800-1200 hermaphrodite blue or purple-reddish florets (*Fig.6*). The peripheral florets of the flower progresses centripetally during the subsequent two or three flowering days. The petals of each floret are fused along the base, forming a somewhat tubular corolla. The androceum is composed by anthers joined to form a tube through which the style extends. The anthers release the pollen into the tube, and as the

style elongates, it pushes the pollen upward out of the tube, making it available to insect pollinators. The stigmatic (pollen-receiving) areas of the style are located on two branches of the style tip and these branches separate after elongation. Self-pollination is avoided by the proterandry of the flowers, in fact the stigmatic surfaces mature two or three days after pollen shedding. Ovary is inferior and monovular. Pollen is ivory-colored, strongly glutinous and re-united in small compact masses. Cultivars vary considerably the amount and quality of their pollen. The nectar is collected in the nectariferous bulb (belong the anthers), nectar secretion and bee visits start with anther dehiscence and when the style wilts (Basnizki & Zohary, 1994). Under field conditions, pollen remains viable two or three days. Pollen samples can be kept viable at 2-4˚ C up to eight to ten days (Foury, 1967).

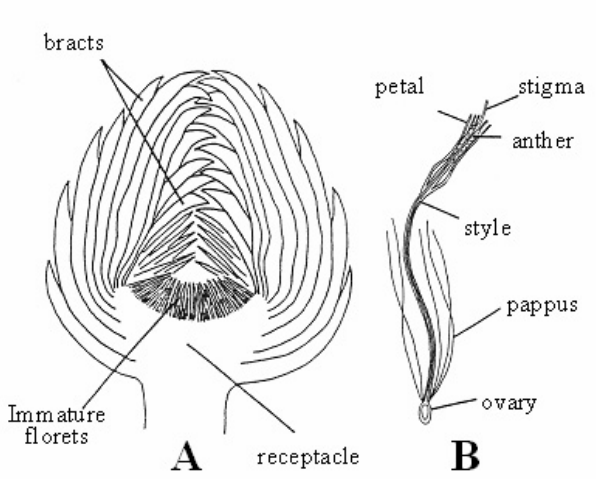

Fig. 6: Characters of the artichoke (A) head with hermaphrodite florets and (B) single floret

The pollination by insects guarantees the allogamy of the species. In the Mediterranean basin, the main pollinator is the honey bee *Apis mellifera* (*Fig. 7*). Xylocopes (*Xylocopa* sp.), osmies (*Osmia* sp.), andrenes (*Andrena* sp), solitary bees and bumble bees also visit the globe artichoke flowers (Pinzauti et al. 1981).

Fig. 7: Globe artichoke pollination by *Apis mellifera*

Seed production per head varies considerably for each variety (*Fig. 8*). Under open pollination, the range observed among cultivars is represented by 150-700 seeds for head (Bianco 1990). Seed-set is also influenced by climatic conditions during flowering. The best results are obtained under dry weather conditions. There is also a marked difference between the seed production of primary heads, and that of secondary and tertiary heads. In well-developed plants, the relatively few primary heads commonly account 50% of seed yield (Basnizki & Zohary, 1994).

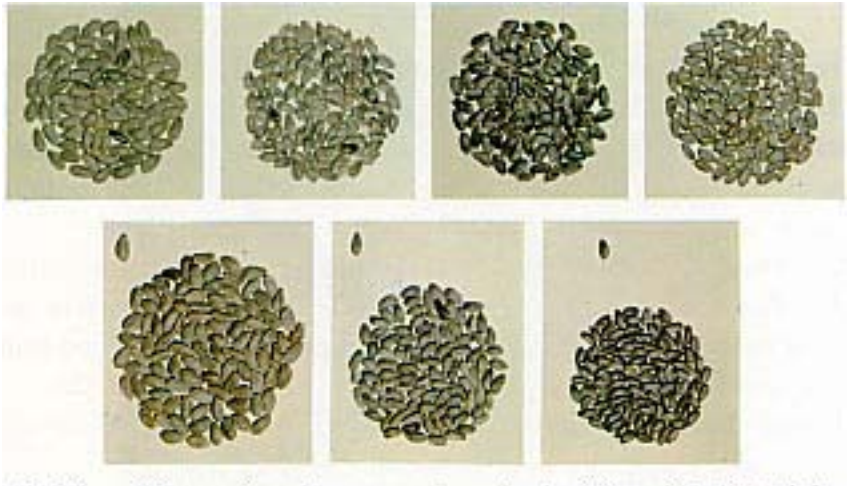

Fig. 8: Differences in color and size of achenes in artichoke varieties.

The fruit is represented by the tipical *Compositae* achene with a prominent pappus that helps the wind dispersion. The 100 seed weight varies in a range of 2,5-7,0 g.

7 Agronomic aspects

Globe artichokes require less fertilizer than other vegetable crops to produce top yields. Fertilizer applications should be made according to the current soil test information. The application rates suggested by Tesi (1994) for Italian globe artichokes are 100-150 kg ha^{-1} of N, 150-200 kg ha^{-1} of P_2O_5 and 150 kg ha^{-1} of K_2O, with a further split application of urea (150-200 kg ha^{-1}) in winter. These quantities should be increased for the long cultural cycle cultivars, due to the increased nutrient uptake by globe artichoke plant. In California, growers apply 112-224 kg/ha of N, 56-112 kg/ha of P_2O_5, 34-112 kg/ha of K_20. In drip-irrigated fields, 34-56 kg/ha of N and half of the P_2O_5 and K_2O are distributed in two bands 5-10 cm apart and about 15 cm below the transplant line. The remaining fertilizer is applied in equal weekly applications throughout the season. Excessive fertilizer reduces yield and quality.

The growth plant regulator gibberellic acid, when applied properly, can increase earliness and uniformity of head development without any significant damage to plant development or reduction of yield (Mauromicale & Ierna, 1995; Garcìa *et al.*, 1999; Calabrese & Bianco, 2000). For annual production, growth regulator treatment should consist of 2 to 3 foliar sprays of gibberelic acid at two week intervals. For perennial productions, gibberelic acid treatments are sprayed on the field 6 weeks before the expected first harvesting.

The globe artichoke field should be leveled for furrow irrigation and the soil well pulverized and smoothed; leveling is not necessary, if sprinkler irrigation is used. In general, transplantation is made manually and, in uncommon cases, mechanically during spring-summer seasons, depending on the choice of the variety and on the climate conditions. Plant density is between 7,000 and 10,000 plants per hectare with 0.8-1 m in row spacing and 1-1.4 m between rows. In USA, wider spacings result in populations of 2,500-3,500 plants ha^{-1}. Direct seeding is a popular method in Southern California deserts, while the non uniform seed germination and the long growing season favor the use of transplants in southern coastal California as well as in other countries.

The ‘bud cuttings’ tend to eliminate the offshoots around the plant; this is a hand-made operation that requires a lot of labor and constitutes 30% of the total cultivation costs. The number of ‘bud cuttings’ can vary between 1 and 3, depending on the choice of cultivar, on the plant age and on the propagation method. The number of the remaining offshoots per plant (1 for Romanesco type and 3-4 for the re-flowering varieties) depends, on the globe artichoke typology. This operation reduces the number of heads per plants, but offers an important advantage for the optimization of the head size.

The ‘stalk removal’ is a common practice for all cultivars and consists in cutting off the top of the plants slightly below the soil level. As result, the artichokes have summer dormancy and are not productive for a short period (about 30-50 days).

Globe artichoke is usually considered a renewal plant. Moreover, the plant should not be planted after another *Asterales*. Due to their disease suppressive properties, broccoli might be considered as a good rotational choice for Verticillium control, when incorporated into the soil at the end of the season. However, market considerations make economically impractical this type of rotation.

Considering all typologies, head production can occur throughout the year, thus making globe artichoke well adapted to climatic conditions and market needs. Depending on market requirements, the heads have to be harvested, with about 10 cm long stems, when the floret primordial are rudimentary, and the heads are fresh, compact, clean, and free from defects such as scratches, bruises, and split bracts. However, not all the heads produced per plant are harvested and marketable; small heads are often used for processing. The sequential head development in each plant makes not feasible the mechanical harvesting of globe artichoke. This could be allowed by the use of some seed-propagated plantings. In general, the temperature can strongly influence harvest frequency.

To retain all qualities, heads must be handled carefully during harvesting, packing-shed and cooling operations. After harvest, heads have rapidly to be stored at 0°C and 90-95% of R.H. (storage potential of artichoke is generally less than 21 days) and to be inspected visually to remove those with insect injuries, mechanical damages, or aesthetic defects. Marketable heads, classified according to their size, must be packed by hand in wax-impregnated, corrugated, paper-board cartons. Heads must be kept refrigerated as they move through the distribution channels. They have a low sensitivity to exogenous ethylene, therefore not considered as an important factor in post-harvest handling and distribution.

8 Biotic stress and weed control

Although considered a rustic plant, globe artichoke is attacked by several pathogens and parasites (Cirulli *et al.*, 2000; Greco *et al.*, 2000; Martinelli *et al.*, 1979; Pasquini and Barba, 2000). Among the pathogens of the aerial part of the plant, *Leveillula taurica*, *Ascochyta cynarae, Ramularia cynarae*, *Bremia lactucae*, and *Botrytis cinerea* must be mentioned. *L. taurica* can be found on the underside of older leaves which can be died, if severely infected. Spores are transmitted from field to field through the air, which makes the spread of the disease difficult to control. No cultural practices have been identified to slow the development of powdery mildew on globe artichoke. Severe outbreaks of wilt, caused by the soil borne root pathogen *Verticillium dahliae*, have been frequently observed on root apparatus in different warm artichoke-growing areas such as South Italy (Ciccarese & Cirulli, 1985), Chile (Fernandez & Bertrand, 1988), and eastern Spain (Armengol *et al.*, 2005). The use of infected stumps could have greatly contributed to the dissemination of the pathogen in

all Spanish artichoke-growing areas, where severe damages have been observed on the cv. Blanca de Tudela as well as on the seed-propagated cv. Imperial Star (Armengol *et al.*, 2005). In general, the disease occurs on a few plants and subsequently spreads throughout the entire field (Cirulli *et al,* 1984) with symptoms that consist in stunting, yellowing, wilting and vessel browning of the stem (Cirulli *et al*., 1994). The strategy for controlling *Verticillium* wilt of artichoke is based on planting on non-infested soils, on the use of disease-free offshoots coming from healthy mother plants, and on long-term rotations with non host-species (Ciccarese *et al*., 1985); the use of resistant cultivars would represent an economical and ecologically sustainable system of control (Cirulli *et al.*, 1994). In the field, *Verticillium* infection can favor the development of *Pythium* and bacterial attacks. Attacks of *Verticillium albo-atrum*, *Sclerotinia sp.*, *Sclerotium sp.*, and *Rhizoctonia solani* could become important under high temperature and humidity conditions (Tesi, 1994).

The viruses, isolated from the artichoke and transmitted mainly by aphids, nematodes, and infected propagation material, are capable of determining symptoms or remain latent, so reducing the growth of the plants (Martelli *et al*., 1979). However, most of the 23 viruses, found in Europe as well as in the Mediterranean basin on this crop, constitutes serious threats to artichoke; a list is reported by Gallitelli *et al.* (2004). Micropropagation techniques for obtaining virus-free artichoke plants are being developed on the "romanesco" and "brindisino" types by Babes *et al.* (2004) and Gallitelli (2006), respectively. Although the damages have never been quantified, the most dangerous viruses are represented by AMCV (Artichoke Mottled Crinkle Virus), for which an efficient controlling method of the propagation material is still required, by ArLV (Artichoke Latent Virus), ACDV (Artichoke Curly Dwarf Virus), TSWV (Tomato Spotted Wilt Virus) and ADV (Artichoke Degeneration Virus) (Lumia *et al*., 2003; Pasquini *et al*., 2004).

Several insects and other pests often restrict the production of globe artichoke in certain areas and require control measures on the part of the growers. Artichoke plume moth (*Platyptila carduidactyla*) is the most serious pest of the perennial crop in California. Economic damages occur when the larvae feed on floral buds, rendering them unmarketable (Bari *et al.*, 1996); if untreated, yield losses could reach 70% (Bari, 1998). Many species of Lepidoptera show trophic activity on globe artichoke in the Mediterranean basin; aphids and larvae of Lepidoptera that dig galleries both in leaves and stems (ex.: *Depressaria erinaceella* and *Gortynia xanthenes*) provoke serious damages to production in South Italy (Ancora,

1986). Other animal parasites are *Larynus cynarae* and *Agromyza andalusiaca* (Tesi, 1994). With regards to animal parasites, remarkable damages caused by the mouses and snails must be remembered.

Weed management is a big problem not only because weeds steal nutrients from the cultivated crop but also because they provide habitat for insect pests, so reducing the efficacy of spray-applied pest control materials. Perennial fields are hosts to a broad spectrum of weeds, including nettle, mustard, chickweed, and oxalis. A possible control system is represented by the chemical one, while there are no known biological controls for weed management in artichoke fields; mechanical cultivation and hand hoeing are practiced. Fields growing annual globe artichokes have the same types of weeds, as above described for perennials. In California, winter weed varieties on the southern coast include shepherdspurse, stinging nettle, malva, groundsel, chickweed, and oxalis; their abatement is done by hand after the pre-plant application of pronamide. In California desert, weeds include goosefoot, purslane, lambsquarter, malva, and watergrass and, on some organically maintained fields, the ground is "solarized" for about 30 days before planting.

9 Genetic resources

The number of globe artichoke cultivars grown in the Mediterranean basin and in other parts of the word cannot be easily determined. A cultivar grown in one location is frequently known by other names in other localities (ex.: about 14 to indicate ‘Catanese’ globe artichoke; Bianco *et al.*, 1990). Therefore, the number of names exceeds that of the actual cultivars. The cultivar composition in Italy, Spain, and France has been extensively studied but it is much less known in other Mediterranean countries.

The number of distinct globe artichoke cultivars (populations, clones or groups of similar clones) seems to be quite small, perhaps only 80-100. Thirty-seven economically important globe artichoke types have been examinated by Dellacecca *et al* (1976). Only 11-12 of them can be considered as being of major commercial importance (Basnizki and Zohary, 1994).

Although the number of cultivars is rather limited, they differ markedly from one another in many traits. Based on the artichoke word collection assembled in Bari (Italy),

Dellacecca *et al* (1976) described the range of variation in 20 different morphological and production traits and prepared a set of descriptors for the variation found.

The artichoke world collection in Bari was also subjected to a detailed study of cultivar divergence by multivariate analysis (Porceddu *et al* 1976; Vannella *et al,*1981). The analysis showed that the majority of the accessions analysed fall into the following four main groups:

A) 'Spinoso', characterized by the presence of long sharp spines on the bracts and leaves;
B) 'Violetto', with medium-sized, violet-colored and less spiny heads;
B) 'Romanesco', with spherical or sub-spherical non-spiny heads;
C) 'Catanese', with relatively small, elongated and non spiny heads,

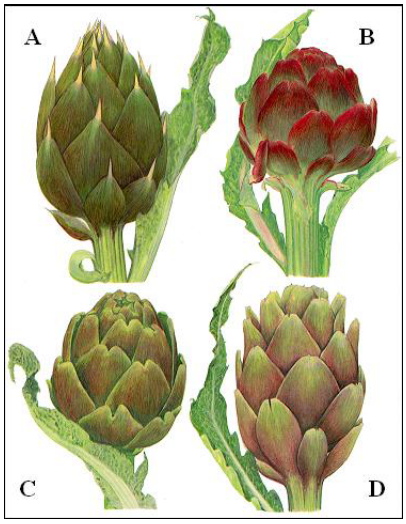

Fig. 9 - Typologies of globe artichoke cultivated in Italy.

On the basis of the harvest time, the same germplasm may also be distinguished as early or 're-flowering' (ex.: 'Spinoso sardo', 'Spinoso di Palermo', 'Violetto di Sicilia'), if produces heads between autumn and spring after watered "ovoli" during summer, and as late (ex.: 'Romanesco', 'Violetto di Toscana'), if produces heads only during spring and early summer (Mauromicale, 2000). In recent studies on AFLP molecular markers (Sonnante *et al.*, 2002; 2007; Lanteri *et al.*, 2004b; 2006; Crinò *et al.*, 2007;), consistent agreement between genotypic and phenotypic classifications based on head characters has indicated that the cultivated morphotypes play an important role in determining variation within the species.

Other cultivars (ex.: Spanish cv. 'Tudela') have been considered to belong to an intermediate group (Dellacecca *et al.*, 1976; Porceddu *et al.*, 1976), while French cultivars have been categorized as (a) 'Brittany' types with large green heads (ex.: Camus de Bretagne, Caribou, Camerys), these including a purple variety so named because of its truncated and spherical shape; (b) 'midi' types with violet leaves coming from South France (ex.: Violet de Provence. Violet de Hyères); (c) 'secondary' types (ex.: Blanc Hyerois) that are intermediate between Camus and the purple variety (www.innvista.com). In the Central Coastal Region of California, perennially grown "Green Globe" is the dominant variety, but annual varieties are grown there as well; in other regions, only various annual varieties, these including "Imperial Star", "Big Heart", and "Desert Globe" are generally grown.

10 Propagation system

- AGAMIC

In general, globe artichoke is vegetatively propagated via 'ovoli' (underground dormant shoots), offshoots or suckers, stumps or dried shoots. Growers find advisable to renew every 2-3 years artichoke plantings because, after a number of years of growth and re-growth, the roots area become congested and the plants lose the vigor.

Generally the techniques for the production of 'ovoli' and shoots are not particularly specialized. They come from plants destined to the production of heads. This fact reduces significantly the plant multiplication potential (Mauromicale *et al.*, 1986).

The low rate of multiplication is due to the inhibition role of apical dominance, which allows the lateral buds growing only after the differentiation of the main head, which corresponds to the beginning of apex degeneration. Thus, the first buds play a strong competitive role against the others that start the differentiation, slowing down their development. Therefore, apical dominance and competition among buds are liable for the different time of growing of the buds and, consequently, for the morphologic and physiologic heterogeneity of the propagation material used for transplanting.

1) Shoots

Shoots come from rhizome buds. At the beginning of the season, globe artichoke plants can produce 8-10 shoots depending on their genetic attitude. Normally 5-6 of them are

valid for transplanting while the others are removed, living only one (for the "Romanesco" type) that will constitute the epigeal part of the new plant. A good shoot will have an adequate number of roots and 4-5 leaves with entire blade; the leaf trait seems to be associated with a higher and earlier production of heads (Elia and Miccolis, 1996). In order to improve the yield property of plants, Eser *et al* (1996) verified the effects of decapitating rooted shoot before planting to increase total yield (Eser *et al* 2005).

This propagation system is widely used from autumn to spring; the percentage of rooting depends on the original material. If the transplanting is made in autumn, plants will start to produce only during the second year (Bianco *et al*., 1990).

2) 'Ovoli'

The 'ovoli' are dormant branches inserted on the rhizomes that interrupt their development because of high temperatures stress and water deficit. They are 13-14 cm long with a cylindrical shape and have one apical and various lateral buds. One plant can produce more than 20 'ovoli' but not all are adequate. Apical bud elimination increases the production of 'ovoli' and their size.

The 'ovoli' are collected from the mother plants at the end of July. They are disposed in straw layers and made pre-sprouted in warm-humid conditions, so allowing the radical system development. After one week, they are ready to be transplanted (Bianco *et al*., 1990).

3) "Zampe"

The stumps are harvested from commercial fields at the end of the production cycle and then are divided in portions called "zampe" (=legs). Every "zampa" has got roots and an adequate number of buds. This is a propagation system that is not spread anymore because it gives heterogeneous plants with a lot of phytosanitary problems.

Although vegetative propagation is economically advantageous, the potential for the spread of diseases (attacks of nematodes, fungal pathogens, and viruses), leading to plant damages and significant economic losses, is very high (Ancora *et al*., 1981). The effectiveness of this procedure is very limited and constitutes a serious obstacle to the development of globe artichoke as a modern crop. In 2004, Mauromicale *et al.* developed a nursery procedure for producing globe artichoke plantlets; the planning consisted of three phases: 1) obtaining offshoots of the same age from the mother plant, raised in the nursery, by

removing the apex and epigeal part of plant; 2) preparation of the offshoots for transplantation; 3) offshoots transplantation and plantlet production in the nursery. This technique, increases the percentage of plantlets surviving from offshoot transplanting, reduces the time of plantlet production, allows to obtain plantlets during all the year so making more elastic the cultivation calendar (planting time).

In 2003-2004, Morone- Fortunato confirmed the influence of mycorrhizal inoculum (*Glomus viscosum* A6) in the plantlet rooting ; an effective mycorrhizal symbiosis between fungi and plantlets of globe artichokes is possible. Mycorrhized plantlets are more efficient for nutrient acquisition and are better equipped to cope with transplanting stress (Morone-Fortunato *et al*., 2006).

4) *In vitro* culture

Although expensive, *in vitro* propagation of globe artichoke has solved many of the disease problems related to the traditional vegetative propagation, especially on 'romanesco' type (Ancora, 1981; 1986). Researchers are under-going for the re-flowering varieties (Harbaoui, 1982; Morzadec and Hourmant, 1997; Morone *et al.*, 2005). A vegetative propagation technique, preserving *in vitro* propagation advantages (disease-free plants, plant uniformity) and reducing plant costs to growers, was developed by Cardarelli *et al.* (2005a e b). This technique involves the following steps: 1) at the end of summer, transplantation of pathogen-free micropropagated plantlets under soiless culture and their growing all the year-round under greenhouse conditions; 2) periodical treatment of stock plants by chemical growth regulator [6-benzylamino purine (BA)] and 'cutting back' of the plant at collar level to promote offshoot production; 3) periodical harvesting of offshoots that are cold stored; 4) at the end of spring, rooting of cuttings under high humidity conditions in multi-pack trays to be ready for further summer transplantations. The same technique has been also applied to obtain virus-free plants of globe artichoke (Babes *et al.*, 2004).

- GAMIC

Globe artichoke can be also propagated by seeds; it is possible to develop from the clones currently cultivated new genotypes (inbred lines, F_1 hybrids, and synthetic varieties) with a different genetic structure. They are able to express their productive potential since the first year. The F_1 hybrids can benefit from heterosis, which is responsible of more vigor, earliness and productivity of the young plants (Tesi, 1976).

Seed-planted cultivars have the following major advantages:

1) Compared to sucker or meristem-produced plants, mechanical seed sowing is a labor saving and therefore a cheaper operation; in 1987, Basnizki and Zohary reported that it takes about 4 to 5 working days to produce 1 t of fresh heads from annual seed planted cv. Talpiot, against 11 from perennial vegetatively propagated clones.
2) In a Mediterranean climate, and if sown early enough, most artichoke genotypes bolt fully the following production season. Thus, by shifting to seed-planting, this perennial plant can be converted into an annually grown crop, which may therefore be introduced into crop rotation. The time spanning from seeding to the end of the harvest is represented by seven to eight month.
3) Direct seeded plants develop long vertical tap roots, which penetrate into the soil deeper than the adventitious roots produced by planted suckers. Therefore, compared to vegetatively propagated plants, they utilize more efficiently both moisture and fertilizers (Foti *et al.,* 2005).
4) For pest and disease control, the shift to seed-planting prevents the transfer and spread either of soil-borne and other pathogens (such as bacteria and viruses) or of pests. Such contaminations are common when vegetative propagation is implemented to start new plantations. Seed planting also prevents virus infections, because most plant viruses are not transmissible via seeds. Thus, annual cultivation, coupled with crop rotation, protects the grower from the ravages of pests and epidemics much better than perennial planting.
5) All these elements contribute to more vigorous plant growth, healthier plants, and lower input of pesticides, fungicides and fertilizers. So, seed-planting makes globe artichoke a valid rotation choice in an organic cultivation system.

Most of the globe artichoke cultivars are highly heterozygous. Those commercial varieties that are propagated by cloning a single selected genotype can show in their yield either additive or dominance-epistatic effects (Hayward & Bresse, 1994). Therefore, when they have been propagated by seed, the yield of their progeny is frequently different from that of the parental generation. The market requires homogeneous heads of commercially acceptable uniformity from open-pollinated seed propagated varieties as well as from inbreed lines and F_1 hybrids. At the present time, seed for varieties of good quality and uniformity is scarce in the globe artichoke market. Consequently, the production of seeds for high yielding varieties is an important objective of globe artichoke breeding programs.

The researches on the development of seed-planted cultivars started in France in mid ‘60s and the biggest progresses were made in 90’s by Israeli investigators (Mauromicale, 1994). Yehuda Basnizki is the father of Israeli seed-planted varieties, and he built the basis of artichoke genetic improvement for seed production.

The majority of seed-planted varieties were produced during the last 25 years in Israel and United States. The first one was Talpiot at the beginning of ’80s. This inbred cultivar was obtained after five generations of self pollination and is characterized by green globose heads and a late maturation (Basnizki and Zohary, 1987). Actually, there are others inbred and open pollinated seed-propagated cultivars. The most important are:

- Imperial Star (commercialized in Italy by the seed company SEMIORTO Sementi srl with the name “Istar”) is one of the most uniform and specifically bred for annual production; in fact it is really precocious. It produces round, big, spineless, green heads, and is harvested 150 to 160 days after sowing.
- Emerald is a high yielding, medium to late variety that is about 2 weeks more delayed than Imperial Star. The heads are large globe in shape, harvested in about 190 -210 days.
- Green Globe is an old variety that can be grown as either an annual or a perennial crop. Plants grow up to 1.5-2 m tall and 3 m wide. It produces green-purple globes 7.5-8 cm in diameter.
- Colorado star has a similar size or is slightly smaller than Imperial Star; this variety cannot be sold commercially at present.

These varieties are produced and commercialized in United States by the Paramount Seeds Inc. They are protected by PVP (Plant Variety Protection Act) and may not be multiplied. The limit for the seed companies on the commercialization of inbreed and free pollination cultivars is that they can be easily reproduced and stolen. The best solution is the production and commercialization of F_1 Hybrids.

In the ‘80s, HU #044, HU 137, and HU #223, all similar to cv. Talpiot, were released to growers (seed producer: Hazera Seed Co., Israel). In addition, HU #271 was commercially developed by Nunhems Zaden BV with the name “Orlando”; it is characterized by elongated, purple and small heads. Nunhems is the most important seed company for the development and the commercialization of globe artichoke F_1 hybrids such as Opal, Concerto, Tempo, harmony, Madrigal.

The biggest problem of these hybrids consist in their their low capability of adaptation to different environmental conditions and in the different demands of Italian globe artichoke market, which is the most heterogeneous both for varieties requiring and production calendar.

In the last 15 years, a lot of researches on these hybrids has been started, not only in the countries where the globe artichoke is traditionally cultivated but also in others in which this species is still little known (Pesti *et al*., 2004; Bucan *et al*., 2005; Halter *et al*., 2005; Jani *et al*., 2005).

At present, researches on F_1 hybrids are aimed mainly at optimizing the cultivation techniques, evaluating the yield along with the production quality, the biochemical composition, and the capability to be processed (Calabrese *et al.* 2005a, b). Some of the researchers focused their attention on the effect of transplant age and type on the growth, the harvest time and the yield of seed propagated plants (Mauromicale, 1994, Tesi e Lenzi, 2005; Baixauli *et al*, 2007) as well as on the ecophysiological and crop yield responses to different levels of fertilization and water supplies (Foti *et al* , 2005; Paradiso *et al* , 2007)

The effects of treatments with giberellic acid GA3 on harvest time and yield quality of seed propagated plants has been carefully investigated (Mauromicale and Ierna, 1995; García *et al* 1999; Calabrese and Bianco, 2000; Esteva *et al*., 2004; Paradiso *et al* , 2007; Miguel *et al*, 2004, Schrader 2005; Maroto, 2007). In fact, all the cultivars seed propagated produce, without any GA3 treatment, at the end of winter and during spring depending on the transplanting date (summer or beginning of autumn) (Basnitzki e Goldshmidt, 1994). Studies on new technologies for the development of F_1 hybrids, their seed production and the evaluation of new seed propagated cultivars were made by Gil Ortega, (2004) and Stamigna *et al.* (2004).

The most important step for the production of new F_1 hybrids consists in optimizing the technique of seed production, minimizing the costs of the manual operations. In 1967, Foury proposed a pollination system for selfing the plants consisting in the following steps:

1. covering of the heads with isolation tissue before the beginning of flowering to protect them from the open pollination.
2. collection of the pollen with a brush;
3. storage of the pollen at the temperature of 2-3˚C until the stigmas are ready;
4. self pollination with the brushes;
5. repeat of the operations, living covered the heads, until the end of flowering.

For the cross pollinations, in 1974, Perrino and Parducci proposed to wash the stigmas, to clean them from the auto pollen, although Pouchard *et al* (1967) already demonstrated that this practice is not useful.

As alternative, to eliminate the problem of self-pollination and decrease the costs of manual operations for hybrid seed production, it might be useful to recur to malesterile lines as female parents. A strategy to produce F_1 hybrids using malesteriles plants, proposed by Basnizki and Zohary, in 1994, is based on the following steps:

1. extraction of uniform, homozygous lines from commercial clonal cultivars by successive self pollination and selection;
2. testing of the combining ability of inbred lines;
3. incorporation of genetic malesterility into some of the inbred lines to obtain female parents;
4. qualitative and quantitative testing of F_1 hybrids combination among inbred lines.

11 Genetic improvement

- Heterozygosity, inbreeding, gigantism

The process of domestication has often favored plants capable of expressing traits associated to production; this implies that different end uses of each crop have oriented the domestication of that plant (Gepts, 2002). When the part used of the plant is not the seed, human selection has often favored the maintenance of high levels of heterozygosity (Zohary and Hopf, 2001; Hancock, 2004) and the affirmation of specific QTLs. In a vegetatively propagated crop like globe artichoke, this has meant that clonal multiplication of plants shows at the same time desired Mendelian (e.g. absence of spines) and complex (e.g. big heads) traits (Porceddu et al., 1976); these latter traits might have high levels of heterosis (Hammer, 1988; Balloux *et al.*, 2003). The high level of heterozygosity in artichoke has been demonstrated by Basnizki and Zohary (1994) who reported that selfing of clonally propagated artichoke varieties leads to a high level of morphological segregation in offsprings, accompanied by considerable inbreeding depression. Pecaut (1993) reported that commonly the fourth and fifth selfing generations seem to be a practical compromise to obtain vigor, seed production, and homogeneity.

Progenies of globe artichoke and wild cardoon crosses generally show a high degree of variation for many quantitative and qualitative characters (Portis *et al.*, 2006; Sonnante, Pignone and Hammer, pers. commun.), thus confirming that modern cultivars of artichoke retain a high degree of heterozygosity (Sonnante *et al*., 2007b). Investigations based on molecular markers such as simple sequence repeats confirm this (Acquadro *et al.*, 2005; Sonnante *et al.*, 2006). A great deal of variation has been observed within artichoke germplasm for agronomic characters, mostly regarding the heads (color, shape, weight,, lower number per plant, presence/absence of spines on bracts, flowering time as early = reflowering vs. late, etc.), while the vegetative part of the plant generally shows a lower level of variation (Dellacecca *et al.*, 1976). Vegetatively propagated crops are easily and quickly domesticated (Gepts, 2002); this also can account for the high degree of variation present in globe artichoke germplasm.

Conversely, the level of variation observed in leafy cardoon is quite limited and only few landraces of this crop are known (Sonnante *et al*., 2007b). These cardoon landraces differ slightly for minor characters relevant to domestication, such as the size of leaf stalk, which represents the edible part of this crop. Moreover, during domestication, the average number of heads per plant has slightly increased compared with wild cardoon (Dellacecca, 1990; Portis *et al.*, 2005). Within this framework, the gigantism observed for head traits in globe artichoke and for leaf traits on leafy cardoon might be due to the action of a limited number of QTLs. Fixed heterosis cannot be underestimated in artichoke (*Fig. 10*), as data from molecular markers confirm (Sonnante *et al*., 2006).

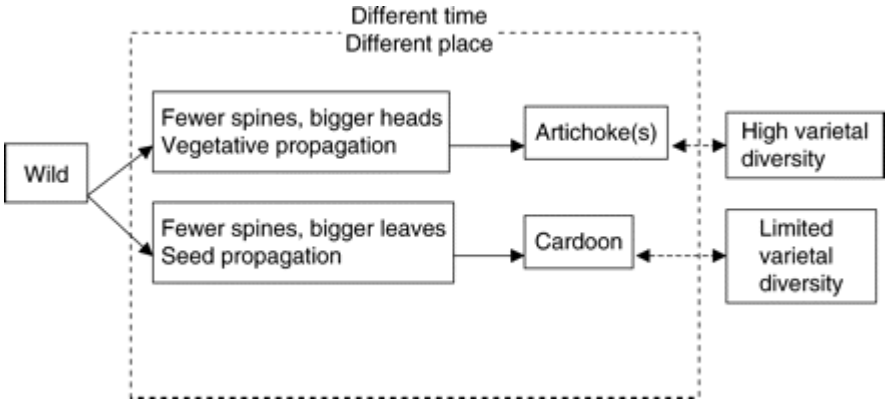

Fig. 10- Diverging domestication patterns in artichoke and leafy cardoon
(from Sonnante *et al.*, 2007b)

- Breeding

Globe artichoke breeding history is limited to a few studies on the inheritance of few main characters (Pécaut, 1993; Lòpez Anido *et al.*, 1998). Head size, shape and weight, plant size, peduncle length, and earliness have a polygenic determinism, while absence/presence of spines (*Sp*/*sp*) together with "yellow leaf" (*j)* and "white flower" (*b*) are recessive mutations. The genetic basis for the anthocianic head, also influenced by the temperature, is made quite complex by a series of modificator genes added to 1 or 2 major genes (Bazniski and Zohary, 1994). Also the male-sterility determinism has been studied. Principe (1984) reported that male sterility is governed by a single recessive gene designed as ms_1, which he detected in globe artichoke material grown in California. Bazniski and Zohary (1994) discovered two additional non-allelic recessive male-sterility genes, ms_2 and ms_3.

Breeding of globe artichoke has been largely hindered by the fact that it is a cross-pollinated species. At present, commercial production is mainly based on the cultivation of heterogeneous clones. Homogeneity of plant material has been obtained by optimizing tissue culture procedures for the micropropagation of 'Romanesco' (Ancora, 1986), 'Violetto' (Morone *et al.*, 2004), and 'Spinoso sardo' typologies (Tavazza *et al.*, 2004) as well as for the production of virus-free plantlets of 'Romanesco' (Babes *et al*, 2004; Barba *et al*, 2004) and 'Brindisino' globe artichokes (Gallitelli *et al.*, 2006). Seed-propagated cultivars are becoming popular in some parts of the world and, at present, limited results have being obtained by use of male-sterile clones with Italian locally adapted germplasm (Stamigna *et al.*, 2004). In globe artichokes, cross-fertilizations are promoted by protandry and are able to determine high heterosis, expressed clearly in biomass and production.

Breeding programs have been based on (I) clonal selection within landraces, (II) hybridizations or selfings followed by clonal selections to vegetatively propagate, and (III) obtention of F_1 hybrids or inbred lines to seed-propagate. Variability was also induced by mutagenesis programmes (Scarascia Mugnozza, 1967; Stamigna *et al.*, 2005).

After 2000 years, farmers are looking at modifying artichoke from a garden to a field crop (Hammer, 2003); the potential of seed-propagated artichoke appears to be high, especially in a period of global climatic change in which perennial cultivations face important problems, like the increase of salinity in irrigation water due to the rising aridity in lower

latitudes, where production of artichoke is economically important (Bianchimano *et al.*, 2005).

The price to be paid for these advantages consists in a reduction of the genetic base of artichoke. Currently, artichoke germplasm cultivated on a small scale is differentiated into many local varieties that have some economic potential. These varieties differ not only in head characters, but also in production physiology and other possible useful traits. One or few seed-propagated varieties of similar value should become available in the future but the use of all this local traditional germplasm appears threatened by genetic erosion (Hammer, 1984).

Many efforts are in progress to safeguard and recovery these precious local genetic resources. Due to the great genetic variability found within several cultivated populations, the term 'varietal type' seems more appropriate than 'variety' to define the accessions of germplasm at present in cultivation (Lanteri *et al.*, 2004). Assessment of genetic variation within autochthonous populations from Sicily and Tuscany has been reported by Portis *et al.* (2005b) and Romani *et al.* (2006), respectively. Within traditional populations of 'romanesco types' (ex.: "Campagnano" and "Castellammare"), a certain variation has also been recently described (Crinò et al., 2008; Pagnotta *et al*., 2007; Trionfetti Nisini *et al.*, 2007). In the last decade, molecular markers (RAPD, AFLP, ISSR, and SSR) were developed and then utilized for characterizing globe artichoke autochthonous Italian populations (Acquadro *et al.* 2003; 2005a; 2005 b; Lanteri *et al.,* 2001; 2004b; Sonnante *et al.*, 2002; 2003; Mauro *et al* 2008). Construction of genetic maps are in progress utilizing segregant populations coming from inter- or intraclonal hybridizations (Lanteri *et al.,* 2004a and in press; Pagnotta *et al.*, 2004). There are not researches reported in literature, on the analysis of globe artichoke F_1 hybrids with molecular markers.

11 Male sterility for F_1 hybrid development

The failure of a plant to produce functional gametes or viable zygotes under a given set of environmental conditions is known as sterility. The term "sterility" covers all cases of infertility resulting from irregularities on the sexual reproductive systems. This infertility is differentiated from incompatibility, in which there is inability of genetically identical viable gametes or nuclei to undergo karyogamy and fertilization; the same gamete may successfully unite with genotypically different partners. Sterility due to failure of plants to produce

functional anthers, pollen or male gametes is called male sterility. Of the two sterility types such as male and female sterility, male sterility is more prevalent and is a stabler genetic system.

Male sterility plays an important role in plant breeding for the production of hybrid seeds and as a tool to facilitate population improvement, backcrossing, test-crossing for combining ability, interspecific and intergeneric hybridization and other intermediate breeding procedures.

An effective hybridization technique permits the production of hybrid seeds in a quantity such that the F_1 generation can be grown directly by the farmer. Male sterility is used for the production of commercial F_1 hybrid seed, especially for species that have little flowers, difficult to emasculate, few seeds for flower, and when the price of the F_1 hybrid seed does not justify the cost of manual emasculation (i.e. allogamous species like onion, fennel, carrot).

- Male sterility origin

Genetic male sterility is of a wide occurrence in flowering plants and arisen as spontaneous mutant in the majority of the sterile plants; only few of them have been induced by mutagenic treatment. The repeated appearance of genetic male sterility in many plants species is a regular phenomenon; mutation of *Ms* to *ms* genes is of high frequency. This is evidenced by the existence of a vast number of *ms* genes in the cytogenetically intensively investigated species, like maize, tomato, belt pepper, and barley.

The *ms* genes have arisen in high frequency both in self- as well as in cross-pollinating species, and more recessive *ms* genes may be detected if cross fertilized species are inbreed or sub-divided into smaller populations. In the majority of plants, genic-cytoplasmic male sterility is originated by intergeneric and interspecific hybridizations. In fact, a specific combination of cytoplasmic "*c*" genes and nuclear '*fr*" genes is necessary for the expression of this male sterility type. Thus, genic-cytoplasmic male sterility is more frequent in the allogamous species.

Male sterility is detected by germplasm cytogenetic analysis but, if it is not found or if it is not stable or linked with undesired genes, male sterility can be induced by mutagenesis with both chemical or physical agents.

• Male sterility type

There are a lot of classifications of male sterility but the most used is that proposed by Kaul (1988) because it includes all the possibilities (*Fig. 11*). According to this classification, male sterility is divided in genetic or non genetic and it may arise either spontaneously or induced by chemical treatments, environmental manipulations or physiological and biochemical alterations.

✓ Structural Male Sterility. - Sterility is due to structural anomalies predominantly in male sex organs, and very rarely in female organs. The male reproductive organs are either completely absent or deformed, mis-formed or mal-formed so that no microsporigenous tissue is developed or microsporogenesis fails to occur completely.

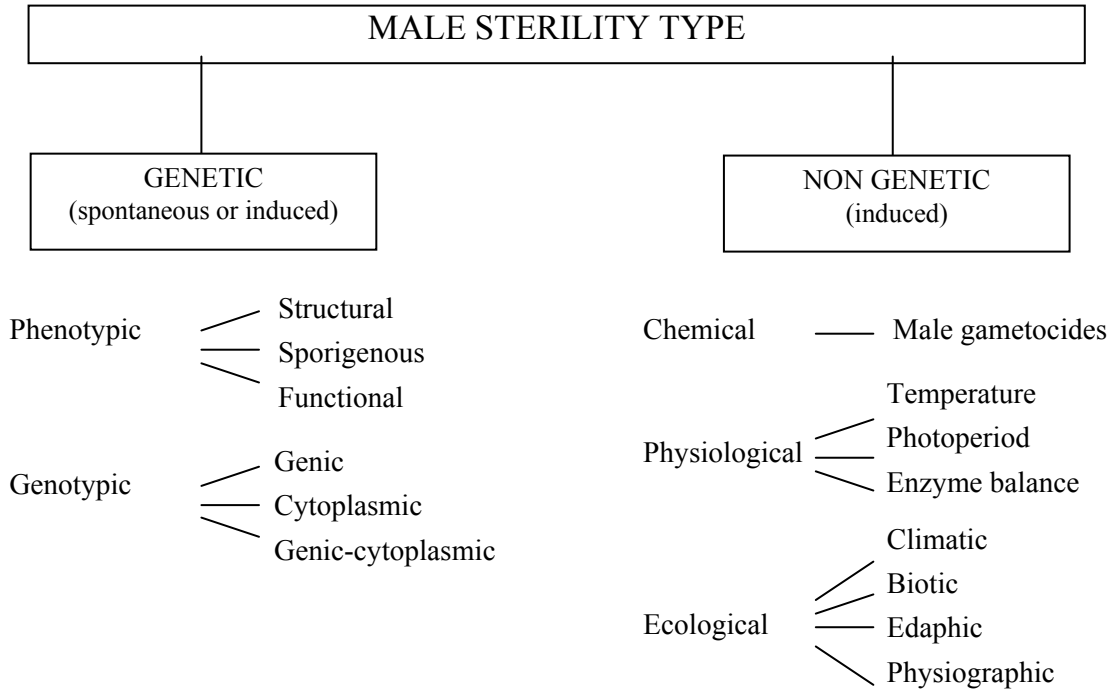

Fig. 11 –General classification of male sterility (Kaul, 1988)

The genetic male sterility can be also classified on the phenotypic and genotypic basis as follows:

✓ Sporogenous Male Sterility. - The stamina develop but the sporogenous tissue is either mis- or mal-formed or develops normally, but microsporogenesis or gametogenesis is

impaired so that either non-, mis- or mal- formations of pollen or its premature abortion occur in otherwise normal homomorphic flowers. Hence, the pollen is either completely absent or non functional or extremely scarce. Abortion of microsporigenous cells may take place before, during or after the meiosis. Thus, it includes predominantly premeiotic, meiotic and post meiotic male sterile mutants.

- ✓ Functional Male Sterility. - Viable pollen is formed but it is incapable of fertilization due to some barriers that render it unable to reach its own or foreign stigma. The main barriers are anther indehiscence, faulty or no exine formation, inability of pollen to migrate to stigma or to affect fertilization.

Genic Male Sterility. - Sterility is controlled by nuclear genes whose action is not influenced by the cytoplasm. Therefore, inheritance patterns and expression of the sterility are entirely of Mendelian type. It exhibits minimum reciprocal differences and is conditioned by recessive genes in the majority of the cases (*Fig. 12*). Expression of male sterility may be the result of the interaction of those genes with nuclear epistatic or inhibitors genes (Lewis, 1941)

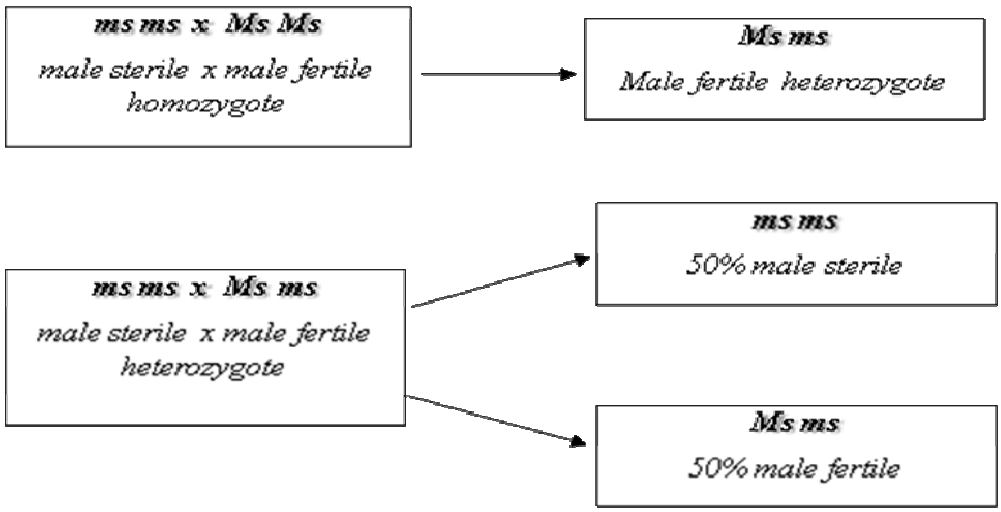

Fig. 12– Genic male sterility

- ✓ Cytoplasmic Male Sterility. - This sterility, unlike the genic type, is controlled by a specific male sterility-inducing cytoplasm known as sterile (S) cytoplasm. Thus, at least two cytoplasm types exist exhibiting male sterility, normal fertile (N) and sterile (S). Irrespective of nuclear genotype, the plants having S-cytoplasm are male steriles and those with N-cytoplasm are male fertiles. Thus, the influence of the nuclear

genotype on this type of S-cytoplasm is negligible and the sterility is inherited maternally.

- ✓ Gene-Cytoplasmic Male Sterility. - The male sterility results from the cooperative action of certain particular nuclear genes designed as *fr* genes and a specific sensitive cytoplasm type termed as S-cytoplasm. Plant species exhibiting gene-cytoplasmic male sterility possess both *Fr* or *fr* nuclear genes as well as N- and S-cytoplasm type. The cytoplasm-specific *fr* genes are ineffective to produce male steriles in a N-cytoplasm but, in a S-cytoplasm, the combination of *fr* nuclear and S-cytoplasm leads to male sterility. The S-cytoplasm possesses a specific type of cytoplasmic genes termed as *c* genes which, in association with *fr* genes, cause male sterility. Combination like N $_{frfr}$ or S $_{Frfr}$ are male fertile. Since the *Fr* nuclear gene interacts specifically with S-cytoplasm plants and revokes male fertility in S-cytoplasm, these genes represent the fertility-restoring *Fr* genes.

A lot of fertility-restoring genes have been found in the cultivated plants. Sometimes those coexist in the same parent but independently segregate and do not interact with their individual expression (Evenor *et al*., 1984). The interaction between "*ms*" genes and S-cytoplasm has never been found.

Globe artichoke male sterility is still not clearly explained. There are only two papers about inheritance of this character in globe artichoke (Principe, 1984; Basnizki and Zohary, 1994), and they reach different results (one or two recessive genes).

The morphohysiologic structure of the flowers was studied in France. Lannunzel (1999) tried to find some phenotypic or functional differences between male sterile and male fertile plants, but did not get any clear result. Morrison *et al.* (1999) studied the pollination of globe artichoke by honey bees (*Apis mellifera* L.) and demonstrated that bees are attracted in a different way by different globe artichoke varieties flowers. The number of seeds per plant produced by bee pollination was lower in male sterile genotypes than in the male fertile ones but some fertile lines were also found to be unattractive to insects. Differences in attractiveness among these lines were supposed to be of genetic origin and probably resulted from differences in nectar availability or composition (Chatelet, 2005).

EXPERIMENTAL PART

INTRODUCTION

Globe artichoke gives an important contribution to the Mediterranean agricultural economy.

Recently, in the Mediterranean countries, where this crop was first grown and its cultivation developed and increased over the centuries, production has remained stagnant since the end of last century. This crop still has several problems that make the cultivation not specialized and rationalized as a modern crop. Hammer (2003) defined globe artichoke as a "garden crop" in order to emphasize that agronomical practices used in its cultivation do not allow the crop to become as a "field crop". The principal problems related to the development of globe artichoke as a modern cultivation consist in:

- the irregularity of the production and the commercialization calendar; in Italy, 50% of the commercialization is mainly concentrated in the period February-April (Sportelli 2001);
- the traditional vegetative propagation system, which requires expensive agronomical practices and does not guarantee well- being propagation material.

The activity of globe artichoke genetic improvement has relatively recently started, this especially due to the studies of globe artichoke floral biology that were acquired and developed only during the last thirty years (Jannaccone, 1967)

No intensive breeding programs have been carried out for globe artichoke, in contrast to what done for other horticultural crops. The only real novelty in this sense is represented by the development of few seed propagated hybrid lines (Basnizki and Zohary, 1994). After Principe (1984), the male sterility was used to enable the production of many experimental F_1 hybrids. In fact, the use of male-sterility in the process of F_1 hybrid constitution reduces the hybrid production costs because it eliminates the long and expensive operation of floral emasculation. To date, F_1 crosses proved interest and further development of the program was taken by Nunhems seed company (Foury *et al*., 2005). However, there are some remaining problems which still hinder the spread of F_1 hybrid seeds in globe artichoke: (i) the correct management of the cultural cycle for seed-propagated plants, (ii) the adaptability of the new F_1 hybrids to the different cultivation environments and to the different production and commercialization calendars, and (iii) the economical production of F_1 hybrid seed because the adequate pollination of male-sterile plants still remains difficult to achieve.

Moreover, two new horizons have been opened for this vegetable in other parts of the world: one in the east (China) and the other in the west (South America). The rapid

expansion of this crop in completely new areas could be favoured by the use of seed, which allows a large number of plants to be grown in a short period of time; this is in contrast to the use of traditional vegetative propagation systems, which limit the number of new plants (Macua, 2007). Therefore, a better knowledge of artichoke genetics is a prerequisite to plan new strategies for breeding this crop. There has been a growing interest for artichoke genetics, as testified by the increasing number of scientific publications produced in the recent years in this field (Sonnante *et al.,* 2007).

The most important studies on the use of F_1 hybrids and seed propagated varieties of globe artichoke in the previous decade aimed at evaluating:

- the agronomic behavior, yield and quality of the seed propagated *cultivars* in Spain (Baixaulli *et al*., 2007a) and in Italy (Calabrese *et al*., 2005a; Calabrese *et al.,* 2007a);
- the effect of some cultural practices on yield (Foti *et al.*, 2005; Bucan *et al.*, 2005);
- the effect of the giberellic acid application to the modification of the calendar cultivation (Mauromicale and Ierna, 1995; Mauromicale *et al.*, 2005; Calabrese *et al.*, 2007b; Baixaulli *et al.,* 2007b; Condes *et al*., 2007);
- the effect of some cultural practices on yield and quality of the seed (Jani *et al.*, 2005; D'Amato *et al.,* 2007a);
- the evaluation of seed germination percentage and the effects of some treatments like osmoconditioning and temperature in the germination of globe artichoke seed (D'Amato *et al.*, 2007b).

In Spain, some researches were carried out on breeding with the aims of improving earliness in some seed propagated cultivars (Gil and Villa, 2004). The only studies focused on genetic improvement for seed production were made by INRA and BBV (Foury, et al 2005), in France, and by University of Tuscia and ENEA (Stamigna *et al.*, 2004), in Italy. All the researches were connected with studies on morphological, histological and functional differences between male-fertile and male-sterile floral structures (Lannunzel, 1999; Chatelet, 2005), and also on the differences of the level of attractiveness between the flowers of male-fertile and male-sterile genotypes.

Up to now, no references of researches on the application of molecular markers on breeding activities for the constitution of F_1 hybrids have been recovered in literature. F_1 hybrids were used by Lanteri *et al.* (2006) to generate a linkage map of globe artichoke based on AFLP, S-SAP, M-AFLP and microsatellite markers.

Moreover, in the globe artichoke cultivation, the use of local varieties propagated with the traditional vegetative system hindered the development of a nursery technique. In

Italy, only in the last ten years, a globe artichoke nursery technique began to develop. The more advanced studies for the development of nursery techniques concern the application of micropropagation systems (Caldarelli *et al*., 2005), the rationalization of the vegetative propagation system (Mauromicale *et al*., 2004), and the use of mycorrhization for adaption of the plantlets to the greenhouses conditions (Morone Fortunato *et al*., 2005); a rationalization of a nursery technique for seed propagation not yet started.

For the development of globe artichoke as a modern crop, it is important to produce new stable seed propagated hybrid lines, but also to study all the techniques necessary for the rationalization of the cultivation system.

OBJECTIVES

1. Development of stable male parental lines for the production of globe artichoke F_1 hybrids by
   - evaluating the clones already available;
   - improving their stability after self-pollinations and sib-crosses;
   - evaluating the homogeneity of their self and sib-crossed progenies;
   - evaluating their ability to produce pollen (hybrid seed production);
   - evaluating the stability of the F_1 hybrids.

2. Development of stable female parental lines by
   - evaluating the clones already available;
   - finding new male-sterile clones.

3. Deeping of the knowledge on the morphological and functional differences between male-sterile and male-fertile globe artichoke flowers.

4. Development of homogeneous F_1 hybrid lines ready to be commercialized by
   - crossing the male-sterile and male-fertile lines already available;
   - crossing the new parental lines obtained during the PhD program.

5. Development of a system of morphological and molecular evaluation for the globe artichoke F_1 hybrids on the basis of the system already available.

6. Development of an effective pollination technique for the production of F_1 hybrids by comparing the different pollination systems available.

All the strategy used to the develop the PhD work activity is reported in the Fig. 2.1.

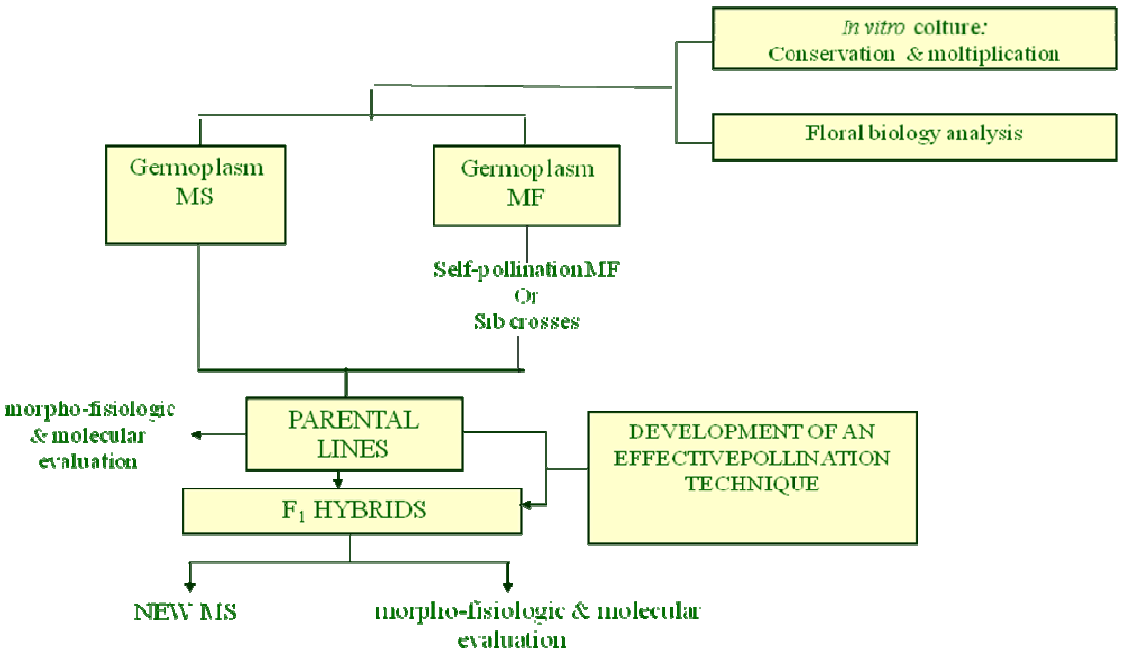

Fig. 2: Work activity diagram of the PhD program

Section A

PARENTAL GENOTYPES

Chapter A1

Floral biology

A1.1 INTRODUCTION

With the aim of shifting from globe artichoke vegetative propagation to seed planting and obtaining F_1 hybrids, the knowledge of the floral biology of this species is crucial, especially if extended to both male fertile (MF) and male sterile (MS) genotypes. Moreover, it is important to unravel the causes and the expressivity of male sterility.

As the mutation for male sterility is likely controlled by a single (ms_1) or two recessive genes (Principe, 1984; Basnizki and Zohary; 1994; Pécaut and Martin, 1994; Morison *et al.*, 2000 Stamigna *et al.*, 2004), its potential in developing hybrid artichoke varieties is quite high. A deep knowledge either on its nature or on its environmental and hormonal sensitivity will greatly facilitate the adoption in hybrid production schemes. To date, only genic male sterility has been detected in globe artichoke.

The floral biology of *Cynara* was studied in detail by Foury (1967) but, unfortunately, the morphological and functional differences characterizing MS and MF genotypes have not yet been defined. These studies were made, only in the last 15 years, by BBV (Bretagne Biotechnologie Végétale, Saint Pol de Léon, France), Stamigna *et al.* (2002)and Morrison *et al.* (2000). Morison *et al*. (2000) demonstrated that the bees were less attracted by the male sterile flowers, probably due to differences in nectar availability or composition. Chatelet (2005) affirmed that the lack of pollen is not enough to explain the flower unattractivity to nectar-gathering individuals, and he also hypothesized differences in the nectar producing structures. Although any structural difference has not been found, possible qualitative differences in the nectar samples of male-sterile and male-fertile genotypes have been observed. Stamigna *et al.* (person. communication.) demonstrated a post-meiotic block of the sterility but when the pollen grains starts to lose their functionality and vitality in the male sterile plants has not yet been demonstrated.

In this study, differences in flower development of two male-fertile and two-male sterile clones have been analyzed. The staging of artichoke flowers was based on the length of external flowers in the first order inflorescences. On this basis, the size of floral organs and the stage of development have been analyzed spanning the stages from pre-meiosis to anthesis.

A1.2 MATERIALS AND METHODS

This work was carried out during the years 2006 and 2007 at the Agrobiologia e Agrochimica Department of Tuscia University, in Viterbo (Italy), under the supervision of Prof. Andrea Mazzucato and with the collaboration of Dr. Irene Olimpieri.

GENOTYPES USED

In this study, the differences in flower development of two male-fertile (*MF-1; MF-2*) and two male-sterile (*MS-6; MS-16*) clones, selected within a F_2 INRA population coming from crosses among French MS clones and genotypes of the Romanesco and Violetto di Provenza types (Stamigna *et al.*, 2004). MS clones and MF genotypes were grown in the experimental fields of ARSIAL (Regional Agency for the Development and the Innovation of Agriculture in Latium), in Tarquinia (Viterbo, Italy).

STAGING

In the spring 2006, the inflorescences of the four clones have been collected at several developmental stages and florets have been used for studies both at the phenotypic and histological level.

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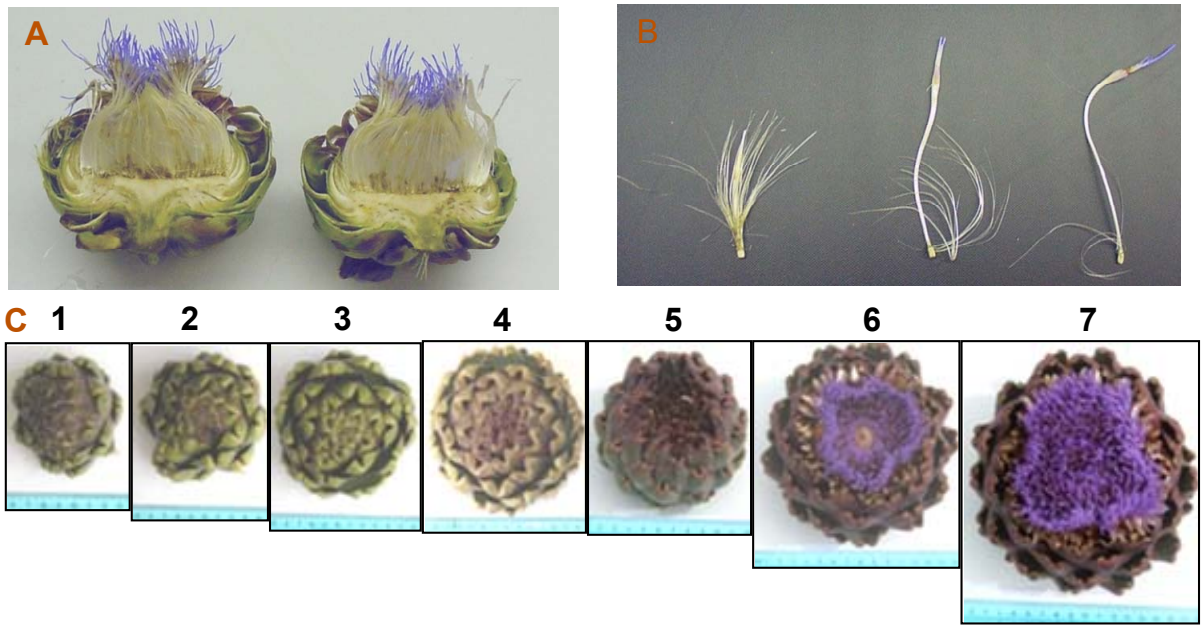

Fig. A1.1 A) Longitudinal section of an artichoke head near anthesis. B) Inner (left), middle (centre) and outer (right) florets from the same head. C) Representative heads collected at seven developmental stages (coded 1 to 7) and spanning 7 to 17 cm of diameter.

The staging of globe artichoke flowers was based on the length of external flowers (*Fig. A1.1 A and B*) in the first order inflorescences. The primary heads at seven stages (coded 1 to 7), spanning 7 to 17 cm of diameter (*Fig. A1.1* C) have been collected. The florets collected at these stages fell into classes of length that were comparable for the four genotypes. On this basis, the size of flower organs and their stage of development have been analysed spanning the stages from pre-meiosis to anthesis

CYTOLOGICAL ANALYSES

Three primary heads per genotype and three florets per head have been sampled .

The florets were fixed for 48 h in FAA (95% ethanol: glacial acetic acid: 37% formaldehyde: H_20; 50: 5:10: 35) and then stored in 70% ethanol at about 4˚C. The entire flower, the anthers, the ovary, and the ovule length for all the samples of each genotype have been measured.

The developmental stage of ovules and anthers, relative to the progression of sporogenesis and gametogenesis, has been investigated through staining with aniline blue and DAPI. Aniline blue is a colorant used to reveal callose deposits or structures during cytokinesis and in plant tissue studies. DAPI (diamidino-2-phenylindole) is a blue fluorescent probe that fluoresces brightly when it is selectively bound to the minor groove of double stranded DNA (its fluorescence is approximately 20-fold greater than in the non bound state). This selectivity for DNA, along with cell permeability allows staining of nuclei with little background from the cytoplasm, making DAPI the classic nuclear counter stain for immunofluorescence microscopy. It has an excitation maximum at 345 nm and an emission maximum at 455 nm (Morikawa and Yanagida, 1981).

Pollen grains were stained by aniline blue to detect the meiosis phases. Each sample (pollen or ovules) was stained with a drop of aniline blue (stock solution) directly on the microscope slide. The sample was kept for 10 min in the dark to let the colorant go through the tissues.

The nuclear development was studied using the DNA specific fluorochrome DAPI (Sigma D-8417), according to the procedure suggested by Coleman and Goff (1985). DAPI was stored as a stock solution (1 mg ml^{-1} of 6-diamidino-2-phenylindole in distilled water) at 4°C. One microliter of stock solution was diluted in 1 ml distilled water to prepare the working solution.

Pollen viability has been analyzed by acetocarmine and FDA (Fluoresceine Diacetate). For the preparation of acetocarmine, 10 g of carmine (Fisher C579-25) was dissolved into 1 L

of 45% glacial acetic acid, added boileezers, and refluxed for 24 h. After filtration into dark bottles, the solution was stored at 4°C. To evaluate viability and size of pollen, three slides per floret were prepared by squashing three anthers into a drop of acetocarmine. Both diameters (minimum and maximum) of each pollen grain, for a total of 300 grains per each floret, have been measured. Acetocarmine is a chromosome- specific stain that binds to DNA of all living cells. It stains chromatin of the nucleus and cytoplasm organelles but not aborted and infertile pollen.

The fluorochromatic reaction test (FCR) was performed at ENEA, under the supervision of Dr. Sergio Lucretti. Pollen from fresh florets of the clones MS-16 and MF-1 has been sampled. The pollen was previously mixed to an iso-osmotic solution of mannitol and sucrose. Few drops of a solution of FDA (1 mg FDA in 10 ml acetone) were distributed on a slide and, after 30 seconds (the time necessary for the acetone evaporation), few drops of the pollen suspension were added into the sucrose and mannitol solution. After five minutes, the samples were observed with a fluorescence microscope.

The florochromatic reaction test (FCR) verify the cytoplasmic membrane integrity and the presence of esterases activity in the vegetative cells; FDA (Fuorescein Diacetate), which is used for this test, is a polar and not fluorescent compound that easily go through the biological membranes. The cytoplasmatic esterases hydrolyze the FDA that release fluorescein. The flurescein produced by the FDA hydrolysis and cumulated in the cytoplasm of the viable pollen grains is visible on the fluorescence microscope, thus giving a yellow-green fluorescence only to the viable grains (Shivanna and Rangaswamy, 1992). While the acetocarmine, binding to the cellular DNA, highlights the structural differences between viable and unviable pollen grains without estimating a cell activity, the FCR test is directly correlated with the functional activity of a viable cell.

Microscopic analyses were done by a Zeiss Axioskop Fluorescence Microscope connected with a Canon digital camera to a computer. The microscope had an UV lamp and filters for various wavelengths; in the study, the length of 365 nm with DAPI and aniline blue filters was used. For the acetocarmine staining, an optical microscope has been utilized.

All data were analyzed by ANOVA test and the means referred to the size of the pollen of MS and MF genotypes were significatively differentiated by Duncan test.

A1.3 RESULTS

FLOWER DEVELOPMENT

The artichoke head is an inflorescence compound where hundreds of florets are inserted in the fleshy receptacle (*Fig. A3.1 A*); outer flowers are the oldest in development. Single florets assume a tubular shape, with a calix transformed into pappus, rudimentary petals, and thin anthers attached to pistil that expands from the anther cone with its violet stigmatic tissue (*Fig. A1.1B*).

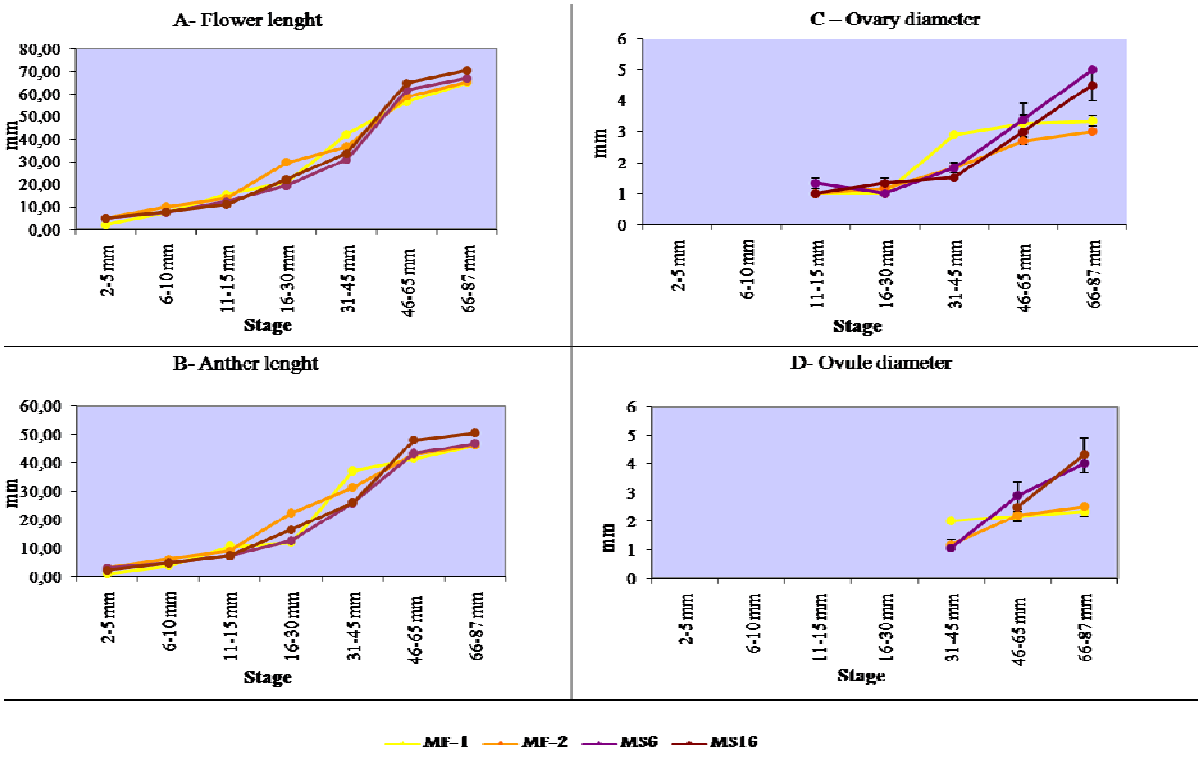

Fig.A1.2. Referring to the outest most florets at the seven stages described above, length in mm of the entire flower (A), of the stamen (B), of the ovary (C) and of the ovule (D).

The outest flowers collected at these stages falled into classes of length that were comparable in the four genotypes analyzed (*Fig. A1.2 A*). The two male-sterile clones showed flowers of the same length of the wild-types (WTs), this indicating that mutation does not affect general flower development. Similarly, all genotypes showed a comparable development of stamina, as judged by the anther length at the seven stages (*Fig. A1.2 B*). Thus, the male sterility was not of staminal type. When ovary (*Fig. A1.2 C*) and ovule (*Fig. A1.2 D*) diameters were measured, both organs resulted more elongated in mature flowers of male sterile clones. This was in agreement with the tendencies reported in other species for male sterile clones such as

(a) more developed female organs and (b) parthenocarpy (Gomez *et al*,. 1999; Yao *et al*., 2001; Ampomah-Dwamena *et al*., 2002).

POLLEN DEVELOPMENT

To assess whether male-sterility was of pollinic or functional type, pollen of the four genotypes was first studied at the three latest stages of development. Within the same genotype, pollen grains had similar diameter across the three stages, this indicating that from the stage 5 onwards the pollen had already reached its maximum size. Therefore, data of the three stages were pooled.

The *Table A1.1* shows the results of both the ANOVA analysis and the Duncan test.

Table A1.1: Mean pollen diameter and index shape (D min/max) at the stages (pooled) 5 to 7 for two male sterile and male fertile clones

Clone	Pollen diameter (mm)	Dmin/Dmax (mm)
MS 16	43,01±0.41 c	0,74±0.023
MS 6	46,96±0.31 b	0,80±0,021
MF 1	52,32±0.31 a	0,74±0,025
MF2	53,42±0.22 a	0,72±0,022
F	249,553***	12,913 n.s.

ANOVA showed that there is a highly significant difference among the average pollen diameters of the four clones, but the differences in the shape of the pollen grains, evaluated as a ratio between the two measured diameters, are not significant.

Compared to the WT, pollen grains of the male sterile clones had a lower diameter. The Duncan test indicated that the average pollen diameter of the two WT clones were not significantly different amount them, but they were significantly different respect to male sterile clones. In addition, whereas the 97-99% of pollen grains from WT clones were stainable by acetocarmine (*Fig. A1.4 A, C*), the 100% of grains from male sterile clones were not stainable, this indicating their very low viability (*Fig. A1.4 B, D*). At these stages, WT pollen grains had a well developed and sculptured exine, as usually found in cross-pollinated species. The thick pollen cell wall hampered the sight inside the grain, so that it was not possible to recognize at which step of microgametogenesis the grain was. Unstained male sterile grains showed a destructured exine pattern that lacked the callose fluorescence (*Fig. A1.4 F*) that well characterized the WT pollen grains (*Fig. A1.4 E*). Thus, the mutation could affect the deposition of cell wall components.

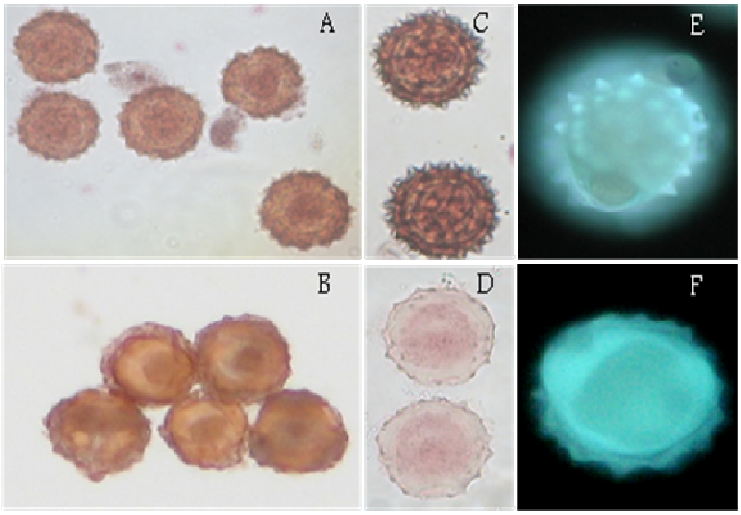

Fig. A1.4: WT (A) and male sterile (B) pollen grains at the stage 5 stained by acetocarmine; WT (C) and male sterile (D) pollen grains at the stage 7 stained by acetocarmine; WT (E) and male sterile (F) pollen grain at the stage 7 stained by aniline blue.

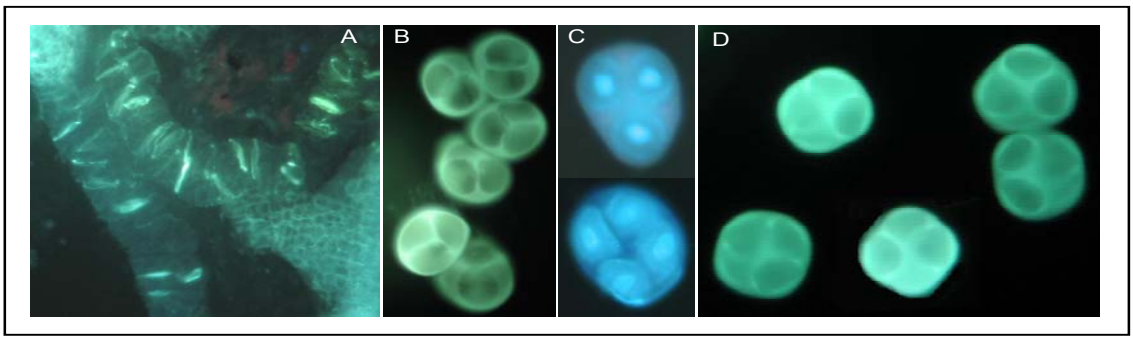

Fig. A1.5: Sporigenous tissue before meiotic division from a WT anther at the stage 1 (A), tetrads from a WT floret at the stage 2 stained with aniline blue (B) and with DAPI (C), tetrads at the same stage from a male sterile flower stained with aniline blue (D).

To observe occurrence of male meiosis, florets from the stages 1 to 3 were studied and dissected anthers analysed after aniline blue staining. In florets from the stage 1 (2-5 mm-long florets), the anthers revealed to contain a compact tissue, that the presence of callose in the septa revealed to be the sporigenous tissue (*Fig. A1.5* A).

Florets from inflorescences at the stage 2 usually showed microspores at the tetrad stage that could be distinguished by both aniline blue (*Fig. A1.5* B) and DAPI (*Fig. A1.5* C) stainings. Anthers from male sterile clones at the same stage showed microspores of similar developmental stage, as the WTs, and no abnormality could be evidenced as following the meiotic division (*Fig. A1.5* D).

In the FCR test, any difference in the fluorescence of the male-sterile and male-fertile clones has not been found. The fluorescence was lower in the male-sterile florets but this

difference was not significant. Probably, FDA did not penetrate enough in the pollen grains, even though we tried to improve the time of staining and we doubled the FDA concentration.

2.2.3 DISCUSSION

For staging, some differences among flowers of the male-sterile and the male-fertile clones have been defined. Male sterility do not produce any effect on the extent of the anther development and does not affect the time of the development. Delay in the anther development of the male sterile florets has not been found.

In the clones studied, male sterility is of pollinic type and the causing defect occurs during microgametogenesis. Presence of normal tetrads in the second stage has been confirmed for all the genotypes, so male-sterility dose not provoke any delay in the microgametogenesis, until the caryogamy; the post-meiotic block, previously hypothesized by Stamigna (2002), was not individuated. Although there are not morphological differences in the male structures, pollen did not result stained by acetocarmine and, so, not functional. From the morphological aberrations observed, it is possible to hypothesize that mutation could affect directly or indirectly exine deposition, this in agreement with the hypothesis that the post-meiotic block of the sterile pollen could be due to a low nutritional activity of the anthers tapetal cells proposed by Stamigna (unpublished data) for the globe artichoke and already observed in other species by Kaul (1988).

Moreover, some differences in the female reproductive organs have been found. In fact, ovaries and ovules of mature flowers were more elongated in the male sterile clones than in the male fertile ones. This was in agreement with that reported in other species: male sterile clones present more developed female organs and have a tendency to parthenocarpy (Gomez et al. 1999; Yao et al. 2001; Ampomah-Dwamena *et al*., 2002).

Chapter A2

Development of parental genotypes

A2.1 INTRODUCTION

In the last 15 years, the seed companies produced F_1 hybrids with a good homogeneity; they are still not enough but well suitable for the industrial production. Unfortunately, they are not widely adaptable to the Italian cultivation conditions and to the market demands. Producing seed propagated varieties for fresh consumption is still a future goal to reach. In Italy, the only research on breeding activities aimed at the production of globe artichoke F_1 hybrids started fifteen years ago at the University of Tuscia and ENEA.

The first step for the development of new F_1 hybrids is the development of stable male and female parental lines.

During the three years of the PhD program, the parents already available in the field were analyzed and new parental lines were developed. In the first two years, the activity was focused on the analysis and selection of single plants to develop as stable lines or to use directly as parents in the crosses. In the third year, the number of plants available was high enough to study their genetic stability. We tried to develop a system for the selection of new lines, which is useful to predict the progresses in stability of every single line throughout the years.

A2.2 MATERIALS AND METHODS

During the first two years of the PhD program, all the open field activities were carried out in the experimental fields of Regional Agency for the Development and Innovation of Agriculture (ARSIAL), in Tarquinia (Viterbo, Italy). In the third year, the open field activities were carried out in the experimental fields of Big Heart Seed Company (BHSC), in Brawley (California, USA).

GENOTYPES

Male fertile (MF)

- **2006**

We started to select the material already available from the previous experiments, in collaboration with Dr. Fabio Micozzi. The different genotypes were located in the experimental fields of ARSIAL, in Tarquinia.

We selected the plants to be used for cross-pollinations (in the text named as *crosses*), and some of these plants were self-pollinated.

The number of heads covered for self-pollination (in the text named as *selfs*) are indicated in the *Table A2.9*(see Results). The genotypes AM 1 and AM 2 are two American genotypes derived from seed propagation of a population owned to Big Heart Seed Company (BHSC, USA). SP1 is a genotype selected by Prof. Saccardo and Dr. Micozzi within a Spanish population while S. Erasmo, Brindisino, and Terom are Italian materials (*Fig. A2.1*) evaluated also in the experimental fields of ARSIAL.

Fig. A2.1: Italian genotypes selfed and used as MF parents.

Apollo and Exploter (*Fig. A2.1*) are two 'Romanesco'-like varieties, obtained by seed propagation and owned to VITROPLANT Italia s.r.l. company.

- **2007**

We selected some male-fertile genotypes on the basis of the previous year results. We repeated some crosses and made new selfs; the number of heads covered for the selfs are indicated in the *Table A2.9* (see Results). We also used the 2006 selfs as new male parents.

In 2007, within a collaboration between BHSC and the University of Tuscia, self-pollinations have been carried out on the best plants selected within the best BHSC stable and productive globe artichoke lines. The seeds from selfs were then sown in the Company's fields and the plants were evaluated during the subsequent year.

- **2008**

During the third year, all selection and crossing activities were carried out in the BHSC experimental fields located in the south of California. The self-lines I_1 , $I_2$and sib-lines were evaluated and the best ones were then selected. These selected lines were crossed with MS plants. Moreover, each of the best self and sib-lines was sib-crossed and a few plants from the selected lines were selfed again (I_2 and I_3).

Male sterile (MS)

- **2006**

The MS clones MS6 and MS16 (*Fig. A2.2*), previously selected (Stamigna *et al.*, 2004) within the segregant F_2 population coming from crosses between a male-fertile "Romanesco"

type and a French (INRA) male-sterile clone, have been used as parents in the hybridization programs.

Fig. A2.2: MS clones used in the hybridization programs

We selfed some F_1 hybrids obtained in 2005 with the aim of finding new male-sterile plants within the F_2 population. The number of heads covered for selfing are indicated in the *Table A2.9* (see Results).

We collected offshoots for the micropropagation of male sterile clones (MS6, MS16) and we started to multiply under *in vitro* conditions the parental lines. We sent the micropropagated plants to BHSC, which multiplied in a broad number the plants in a nursery.

- **2007**

Some F_1 hybrids realized during 2006 were selfed in order to obtain a F_2 population. The number of heads covered for selfing are indicated in the *Table* A2.9 (see Results).

The clone MS6 was multiplied by BHSC, in their *in vitro* culture laboratory, to be used as female parent in the hybridization programs.

- **2008**

Segregation for some traits was evaluated within the F_2 progenies obtained by self-pollination of F_1 hybrids in 2007. From these F_2 progenies, sown at the BHSC, new male sterile plants were selected.

POLLINATION TECHNIQUE

Self-pollination

Before the beginning of flowering, every single head has been covered with an "anti-aphid" net. Because of the gradual centripetal flowering, pollen has been collected two times a day with a brush, during the entire blossoming period (*Table A2.1*). Stored in the refrigerator (4°C), pollen was brushed back once every two-three days onto the flowers; because of the proterandry, the stigmatic region of each floret remains receptive three days

after anther dehiscence. Normally pollen was collected and brushed back in the morning, before the day became hot.

When the flowering finished and the head started to dry, the "anti-aphid" net has been removed to protect the development of the seeds against the high temperatures.

Tab. A2.1. Localities and dates of pollination activity

Year	Locality	Date of flowering	Last flowering day	Date of collection
2006	ARSIAL, Tarquinia (Viterbo, Italy)	June 4th, 2006	July 1th, 2006	August 7th,2006
2007	ARSIAL, Tarquinia (Viterbo, Italy)	June 8th, 2007	July 1th, 2007	August 7th,2007
2008	BHSC, California (USA)	May 1th, 2008	June 5th, 2008	July 10th,2008

One month after the end of flowering, the seeds were already mature enough to be collected, so the heads were cut and stored in a shady dry place for other 10 days in order to allow drying completely.

Sib-crosses

In 2008, each of the most stable lines was also sib-crossed. The sib-crosses were performed by bees in tents (*see* the paragraph on Cross Pollination Technique, in Chapter 3).

The seeds were collected manually both in 2006 and 2007. During the last year of the experiments, the seeds were collected by combine harvester machine.

SOWING

In 2006, seeds were sown on August 11th, in Albani e Ruggeri Nursery of Civitavecchia (Italy), inside plastic containers with 180 spots. Forty-five days after sowing, the plantlets were transplanted in ARSIAL fields. Whereas, plants of the male sterile clones and those of the other vegetatively propagated male parents were obtained by offshoots, which were collected from the mother plants during the spring 2005. The offshoots were grown in the nursery until the end of summer and, in September 2005, were transplanted in ARSIAL experimental fields. The fields were previously prepared by ploughing 40 cm deep, with an application of phosphorus (P) and potassium (K) amendments. Few days before the manual transplanting, the soil was superficially cultivated (disked and rototilled). Nitrogen fertilizers were given to the culture in four parts. The first part was incorporated into the soil just prior to planting, and the others were given during the culture development.

Transplantation was made in 20 plant plots formed by five rows with 120 cm apart; every row had four plants 100 cm apart. Globe artichokes were frequently drip irrigated during plant growing, flowering and seed growing seasons. Moreover, weeds were regularly controlled.

In the USA, sowing was made directly in the field on November 1st, 2007. To enhance drainage, the site was deep chisel plowed, and a broadcast application of phosphorus (P) and potassium (K) amendments was given. The field was disked and raised beds were formed. Nitrogen was incorporated into the raised beds just prior to planting and after, during the culture growing. Beds were formed in rows of 1.80 m apart and raised 12.7 cm high. Plants were spaced 0.9 m in each row. The selfed plants were planted in 100 plants blocks (25 x 4), 100 plants for each self. Two seeds per spot have been sown in order to have not empty spaces. After germination, the double plants were removed. Every two rows of male fertile line was alternated with a row of 25 plants of MS6. The micro propagated MS6 plants, coming from the nursery Baroda's Farm, in South California, were transplanted two weeks after sowing.

The F_2 plants were sown in plots of 20 plants each with the same distances.

All plants were furrow irrigated during plant growing, flowering and seed growing seasons. Moreover, weeds were regularly controlled.

ANALYSIS SYSTEM

The morphological traits were evaluated on the basis of the ***Protocol for Globe Artichoke*** European Union CPVO (2004). The following traits indicated by literature as the responsible of the highest percentage of variance (De Pace *et al*, 1979; Crinò *et al.*, 2008) and as the most important for the market have been chosen for the morphological analyses of the plants:

1. Plant Height (PH), as reported in *Fig. A2.3*: height, including main head (cm);
2. Plant diameter (PD), as reported in *Fig. A2.3*: maximum plant width (cm);
3. Number of lateral shoots (SS), as reported in *Fig. A2.3*;

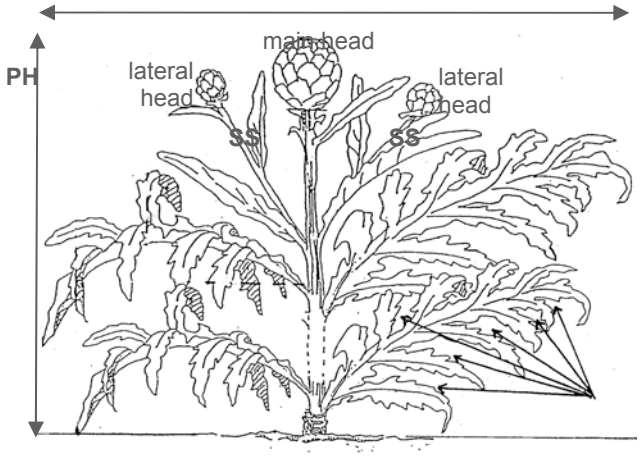

Fig. A2.3: Plant height and width, as evaluated on the plant

4. Main head time (MHT): date of the main head harvesting;
5. MHD: diameter of the main head (cm);
6. MHL: length of the main head (cm);
7. MDL: MHD/MHL (Shape Index);
8. MHS: main head shape (*Fig.A2.4*);

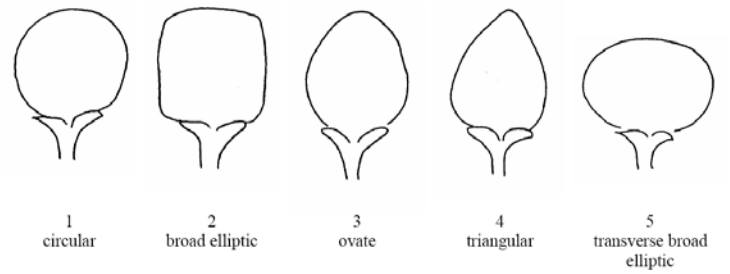

Fig. A2.4: Main head shape (CPVO descriptors)

9. Main head point (MHP): 1= sharp point; 2=round point; 3=flat point; 4=concave point.
10. External bract color (MHC) of the main head (*Fig. A2.5*): 1= green; 2=green purple thinly striped; 3=purple green thinly striped; 4= purple.

Fig. A2.5: External bract color of the head (1=green, 2= green purple thinly striped, 3= purple green thinly striped, 4=purple)

11. Main bract color (MBC) with presence of golden background color in the external bracts.

12. Main head spines (MHSp), as described in *Fig. A2.6*: 1=absent or very little, 2=little, 3=big spines in the external bracts.

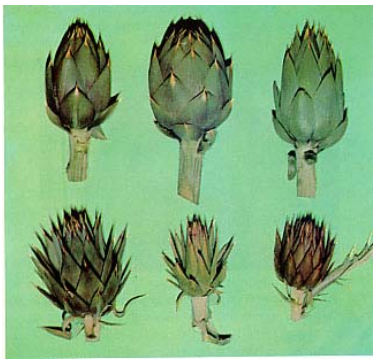

Fig. A2.6: Typologies of spines on the main head

13. Main head mucron (MHM): 1=absent (*Fig. A2.7*), 2=little, 3=big mucron in the external bracts;
14. External bract tip (LT): 1=sharp; 2=concave; 3=round;

Fig. A2.7: Main head mucron (CPVO descriptors);

15. Primary head time (PHT): date of the primary head harvesting;
16. PHN: number of first order of heads on the lateral shoots;
17. PHD: diameter of first order of heads on the lateral shoots (cm);
18. PHL: length of first order of heads on the lateral shoots (cm);
19. PDL: DPH/LPH (Shape Index);
20. THN: total number of heads.

MALE FERTILE PLANTS

In 2006 and 2007, the single plants, to use as male parents in the crosses and to develop as new stable parents by self-pollination and sib-crosses, have been selected and then evaluated.

Whereas in 2008 all the self-pollinated and open-pollinated lines were evaluated with some of CPVO traits (25 plants/line), capable of better differentiating the genotype in this experiment some descriptors have been modified as follows in order to fit them into the analysis:

- PH: the plants were divided in three classes 1=plant height <70 cm; 2=plant height 70-90 cm; 3=plant height > 90 cm;
- External bract color (MHC) of the main head: 1=green; 2=green-gray; 3=green golden; 4=green purple striped.

Trait frequencies and discriminant analysis have been calculated with SPSS software (Field, 2005); the most stable and uniform self-pollinated (I_1) and open-pollinated (OP) lines have been chosen. The result frequency analysis has been combined with the empiric selection. The results have been also combined with the observations made directly in the field where the lines that we wanted to improve and to use as male parents in the crosses have been chosen.

The off-type plants of each line selected were eliminated. One plant of each line has been chosen both for manual self-pollination and cross-pollination with the clone MS6. Moreover, the I_1 and OP lines selected in tents have been isolated and then sib-crossed and crossed with the clone MS6.

The total seed production was evaluated and the average seed production per head was calculated. If the number of seeds was higher than 150, the total number of seeds was estimated counting and weighting 100 seeds for three times and calculating an average weight of 100 seeds. Then, the estimated total number of seeds dividing the total weight for the average weight of 100 seeds has been calculated.

MALE-STERILE PLANTS

In 2006, the clones MS6, MS16 and MS14 were evaluated with some of CPVO traits. In 2008, the F_2 generation was evaluated for the segregation of two traits: spineless, and male-sterility. The segregation ratio was verified by X^2 test. Pollen vitality was estimated by acetic carmine staining (see Paragraph 2.2). The plants were considered as male sterile only if the 100% of pollen grains were not stained in acetocarmine; all the other intermediate cases were excluded. Staining of three florets/plant was evaluated. Moreover, the new male sterile plants were selfed to confirm their pollen-sterility. Seeds were collected after open-pollination to evaluate the plant attitude to be crossed and to produce seed.

All BHSC data were collected with the main collaboration of Mrs. Alyssa Jordan, and with the constant help of Mr. John Rusty Jordan.

MICROPROPAGATION

In order to conserve the male sterile and the male fertile clones that resulted more stable, in 2006, an *in vitro* propagated collection of clones was carried out at the BAS-BIOTECGEN Section of ENEA, under the supervision of Dr. Raffaela Tavazza.

The micropropagation technique was acquired in the following two phases of explant sterilization and multiplication:

1. Sterilization of the shoot apexes

The offshoots were collected in autumn 2006. In this season correspondent with the vegetative phase of globe artichoke, the offshoots are more responsive to the condition of *in vitro* propagation.

Well developed off-shoots were collected and wrapped in wet paper until their use in the laboratory. After removal of both the dirt and the external leaves, only the central parts of the shoots 2-3 cm long. The shoots were washed with tap water, and dipped into a solution of mercuric chloride ($HgCl_2$) at a concentration of 5g l^{-1}. The shoot apexes were three times rinsed in sterilized water (the last time for 5 minutes) and then dipped into a solution of sodium hypochlorite ($NaOCl_2$) at a concentration of 1.5% for 15 minutes. Few drops of Tween 20 (2 drops in 200 ml of solution) were added to this last solution in order to clean deeper the apexes. Some others rinses in sterile water were made. The last rinse was made with a filter sterilized anti-oxidant solution of citric and ascorbic acids at the concentrations of 150 mg l^{-1} and 100 mg l^{-1}, respectively (Ancora *et al.,* 1981).

The apexes were further reduced with a sterile scalpel in order to eliminate all the parts in contact whit the sterilization solutions. They were subsequently cultured in sterile hermetic plastic containers containing 10 ml of propagation medium.

The basal medium used for the proliferation was that described by Tavazza *et al.* (2004) (*Table A2.2*). The cultures were maintained in an incubator under alternate light (16h) and dark, at temperatures of 25˚C and light intensity of 3000-4000 Lux.

2. Proliferation

When the apex explants started to proliferate, they were transferred into bigger sterile plastic containers with 25 ml of propagation medium and 4-5 explants for every container (*Fig. A2.8A* and *B*), under the growing conditions described above. The sub-cultures were repeated every 15-20 days (*Fig. A2.9 A* and *B*).

Fig. A2.8: In vitro culture of the globe artichoke plantlets.

Table A2.2: Composition of basal medium utilized for the *in vitro* proliferation of globe artichoke

MACROELEMENTS	
NH_4NO_3	400 mg l^{-1}
KNO_3	800 mg l^{-1}
$MgSO_4x7H_2O$	370 mg l^{-1}
KH_2PO_4	170 mg l^{-1}
$CaCl_2$	150 mg l^{-1}
$Ca(NO_3)_2x4H_2O$	1000 mg l^{-1}
MICROELEMENTS	
$ZnSO_4$ x $7H_2O$	8.6 mg l^{-1}
H_3BO_3	6.2 mg l^{-1}
$MnSO_4$ x $4H_2O$	20 mg l^{-1}
$CuSO_4$ x $5H_2O$	0.25 mg l^{-1}
$NaMoO_4$ x $2H_2O$	0.25 mg l^{-1}
$CoCl_2$ x 6 H_2O	0.025 mg l^{-1}
KI	0.83 mg l^{-1}
FeEDTA (Sigma solution)	36.7 mg l^{-1}
VITAMINS	
Meso-inositol	200 mg l^{-1}
Thiamine	1 mg l^{-1}
Pyridoxine HCl	0.5 mg l^{-1}
Nicotinic Acid	0.5 mg l^{-1}
Glycin	2 mg l^{-1}
Sucrose	30 g l^{-1}
Agar (Plant Agar Duchefa)	7 g l^{-1}
ORMONS	
Kinetin	0.5 mg l^{-1}
IBA	0.1 mg l^{-1}

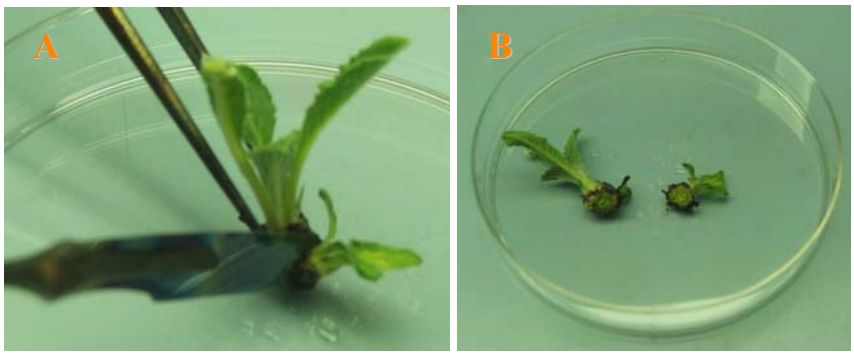

Fig. A2.9: In vitro subcultures (A and B).

A2.3 RESULTS

A2.3.1 MORPHOLOGICAL ANALYSES

The *Table A2.3* summarizes the results of the analyses performed on plants in 2006 and 2007. From the evaluation of these plants, the male parents to be used in the new crosses were chosen. Selection was carried out on the basis both of the head shape and the total productivity. In 2006, the plants selected were AM 1 plants 3, 4 and 7; SP1 and SP2, SP1 I1 05 plants C2, E4 and F2. In 2007, all the plants indicated in the table have been selected. All the selected plants had green and big main heads. The main head shape was circular or transverse elliptic. Only the plants with heads without spines and mucron have been chosen. Evaluation of S. Erasmo, Brindisino, Terom, Apollo, and Exploter genotypes was not performed because it was not of interest in this work.

The evaluation of the male sterile clones is illustrated in *Table A2.4*. MS 6 and MS 14 were characterized by circular purple and green striped main heads, while MS 16 had broad elliptic main heads; all of them were spineless. The average production of MS6 was lower than the clones MS16 and MS 14

The results of the frequencies analysis made in 2008 are shown in *Table A2.5*, where some lines appear more stable than others.

In the group of BN 62-2 genotype, the self-pollinated lines no. 6 and no. 9 (*Fig. A2.10*) were selected. BN 62-2 #9 is a line slightly more uniform in height than BN 62-2 #6; the head had a globe shape but with a sharp point, like the predominant head shape of BN 62-2 #6; BN 62-2 #6 head point is predominantly round (*Tab. A2.5*. The 69% of BN 62-2 #9 plants and the 60 % of the BN 62-2 #6 plants have a golden green color.

Fig. A2.10: Two self-pollinated lines selected in 2008; BN 62-2 # 6 (on the left) and BN 62-2 # 9 (on the right).

In the group of the F-19 11-1 genotype, four self-pollinated lines were selected. The best one was the F-19 11-1 # 3 (*Fig. A2.11*), that produced uniform and short plants with big

and oval dark-green heads. It produced a lower average number of heads/plant than the other lines (*Table A2.6*) but they were bigger respect to the other lines (unshown data). This line could have the chance to be developed in a variety adapted to the mechanical harvesting of the heads and to a higher plant density of cultivation.

Table A2.3: Plants evaluated with some of the European Union CPVO descriptors and used as male parents in 2006 and 2007 hybridizations

Genotype	Plant in map	PH (cm)	PD (cm)	MHT	MHD (cm)	MHL (cm)	MDL	MHS	MHC	MHSp	MHM	PHT	PHN	PHD (cm)	PHL (cm)	PDL	TNH
2006																	
SP1 I1 05	B3	50	130	11/4/06	12	10	1,2	5	1	1	1	14/4/06	4	9	9	1,0	8
	C1	110	140	11/4/06	10	9	1,1	4	1	1	1	14/4/06	3	7,5	8,5	0,9	7
	C2	80	170	11/4/06	12	9,5	1,3	5	1	1	1	14/4/06	3	10	6,5	1,5	9
	C3	100	160	11/4/06	10	9,5	1,1	1	1	1	1	14/4/06	4	8	6	1,3	5
	E2	110	160	11/4/06	10	9	1,1	2	1	1	1	14/4/06	3	9	9	1,0	6
	E4	100	180	11/4/06	13	10	1,3	5	1	1	1	14/4/06	3	10	8	1,3	9
	F1	80	150	11/4/06	12	9	1,3	5	1	1	1	14/4/06	3	9	7,5	1,2	9
	F2	100	120	14/3/06	10	9	1,1	1	1	1	1	30/3/06	5	11	10	1,1	8
	F3	70	160	14/3/06	10	11	0,9	1	1	1	1	30/3/06	3	9,5	9	1,1	6
AM 1	1	100	190	13/4/06	12	10	1,2	5	1	1	1	19/4/06	2	11	9	1,2	5
AM 2	2	107	170	13/4/06	12	9,5	1,3	5	1	1	1	19/4/06	3	10	8,5	1,2	6
	3	60	120	10/3/06	10	9	1,1	5	1	1	1	16/3/06	3	7,5	7	1,1	7
	4	80	190	10/3/06	11	9	1,2	1	1	1	1	16/3/06	4	7	9	0,8	9
	5	78	200	10/3/06	10	8	1,3	1	1	1	1	16/3/06	3	7	9	0,8	10
	6	66	200	*offtype*									0				0
	7	95	150	13/4/06	14	11	1,3	5	1	1	1	19/4/06	3	8	7,5	1,1	9
SP1		65	180	8/4/06	10,5	8,5	1,2	5	1	1	1	14/4/06	3	9	8	1,1	7
SP2		70	160	8/4/06	10	8	1,3	5	1	1	1	14/4/06	3	8,5	7	1,2	8
2007																	
SP1 I106	A2	85	150	24/4/07	13	11,5	1,1	5	1	1	1	2/5/07	3	9,5	7	1,4	7
	A3	70	170	24/4/07	14	9,5	1,5	5	1	1	1	2/5/07	1	11	8	1,4	3
	B1	65	140	24/4/07	13	9	1,4	5	1	1	1	2/5/07	2	9,5	6,5	1,5	5
AM1I106	B2	65	140	24/4/07	12	9,5	1,3	5	1	1	1	2/5/07	2	10	8,5	1,2	5
	C4	70	170	24/4/07	13	9,5	1,4	5	1	1	1	2/5/07	1	12	9,5	1,3	3
	D2	65	140	24/4/07	12	9	1,3	5	1	1	1	2/5/07	2	10,5	7,5	1,4	5

Table A2.4: Male sterile plants evaluated with some of the European Union CPVO descriptors in 2006

Genotype	PH (cm)	PD (cm)	TMH	DMH (cm)	LMH (cm)	DL	MHS	MHC	MHSp	MHM	TPH	PHN	PHD (cm)	PHL (cm)	PDL	TNH
MS6	71	120	11/4/06	9,5	8	1,2	1	3	1	1	14/4/06	3	9	8	1,2	5
MS16	84	135	11/4/06	10	10	1	3	3	1	1	14/4/06	5	9	9	1	8
MS14	75	130	11/4/06	9,3	8,7	1,1	1	3	1	1	14/4/06	5	9	9	1	8

Table A2.5: BHSC self-pollinated lines (I_1) and Sib-pollinated lines (OP) evaluated in 2008 with frequency CPVO traits analysis

Line (I_1)	PH			MHS					MHP				MC				MHSp			MHM			LT		
	1	2	3	1	2	3	4	5	1	2	3	4	1	2	3	4	1	2	3	1	2	3	1	2	3
Self-pollinated lines																									
F-19 23-1 #1	0	100	0	0	0	67	33	0	17	83	0	0	63	13	25	0	54	42	4	100	0	0	58	42	0
BN 62-2#10	0	100	0	0	0	67	33	0	17	83	0	0	63	13	25	0	54	42	4	100	0	0	58	42	0
BN 62-2#13	8	40	52	52	28	4	4	12	4	52	40	4	56	12	32	0	100	0	0	24	44	32	0	100	0
BN 62-2#14	32	56	12	16	0	0	0	84	4	40	40	16	28	4	60	8	100	0	0	64	36	0	0	100	0
BN 62-2#9	79	21	0	85	4	0	11	0	79	21	0	0	39	0	61	0	46	46	7	96	4	0	7	14	79
BN 62-2#5	44	52	4	56	0	0	0	44	44	48	8	0	56	12	32	0	96	4	0	40	60	0	4	84	12
BN 62-2#6	52	40	8	76	0	24	0	0	36	52	8	4	32	0	60	8	100	0	0	8	92	0	0	100	0
BN 62-2#7	54	46	0	88	0	8	4	0	13	71	8	8	25	0	71	4	96	0	4	17	54	29	0	100	0
BN 62-2#8	76	24	0	48	0	20	16	16	16	60	24	0	64	4	28	4	92	0	8	8	88	4	0	100	0
CYL #1	55	40	5	0	0	30	70	0	75	15	0	10	50	0	5	45	85	15	0	100	0	0	95	5	0
CYL #10	0	12	88	0	0	8	92	0	84	8	8	0	56	4	16	24	80	20	0	100	0	0	64	36	0
CYL #11	4	16	80	0	4	8	88	0	84	12	4	0	28	4	12	56	68	32	0	100	0	0	64	36	0
CYL #2	14	86	0	0	0	5	95	0	95	5	0	0	5	95	0	0	67	33	0	100	0	0	57	43	0
CYL #3	58	38	4	0	4	67	29	0	83	13	4	0	54	46	0	0	96	4	0	100	0	0	58	42	0
CYL #4	36	60	4	0	0	68	32	0	96	4	0	0	28	4	0	68	92	8	0	100	0	0	48	44	8
CYL #7	8	79	13	0	0	38	63	100	100	0	0	0	38	0	0	63	88	13	0	100	0	0	83	17	0
CYL #8	4	48	48	0	0	12	88	0	96	4	0	0	32	12	24	32	84	16	0	100	0	0	60	40	0
CYL #9	13	87	0	0	0	9	91	0	87	13	0	0	17	13	9	61	70	30	0	100	0	0	39	48	13
F-19 #1	100	0	0	32	0	56	12	0	16	80	0	4	68	24	8	0	52	20	28	100	0	0	76	24	0
F-19 #10	0	79	21	0	0	4	96	0	75	21	4	0	63	0	29	8	25	54	21	100	0	0	100	0	0
F-19 #2	0	80	20	40	0	56	4	0	8	92	0	0	64	0	36	0	36	60	4	100	0	0	80	16	4
F-19 #3	0	96	4	0	0	96	4	0	60	12	28	0	60	12	28	0	24	48	28	100	0	0	96	4	0
F-19 #4	0	12	88	42	0	58	0	0	8	92	0	0	42	42	16	0	66,7	29,2	4,2	100	0	0	79,2	20,8	0
F-19 #5	41	59	0	91	9	0	0	0	18	73	9	0	96	5	0	0	100	0	0	0	77	23	0	100	0
F-19 #6	0	80	20	80	4	8	8	0	40	56	4	0	56	0	44	0	92	8	0	56	24	20	8	92	0
F-19 #7	38	38	25	81	0	13	6	0	0	56	44	0	31	6	63	0	94	0	6	81	19	0	20	80	0
F-19 #8	38	50	13	17	0	38	46	0	46	46	8	0	46	54	0	0	88	13	0	50	42	8	0	88	13
F-19 #9	12	88	0	8	0	40	52	0	20	72	8	0	52	4	44	0	56	44	0	100	0	0	0	87	13

F-19 11-1 #1	20	52	28	4	0	56	40	0	44	56	0	0	68	0	24	8	12	48	40	100	0	0	88	12	0
F-19 11-1 #10	8	88	4	0	0	76	24	0	44	56	0	0	76	16	0	8	64	36	0	100	0	0	84	12	4
F-19 11-1 #11	70	25	5	90	0	0	0	10	10	85	5	0	55	0	35	10	95	5	0	30	65	5	5	90	5
F-19 11-1 #12	33	67	0	46	42	8	4	0	92	8	0	0	63	4	25	8	50	8	42	58	0	42	4	67	29
F-19 11-1 #14	64	28	8	100	0	0	0	0	72	28	0	0	52	0	20	28	60	32	8	44	56	0	8	52	40
F-19 11-1 #2	36	60	4	0	0	76	24	0	52	48	0	0	60	32	8	0	16	36	48	100	0	0	92	8	0
F-19 11-1 #3	100	0	0	4	0	96	0	0	100	0	0	0	96	0	0	5	87	0	13	17	26	57	17	83	0
F-19 11-1 #4	25	75	0	100	0	0	0	0	100	0	0	0	92	4	0	4	92	8	0	92	8	0	58	42	0
F-19 11-1 #5	36	46	18	100	0	0	0	0	50	50	0	0	59	0	41	0	73	27	0	77	23	0	32	64	5
F-19 11-1 #6	56	36	8	96	0	4	0	0	0	0	100	0	88	12	0	0	64	0	36	36	60	4	0	100	0
F-19 11-1 #7	12	80	8	60	0	36	4	0	60	40	0	0	92	8	0	0	20	24	56	100	0	0	96	4	0
F-19 11-1 #8	8	68	24	12	0	8	80	0	96	4	0	0	72	4	12	12	52	20	28	100	0	0	56	28	16
F-19 11-1 #9	20	64	16	0	0	76	24	0	92	8	0	0	76	0	24	0	8	64	28	100	0	0	100	0	0
WB #1	4,2	95,8	0	4,2	0	95,8	0	0	4,2	95,8	0	0	66,7	25	8,3	0	41,7	58,3	0	100	0	0	87,5	8,3	4,2
WB #5	4,2	66,7	29,1	0	12,5	87,5	0	0	0	83,3	16,7	0	45,8	0	37,5	16,7	100	0	0	100	0	0	29,2	70,8	0
WB #2	12,5	87,5	0	0	0	0	45,8	54,2	50	50	0	0	50	45,8	4,2	0	50	50	0	100	0	0	95,8	4,2	0
WB #3	0	4,2	95,8	0	0	37,5	62,5	0	62,5	37,5	0	0	33,3	20,8	45,8	0	29,2	41,7	29,2	100	0	0	79,2	20,8	0
WB #4	8	92	0	0	0	56	44	0	48	48	4	0	68	20	12	0	44	32	24	100	0	0	96	4	0
BN Tent #2	0	3,8	96,2	88,5	7,7	3,8	0	0	73,1	23,1	3,8	0	38,5	7,7	42,3	11,5	96,2	0	3,8	3,8	73,1	23,1	11,5	88,5	0
BN Tent #4	36	64	0	100	0	0	0	0	8	92	0	0	76	0	0	24	100	0	0	100	0	0	24	76	0
Sib-pollinated Lines																									
F-19 19-2	12	36	52	96	4	0	0	0	56	40	4	0	92	4	0	4	48	12	40	84	16	0	0	48	52
B N 62-2 gr #7	0	96	4	0	0	88	8	4	12	68	20	0	52	0	48	0	16	68	16	100	0	0	88	12	0
B N 62-2 gr #8	0	40	60	8	0	72	20	0	24	76	0	0	88	0	12	0	20	60	20	100	0	0	100	0	0
B N 62-2 gr #10	20	60	20	100	0	0	0	0	40	52	8	0	52	0	40	8	96	0	4	8	76	16	4,2	95,8	0
B N 62-2 gr #11	0	52	48	84	0	16	0	0	52	40	8	0	60	0	20	20	92	8	0	24	76	0	8	92	0
B N 62-2 gr #12	8	52	40	92	0	4	4	0	28	72	0	0	40	8	32	20	100	0	0	4	88	8	4	76	20
B N 62-2 gr #13	0	100	0	16	0	36	48	0	4,2	91,6	4,2	0	20	72	0	8	48	48	4	100	0	0	76	24	0
B N 62-2 gr #14	8	92	0	0	0	48	16	36	4,2	95,8	0	0	36	12	16	36	60	40	0	100	0	0	44	52	4
BN Tent #1.	4	72	24	96	0	0	0	4	8	64	28	0	44	44	12	0	88	8	4	20	80	0	4	96	0
F-19 23-1 #2	0	64	36	0	0	60	40	0	72	28	0	0	100	0	0	0	28	36	36	100	0	0	80	20	0
F-19 26-2 #2	0	96	4	20	0	72	8	0	68	32	0	0	52	4	40	4	28	56	16	100	0	0	92	8	0
F-19 26-2 #3	0	80	20	0	0	69,6	30,4	0	21,7	78,3	0	0	30,4	52,2	17,4	0	56,5	34,8	8,7	100	0	0	69,6	30,4	0
F-19 26-2 #4	0	14,3	85,7	4,8	38,1	42,9	14,3	0	28,6	66,7	4,8	0	71,4	14,3	14,3	0	66,5	33,3	0	100	0	0	81	19	0
F-19 26-2 #6	0	16	84	8	0	40	52	0	44	56	0	0	52	12	36	0	84	16	0	100	0	0	56	44	0
F-19 26-2 #7	20,8	70,8	8,3	100	0	0	0	0	12,5	87,5	0	0	50	0	50	0	91,7	4,2	4,2	33,3	54,2	12,5	4,2	91,7	4,2
F-19 26-2 #8	34,8	43,5	21,7	95,7	0	0	4,3	0	30,4	65,3	4,3	0	82,6	0	17,4	0	90,9	0	9,1	30,4	69,6	0	13	87	0
F-19 26-2 #10	0	33,3	66,7	100	0	0	0	0	13,3	86,7	0	0	86,7	0	0	13,3	93,3	6,7	0	33,3	40	26,7	0	85,7	14,3
F-19 26-2 #11	5	25	70	90	0	5	5	0	55	45	0	0	100	0	0	0	75	0	25	40	60	0	15	50	35

Fig. A2.11: F -19 11-1 #3 self-pollinated line developed in 2008

The F-19 11-1 # 6 was similar to the F-19 11-1 # 3 line but the head shape was rounder (*Table A2.5*). Even if F-19 11-1 # 9 was more uniform than the other clones, its traits showed lesser interest.

In the group of the White Bloomers (WB), the two self pollinated lines #5 and #1 were selected; WB #1 was slightly more uniform and better looking than WB#5, which was principally variable in the color.

Another interesting self-pollinated line was BN tent 4 (*Fig. A2.12*) that had a very uniform green circular head shape with a round point and spineless but, in a few plants, the heads were purple striped on the basis.

In the open-pollinated line BN 62-2 gr op #9 (*Fig. A2.12*), the head shape was also predominantly circular; but the head point was sharp and an high percentage of plants with heads bearing external mucronate bracts have been observed.

Fig. A2.12: BN tent 4 and BN 62-2 gr op 9 developed in 2008

In the *Table A2.6*, the mean productivity per plant and the percentage of the off-type plants for every line is reported. The ranges of THN per each group are different and some

groups, in particular, have lines more productive than the others. For example, in F 19 11-1, the range is variable between 8.42 and 12.0 heads per plant, whereas F-19 has a range between 10.0 and 15.46. The number of off-type plants is also variable. The percentage of off-type plants ranges between 0 and 40%.

Table A2.6: Mean number of heads per plant (TNH) and percentage of off-type plants in BHSC lines evaluated in 2008

Line (I)	THN mean	% off type	**Line (I)**	THN mean	% off type
F-19 23-1 #1	14,4	4	F-19 11-1 #4	10,62	16
BN 62-2#10	13,83	16	F-19 11-1 #5	12,00	16
BN 62-2#13	10,36	8	F-19 11-1 #6	8,57	0
BN 62-2#14	13,36	0	F-19 11-1 #7	11,96	0
BN 62-2#9	10,85	0	F-19 11-1 #8	8,42	4
BN 62-2#5	13,00	0	F-19 11-1 #9	9,72	0
BN 62-2#6	13,50	0	WB #1	-	4
BN 62-2#7	13,00	4	WB #5	-	4
BN 62-2#8	13,00	0	WB #2	-	4
CYL #1	-	20	WB #3	-	4
CYL #10	17,33	0	WB #4	-	0
CYL #11	-	0	**Line (OP)**		
CYL #2	-	16	F-19 19-2	11,46	0
CYL #3	-	4	B N 62-2 gr op #7	13,04	0
CYL #4	-	0	B N 62-2 gr op #8	12,64	0
CYL #7	-	4	B N 62-2 gr op #10	11	0
CYL #8	-	0	B N 62-2 gr op #11	11,32	0
CYL #9	12,67	8	B N 62-2 gr op #12	10,58	0
F-19 #1	15,36	4	B N 62-2 gr op #13	11,64	0
F-19 #10	10,50	0	B N 62-2 gr op#14	11,88	0
F-19 #2	15,44	0	BN Tent #1.	13,36	0
F-19 #3	14,96	4	BN Tent #4	8,71	0
F-19 #4	14,96	4	F-19 23-1 op #2	13,04	0
F-19 #5	10,65	12	F-19 26-2 op #2	15,92	8
F-19 #6	12,00	0	F-19 26-2 op #3	14,48	0
F-19 #7	14,25	36	F-19 26-2 op #4	15,1	16
F-19 #8	13,48	4	F-19 26-2 op #6	14,36	0
F-19 #9	10	0	F-19 26-2op #7	11,23	4
F-19 11-1 #1	10,12	0	F-19 26-2 op #8	11	8
F-19 11-1 #10	9,04	0	F-19 26-2 op #10	10,86	40
F-19 11-1 #11	9,42	2	F-19 26-2 op #11	11,05	20
F-19 11-1 #12	10,26	8	BN Tent #2	13,04	0
F-19 11-1 #14	10,32	0	B N 62-2 gr op #9	8,68	0
F-19 11-1 #2	11,40	0	BN 62-2 gr op#6	13,16	0
F-19 11-1 #3	7,55	4	B N 62-2 gr op #7	13,04	0

All the plants showing a high level of inbreeding depression and those segregating for some traits that were significantly different from the typology of the line, were included in the off-type plants.

Results on the Discriminant Analysis are summarized in the *Tables A2.6* and *A2.7.* The first table reports the percentage of variance explained by each discriminant function. Seven

discriminant functions were necessary to explain the 100% of the variance but only the first three functions explained the 76.5% of the total variance. Function 1 is linked mainly with two traits: the shape of the main head (MHS) and the presence of the mucron (MHM). The second and third functions are linked mainly with the height of the plant (PH) and head point, respectively; the PH explained the 15 % of all the variance.

Table A2.7: Variance explained by each discriminant function and its link with the main traits.

Function	Variance explained (%)	Cumulative variance (%)	Main traits linked
1	48,8	48,8	MHS, MHM
2	15,1	63,9	PH
3	12,4	76,3	MHP
4	9,6	85,9	-
5	6,5	92,4	-
6	5,4	97,8	LT
7	2,2	100,0	MHC, MHSp

Classification functions of the discriminant analysis have been used to study if each plant was classified in its own genotype of provenance. The *Table A2.7* shows the results of the summary classification table. Some of the selected genotypes had a high percentage of cases (plants) correctly fitted in its own group; for example: WB #1 and WB#5 that had both the 87.5% of cases correctly classified as belonging to the genotype WB #1 and WB#5, BN tent#2 and BN tent #4 had a 77 and 74% of cases correctly classified, BN 62-2#9 had the 64%. However, other selected lines have a low number of cases correctly classified in its own genotype group (i.e. BN 62-2#6 and BN 62-2 gr op #6 with 12 and 0% of cases correctly classified). Only 24.1% of the total cases were classified correctly in its own group.

Table A2.8: Percentage of individuals per each line classified in the corrected group (from the summary classification table of the discriminant analysis).

Line (I and OP)	Individuals classified in its own corrected group (%)	Line (I and OP)	Individuals classified in its own corrected group (%)
BN 62-2#5	0	F-19 #5	31,8
BN 62-2#6	12	F-19 #6	0
BN 62-2#7	33	F-19 #7	40
BN 62-2#8	12	F-19 #8	4,2
BN 62-2#9	64	F-19 #9	56,5
BN 62-2#10	33	F-19 #10	12,5
BN 62-2#13	20	F-19 23-1 #1	0
BN 62-2#14	60	WB #1	87,5
CYL #1	30	WB #5	87,5
CYL #2	54,2	WB #2	12,5
CYL #3	24	WB #3	50

CYL #4	32	WB #4	0
CYL #5	0	WB F-19 23-1	4
CYL #6	0	B N 62-2 gr #11	0
CYL #7	0	B N 62-2 gr #12	4
CYL #8	0	B N 62-2 gr #13	4,2
CYL #9	33,3	BN 62-2 op 13	0
CYL #10	28	B N 62-2 gr #14	20,8
CYL #11	40	BN Tent #1	32
F-19 11-1 #1	0	BN Tent #2	76,9
F-19 11-1 #2	62,5	BN Tent #4	74
F-19 11-1 #3	81,8	F-19 23-1 op #2	24
F-19 11-1 #4	41,7	F-19 26-2 op #2	20
F-19 11-1 #5	18,2	F-19 26-2 op #3	0
F-19 11-1 #6	68,2	F-19 26-2 op #4	33
F-19 11-1 #7	45,8	F-19 26-2 op #6	4
F-19 11-1 #8	20	F-19 26-2 op #7	0
F-19 11-1 #9	32	F-19 26-2 op #8	4,5
F-19 11-1 #10	28	F-19 26-2 op #10	14,3
F-19 11-1 #11	0	F-19 26-2 op #11	20
F-19 11-1 #12	0	F-19 19-2 op	60
F-19 11-1 #14	48	BN 62-2 gr op #6	0
F-19 #1	0	BN 62-2 gr op #7	28
F-19 #2	20	BN 62-2 gr op #8	40
F-19 #3	0	BN 62-2 gr op #9	32

A2.3.2 SEED PRODUCTION

2006-2007 Self-pollinated plants (*selfs*)

The *Table A2.9* summarizes the results on seed production obtained after self-pollinations in 2006 and 2007. It was very low in both years varying between 0 and 25 seeds. Italian genotypes did not produce any seed and the American and the Spanish ones produced a very low amount of seeds. The *selfs* of F_1 hybrids were high yielding even though, in 2006, all seed production obtained by self pollination was inconsistent.

Table A2.9: 2006-2007 seed production of self-pollinated male-parents and F_1 hybrids. "I" is the self generation $I_{1=}$ first generation of self

Genotypes	**analyzed plant**	**Self generation (no.)**	**Heads (no.)**	**Seeds (no.)**	**Seeds/head (no.)**
2006 male parents					
AM 1	A3	I_1	5	22	4,4
AM 1	A7	I_1	5	2	0,4
SP	1	I_1	5	25	0,0
S. Erasmo		I_1	0	0	0,0
Terom		I_1	0	0	0,0
Apollo		I_1	5	0	0,0
Exploter		I_1	5	0	0,0

Brindisino		I_1	3	0	0,0
2007 male parents					
SP	2	I_1	2	0	0,0
SP 1 05 I_1	E1	I_2	2	0	0,0
SP 1 05 I_1	C2	I_2	2	0	0,0
SP 1 05 I_1	C3	I_2	1	0	0,0
AM 1	A7	I_1	2	4	2,0
AM 1	A2	I_1	3	4	1,3
F_2 2006					
MS6 X AM1		I_1	2	0	0,0
MS16 X AM1		I_1	2	0	0,0
MS6 X SP 1		I_1	2	3	1,5
MS16 X SP 1		I_1	2	16	8,0
F_2 2007					
MS6 X AM 1 05	A2	I_1	2	94	47,0
MS6 X 12 05	A2	I_1	2	35	17,5
MS6 X SP1 06	1	I_1	2	12	6,0
MS6 X SP1 06	2	I_1	2	26	13,0
MS6 X SP1 06	3	I_1	2	9	4,5

2008 SELF-POLLINATED PLANTS (*Selfs*)

In 2008, the number of seeds produced per head was consistently higher than in the other two previous years and a different tendency to produce seed from the plants belonging to different American selections (*Tab. A2.10*) has been found. In general, CYL is a bad seed producer, especially when it has been self-pollinated; all the plants coming from the different CYL inbred lines (I_1) produced within a range of 0 and 46 seeds per head. On the contrary, BN-62-2 is a better seed-producer even if it is self-pollinated. The 2nd plant of the self-pollinated line BN 62-2#5 (I_1), in fact, produced 351,6 seeds per head. Moreover, White Bloomer (WB) is also a good seed producer, but the inbred line (I_1) number 5 (WB#5) is a worse seed producer than WB#1. Even in the second inbred generation (seeds are I_2), the seed production of some 2008 self-pollinated lines was maintained high.

Table A2.10: 2008 seed production of self-pollinated male-parents. ("I" is the self generation, I_1= first generation of selfing; *= plants selected from the best lines)

Selfing line	Plant	Heads (no.)	Seeds (no.)	Seeds /head (no.)	Selfing line	Plant	Heads (no.)	Seeds (no.)	Seeds /head (no.)
CYL#1 I_1	1	3	4	1,3	CYL#11 I_1	1	3	74	24,7
CYL #1 I_1	2	3	30	100,0	*BN 62-2 #6 I_1	1	5	25	5,0
CYL#9 I_1	1	0	0	0,0	*BN 62-2 #6 I_1	2	2	15	7,5

BN 62-2 #5 I_1	1	3	2	0,7	*BN 62-2 gr #6	1	3	37	12,3
BN 62-2 #5 I_1	2	4	1.406	351,6	*BN 62-2 gr #6	2	3	0	0,0
* BN 62-2 #9 I_1	1	2	2	1,0	CYL #5 I_1	1	3	27	9,0
*BN 62-2 #9 I_1	2	3	49	16,3	CYL#11 I_1	2	2	0	0,0
*F19 #1 I_1	1	3	6	2,0	F-19 11-1#5 I_1	1	3	153	51,0
*F19 #1 I_1	2	2	13	6,5	*F-19 11-1 #9 I_1	1	3	25	8,3
*F-10 #9 I_1	1	4	208	52,0	*F-19 11-1 #9 I_1	2	3	53	17,7
*F-10 #9 I_1	2	3	9	3,0	CYL #6 I_1	1	1	46	46,0
*F-19 11-1 #3 I_1	1	6	51	8,5	CYL #6 I_1	2	1	1	1,0
*F-19 11-1 #11 I_1	1	3	0	0,0	*WB #1 I_1	1	3	308	102,7
*F-19 11-1 #11 I_1	2	3	13	4,3	*WB #1 I_1	2	4	536	134,1
23-1 op self #1 I_1	1	1	26	26,0	*WB #5 I_1	1	7	65	9,3
23-1 op self #1 I_1	2	7	3	0,4	*WB #5 I_1	2	4	4	1,0
*BN 62-2 gr #9	1	3	895	298,3	*F-19 #4 I_1	1	2	222	11

SIB-CROSSES (2008)

The number of seeds per head obtained by sib-crosses in 2008 was higher than the seed-production per head obtained by the same lines after self-pollination (*Tab. A2.11*. For example, two plants of BN 62-2 #9 I_1 produced only 1 and 16 seeds per head, when they were self-pollinated, while the inbred line BN 62-2 #9 I_1, when sib-crossed, produced an average of 161,9 seeds per head. Moreover, two plants from self-pollination of F19 #1 I_1 produced only 2 and 6,5 seeds per head, while the mean production of the F19 #1 I_1 line was 300,6 seeds per head.

There were some lines that produced a low amount of seeds both in self-pollination and sib-crosses. For example, they are WB # 5 I_1, F-19 11-1 #11 I_1 , F-19 11-1 #9 I_1, BN tent #4 and BN tent #2 produced in sib-crosses 35.5, 18.0, 69.0, 38.1 and 63.5 seeds per head, respectively. Therefore, these five lines are the worst seed producers than the other lines and may also be the worst pollen producers.

Tab. A2.11: 2008 seed production from male-parent sib-crosses ("I" is the self generation $I_{1=}$ first generation of self).

Line selected	Heads (no.)	100 seed weight (g)	Total weight (g)	Ext. no. of seeds (if>100)	Ext. no. of seeds/head (if>100)
BN 62-2 #9 I_1	97	4	628	15700	161,9
F19 #1 I_1	68	3,05	314	10295	151,4
F-19 #9 I_1	120	3,59	1295	36072	300,6
F-19 11-1 #3 I_1	70	5,16	534	10349	147,8
F-19 11-1 #11 I_1	82	5,22	77,22	1479	18,0
BN 62-2 gr #9	56	4	668	16700	298,2
BN tent #4 I_1	67	4,9	125	2551	38,1
BN 62-2 #6 I_1	88	4,17	542	12998	147,7
BN 62-2 gr #6	137	4,02	450	11194	81,7
F-19 11-1 #9 I_1	104	3,59	257,59	7175	69,0
WB #1 I_1	62	4,74	494,74	10438	168,3
WB #5 I_1	72	5,05	129,05	2555	35,5
F-19 #4 I_1	120	4,33	1032,33	23841	198,7
BN 62-2 gr #8	80	3,84	357,84	9319	116,5
BN tent #2 I_1	56	4,11	146,11	3555	63,5

2008 F_2 SEGREGATION AND SELECTION OF NEW MALE STERILE PLANTS

From the segregation of some F_2 populations, a study on the genetic determinism for some characters such as male sterility and spineless has been tried. According to the classification proposed by Lannunzel (1999), the plants coming from F_2 segregation have been divided into four fertility classes: 1=0%, 2=1-10%, 3=10-89% F=90-100% of pollen grains stained by acetocarmine. All the plants falling in the first two classes have been classified as male-sterile. In our work, 11 plants fell in the first class, one plant in the second class, and the others had a variable fertility between the third and the F class. Only the plants that fell in the first class have been selected for breeding activity.

Table A2.12: Segregation results for the male-sterility trait

	Cross combination (F_2)	**Total plants analyzed (no.)**	**Segregants for male sterility (no.)**	**Expected value**	**X^2 test**	**Ratio MF/MS**
1	MS6 X AM 1 05	61	0			
2	MS6 X SP1 06 2	5	0			
3	MS6 X SP1 06 1	14	0			
4	MS6 X 12 05	6	0			
5	MS16 X AM 07	44	12	11	0,12 n.s.	3:1

Unfortunately, the first four F_2 populations gave only plants classified as third and F classes, while the fifth gave 12 plants classified as male-sterile plants (*Tab. A2.12*). Tested with X^2, this latter frequency has indicated a 3:1 segregation ratio, which suggests a possible inheritance controlled by a single recessive gene. The populations no. 2, 3, and 4 gave no male sterile plants and a segregation ratio was not established; the number of plants analyzed was not enough for the analysis. The F_2 did not evidence any male sterile.

The segregation results of spineless trait are summarized in the *Table A2.13.*

Table A2.13: Segregation results for the spineless trait

	Cross combination (F_2)	Plants (no.)	Spiny	Expected value	X^2 test	No spiny/spiny ratio
1	MS6 x AM 1 05	56	18	14	1,52 n.s.	3:1
2	MS 16 x AM USA (spiny)	178	29		7,20** 11,13***	3:1 15:1

The F_2 no. 1, which derived from a spiny plant as MF parent, gave a segregation ratio non significantly different from the ratio 3:1, while the F_2 no. 2 gave a segregation ratio significantly different either from the segregation ratio 3:1 or from that 15:1. Only data on the population no. 1 suggested that the bract spineless is governed by a single mutation; it was not possible establish any determinism for the F_2 population no. 2. As reported in literature (Basnizki and Zohari, 1994), the non spiny allele (Sp) should be dominant over the spiny wild type.

The *Table A2.14* summarizes the number of seeds produced after both self- and open pollination by the new male sterile plants selected in 2008. The results of the self-pollination confirmed the results obtained by acetic carmine staining. In fact, these plants were selected

during the flowering period because their pollen grains were stained in acetocarmine. When self-pollinated, all the plants selected did not produce any seed.

Their tendency to produce seeds was evaluated by counting the number of seeds per head produced in open-pollination conditions; the plants 3, 8 and 10 produced a high amount of seeds per head, comparable with the amount produced by the male fertile lines, while the plants 1, 5 and 11 gave a low quantity of seeds.

Table A2.14: 2008 new male-sterile plants [number of seeds produced by selfing (S) and open-pollination (OP)]

Plant	**Map. Pos**	**N° of heads (S)**	**N° of seeds (S)**	**N° of heads (OP)**	**N° of seeds (OP)**	**N° of seeds /head (OP)**
1	46-3	11	0	5	21	4,2
2	46-17	5	0	10	481	48,1
3	46-18	6	0	20	3636	181,8
4	46-20	2	0	3	149	49,7
5	48-0	11	0	14	69	4,9
6	48-1	13	0	1	0	0,0
7	48-16	4	0	16	532	33,3
8	48-30	1	0	17	2282	134,2
9	48-32	1	0	16	238	14,9
10	48-35	1	0	16	1952	122,0
11	48-36	3	0	7	103	14,7

MICROPROPAGATION

The average level of shoot proliferation, calculated in four months of activity, is indicated in *Table A2.15* The highest level of proliferation was obtained by the clone MS6 while the lowest one was obtained by the genotypes AM 1 and AM 2, both coming from USA.

Table A2.15: Average level of proliferation of the shoots calculated in four months of activity.

Genotypes	Micropagation ratio
MS6	1.94
MS16	1.7
MS14	1.5
AM1	1.4
AM2	1.34
SPINOSO SARDO	1.6
MS6 x AM1 A3	1.7

*A2.4 **DISCUSSION***

The first step for the constitution of new F_1 hybrids is the development of stable parental lines. In general, these lines can be propagated vegetatively from one single plant, which resulted as a good parent due to its cross ability efficiency. The genotype choice becomes necessary for the propagation of the female parents; male sterile plants can be propagated only vegetatively. For this reason, an *in vitro* collection of globe artichoke parental line was started in 2006 and is still maintained at ENEA, in Rome (Italy).

Seed propagation of the male parents could be possible with the availability of pure lines. Nevertheless, in globe artichoke, the pure lines are not available because of its high level of heterozygosity, which is strongly connected with the traditional vegetative propagation system used during the crop domestication (*Sonnante et al.,* 2007). Heterozygosity is also responsible for the low homogeneity shown by the globe artichoke F_1 hybrids. The high level of heterozygosity in artichoke has been demonstrated by Basnizki and Zohary (1994), who reported that selfing of clonally propagated globe artichoke varieties led to a high level of morphological segregation in the offsprings, accompanied by a considerable inbreeding depression. Another problem linked to the production of pure lines in globe artichoke consists in the predominant out-crossing of the plants that causes an high level of inbreeding depression too, since the first inbreed generations. The primary consequence of this depression is expressed as a drastic reduction of pollen and seed production.

Therefore, a good male parent has to be stable and also as a good pollen producer. For this reason, in the genetic improvement of male parental lines, it is recommended to alternate self-and sib crosses generations to reduce the inbreeding effects and guarantee a good performance in the pollination. For this reason, in 2008, we have chosen to carry on at the same time sib and self generations of the same plants with the aim of comparing, in the following years, the lines obtained both by selfing and sib-crosses and then compare their level of stability.

In 2008, the number of lines to analyze was very high and we had to find a quick and repeatable system to identify the most stable ones; in addition, the lines to use in developing the new male parents have been selected. The system had to be quick because there were 70 lines under cultivation and they changed rapidly their characteristics. Therefore, the first step was to choose some important traits to consider as descriptors in the genotype morphological characterization. For each trait, the lines showing the highest frequencies of one class and the lowest range of variation have been selected. In this way, only the most stable lines have been chosen.

The statistical analysis has been combined with the requirements of the BHSC and with the direct observation and the experience of Mr. J. R. Jordan. The American Company wanted to select and keep some lines for every group: for example, some lines of the White Bloomer (WB) group, some lines of the BN 62-2 group, and some lines of the F-19 group, etc.

All the plants showing a high level of inbreeding depression and those segregating for traits different from the typology of the line were included in the "off-type" plants. However, in some case, individuating some varieties as a "type" was quite difficult because their stability was low and their segregation for the traits considered was too high (i.e. F-19 26-2 #11 and F-19 #7, that had respectively the 40 and the 36% of off-types).

The discriminant analysis was performed to see which traits affected more the total variability of the groups and which lines were less variable than others.

The classification matrix is a common result that can be useful to determine how well the current classification functions predict group membership of cases. The classification matrix shows the number of cases that were correctly classified (on the diagonal of the matrix) and those that were misclassified. In other words, this matrix summarizes the classification of each particular case. If one case (plant analyzed) is classified in its own group, it is classified correctly. In the work, only the percentage of cases for each group that resulted correctly classified has been reported. Only the 24.1% of the total cases were classified correctly in the groups. This could be understandable if we consider the high number of lines evaluated and the differences in the stability of all the lines. In fact, from the frequency analysis, some lines that were characterized by a high variability of the evaluated traits have been recorded; some lines showed a similar affinity with each other. Moreover, all the traits analyzed might have not been enough to highlight the variability among groups that were very similar and that belonged to the plants of the same selections. To confirm this, we can report that the 76.5% of all the variance was explained by only three traits that were the most variable in the field. For example, PH explained a high percentage of variance but, however, this is a trait that is strongly influenced by inbreeding depression (Baznizki and Zohary, 1994). Consequently, the lines that had a high segregation for PH trait resulted not stable, even if the other traits were homogeneous. This could have significantly weight in the results of the classification summary table related to the discriminant analysis.

On the basis of the results on the frequency and on the discriminant analyses, it is possible to predict which varieties will be the most stable this year. Moreover, combining these results with the seed production obtained in the sib crosses as well as in the crosses with

male sterile plants (*see* the chapter B1 and B2), we can predict also which will be the best male parental lines among all the BHSC lines. In the following year, the evaluation of the homogeneity of the hybrids obtained with these lines will be able to confirm our hypothesis.

Seed production obtained by selfing in the first two years was very low, especially when compared with the production obtained by the selfing during the previous year. This could be partly explainable by the differences of environmental conditions; in fact, it is reported that the dry weather condition give the best results in seed production (Baznizki and Zoary, 1994). Therefore, during the third year, the dry condition of the South-west of California could have favorably influenced the seed production. Nevertheless, during the first year, in Italy, American lines showed better trend of producing seed respect to Italian clones. This could be due to the different tendency of American varieties to produce seeds. In fact, in Europe, because of the traditional vegetative propagation system, selection for good seed producer varieties started only in the past years whereas, in the United States, the selection for a good seed production was always considered as important; in USA, in fact, globe artichoke is traditionally also propagated by seeds. These tendencies have to be confirmed by further studies.

In the majority of the lines, the materials coming from sib-crosses appeared more seed productive than those from self-pollination. This could be explained by a higher efficiency of bees respect to the brushes in the pollination. However, Morison (2000) demonstrated that pollination by brushes had the same or higher efficiency than pollination by bees.

In the self pollinations performed in the thesis program, only two plants for each line have been selected and then self-pollinated. Consequently, the result depended on the single plant that tended to produce both pollen and seed. In the sib-cross experiment, a higher number of plants of the same line have been used. Therefore, the performance of the sib-crosses depended on the tendency of all the plants of the same line to produce pollen and seed and on their inter-compatibility.

In the evaluation of seed production of the sib crosses, some lines producing a low amount of seeds both in self and sib-pollinations have been found. This could be due to a more considerable effect of inbreeding depression that, in globe artichoke as in other allogamy species, can be already observed in the first inbred generations and can provoke a reduction of pollen and seed production (Principe, personal communication).

In 2008, the segregation of traits such as male-sterility and bract spineless were analyzed. About the male-sterility, unfortunately, the results are not completed because male sterile plants were found only in one F_2 progeny and the number of plants analyzed for each

progeny was low. The segregation ratio 3:1 obtained, could confirm the results reported by Principe (1984), but it is in contrast with Baznizki and Zoary (1994) that discovered two additional non allelic recessive male-sterility genes: ms_2 and ms_3, which were widely used in their breeding work. Stamigna *et al*. (2004) also confirmed a segregation ratio corresponding to 2 recessive genes. Data on segregation for bract spineless confirmed what has already been reported in literature (Baznizki and Zohary, 1994; Lanteri *et al*., 2006).

An objective of this work was also to develop new stable male sterile lines that can be used as female parents. In fact, it was important to get a large spectrum of female clones in order to test the possible F_1 hybrid combinations and satisfy all the different needs of the market.

In 2008, eleven new male sterile plants, which have been introduced in the genetic improvement programs of this year, have been selected from the segregation of the F_1 hybrids selfed in 2007. The stability of the clones in the crosses and their productivity will be evaluated this year. Moreover, it was crucial to find new male sterile plants that are able to produce a high number of hybrid seeds per head. A number of the male sterile lines available and used in the breeding programs, although exhibiting an adequate female fertility when hand pollinated, can produce a little or no seeds in industry conditions (Chatelet, 2005). Consequently, the economical production of F_1 hybrid seed after adequate pollination of male sterile plants remains up now difficult to achieve (Foury *et al*., 2005).

In our work, the tendency of male sterile plants to produce seeds, when open-pollinated, was considered as a valid index of their potentiality to produce hybrid seed. In fact, this depends on the plants genetic capability to produce seed and on the attractiveness of the flowers against the pollinating insects. Morison *et al* (2000) demonstrated that the globe artichoke male sterile plants available in the experiment were not attractive for the bees. However, in the same experiment, there were also some male fertile lines that were unattractive for the bees. Consequently, we can assume that some male sterile plants could also have been more attractive than the others. The first indication of this different level of attractiveness was considered properly by the number of seeds per head produced in open-pollination conditions. In this work, we found some male sterile plants that produced, in open pollination, a number of seeds per head comparable with that produced by male fertile plants.

Section B

F1 HYBRIDS

Chapter B1

Pollination techniques

B1.1 INTRODUCTION

Development of cultivars and hybrids are important objectives in globe artichoke breeding programs. Economical use of lines or F_1 hybrids is dependent on seed production and, consequently, on the possibility of carrying out successful crosses (Bernal *et al*., 2005).

Due to the traditional vegetative multiplication, globe artichoke has partially lost its seed production ability (Foury *et al*., 1978). Inbreeding and environmental sensitivity affect both vigor and seed production. Fertility is generally low and the percentage of seeds per head is lower than its potential production (Pécaut, 1993).

Since self incompatibility has not been identified in this species (Chatelet *et al.,* 2005), breeders relied on a highly stable, genic male sterility system to insure the hybrid nature of seeds produced on female lines. However, a number of these lines, although exhibiting adequate female fertility when hand pollinated, yielded little or no seed in seed industry conditions.

In this work, different pollination systems for hybrid seed production were compared in order to find the most efficient pollination system and in order to evaluate its potential in the seed productivity of our male sterile lines.

In 2006, the field experiment were carried out at the Regional Agency for the Development and Innovation of Agriculture (ARSIAL), in Tarquinia (Viterbo, Italy). In 2008 open field activities were carried out in the experimental fields of Big Heart Seed Company (BHSC), in Brawley (California, USA).

In 2006, manual pollination by brushes and pollination by bumblebee (*Bombus terresris*) were compared with an open pollination control.

In 2008, the effectiveness of honey bees (*Apis mellifera*) as pollinators was evaluated in order to verify if, changing the percentage of both male sterile and male fertile plants, there were some differences in the seed production. Moreover, the pollination by bees was compared with the manual pollination by brushes and open pollination. In both experiments, seed production of the male sterile and male fertile plants was compared.

B1.2 MATERIALS AND METHODS

GENOTYPES

In 2006, two BHSC male lines (AM1 and AM2) were considered as good pollinators, and the male sterile clone MS6 was used in the experiment (*see* Chapter A2).

In 2008, five BHSC different lines were used as male parents, while the female parent was the clone MS6 (*see* Chapter A2). The name of the male lines used, the number and the different percentage of male and female parents are summarized in *Table B1.1*

Table B1.1: Cross-combinations and number of plants used in the experiment during 2008.

Cross-combination cross	Name	Plant (no.)		Plants (%)		Tents size (m)
		MF	MS6	MF	MS6	
	Experiment 1					
F-10 #9 I_2 x MS6	Tent3	19	20	50	50	2.7 x 14.3
BN 62-2 #9 I_2 x MS6	Tent1	8	13	38	62	2.7 x 14.3
F-19 11-1 #3 I_2 x MS6	Tent4	10	21	30	70	2.7 x 14.3
F-19 11-1 #9 I_2 x MS6	Tent11	5	29	18	82	2.7 x 14.3
	Experiment 2					
22BD xMS6	tent1	36	36	50	50	9.1 x 18.3
22BD xMS6	tent2	25	47	35	65	9.1 x 18.3
22BD xMS6	tent3	18	54	25	75	9.1 x 18.3
22BD xMS6	tent4	8	64	10	90	9.1 x 18.3
22BD xMS6	manual control	30	30	50	50	9.1 x 18.3

Some other stable lines already tested by BHSC were planted in isolated fields (more than 5 km far from other artichoke fields) and crossed with the clone MS6 under open pollination conditions. The name of the male fertile lines used in the crosses and the total number of both male fertile and male sterile plants are indicated in *Table B1.2*

Table B1.2: Cross-combinations under open pollination conditions during 2008.

Combination cross	Plants no.	
	MF	MS6
F-1 17-1 x MS6	1200	300
F-1 22BD x MS6	900	289
F-1 9BDG x MS6	1600	184
F-1 F-19 Red x MS6	900	300
F-1 BN x MS6	1200	292

F-1 F-19 CXL x MS6	1200	300

SOWING

The male plants used in Tarquinia during 2006 were sown in Albani e Ruggeri Nursery of Civitavecchia (Italy). The planting system was the same already indicated in the Chapter A2 for the sowing of the seed propagated parental plants in 2006. Seeds were sown on August 8th, 2005. The plants of the clone MS6 were obtained by shoots, which were collected from the mother plants during the spring 2005 and then grown in the nursery until September. MS6 plants where transplanted at ARSIAL fields in September 2005. Transplantations were made in plots composed by three male fertile (AM 1 or AM2) and 6 male sterile (MS6) plants. The plants were planted in two rows 120 cm apart and, in each row, the plant were 100 cm apart. There were three plots (pollination by bumblebee, pollination by brushes, open pollination) for every cross-combination (AM1 x MS6 and AM 2 x MS6).

In 2008, sowing was made directly in the field on November 1st, 2007, as indicated in the Chapter A2. The plants were planted in rows 180 cm apart and plants were spaced 0.9 m in each row. For this experiment, the rows of the male sterile plants were alternate to the rows of the male fertile ones.

The plants were separated in blocks with a variable number of male fertile and male sterile plants (*Table B1.1*) to obtain different percentages of the two genotypes per each block.

POLLINATION TECHNIQUE

- Isolation structures

Four adjacent 6 x 2 m tents (*Fig. B1.1*), installed at the beginning of flowering and covered by 1-mm mesh screen, were used to isolate both pollination by bumblebees and pollination by brushesOpen pollinated plants were not isolated to allow pollination by all the globe artichoke pollinator insects.

Fig. B1.1: Tents installed in Tarquinia (Italy) at the beginning of flowering

The isolation structure used in California consisted in tents also covered by 1-mm mesh screen. The dimensions were variable (*Table B1.1*) on the two different experiments (*Fig. B1.2*).

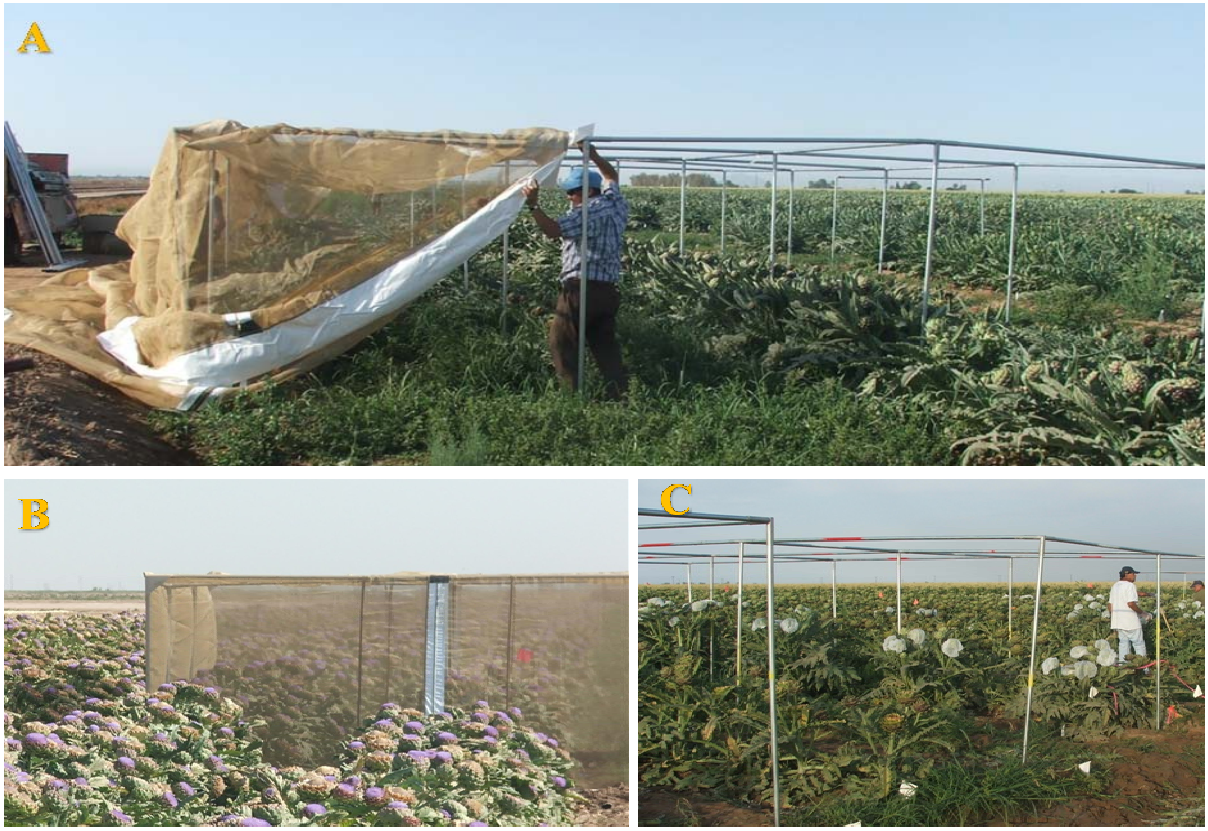

Fig.B1.2: Tents installed in Brawley (Ca, U.S.) at the beginning of flowering

• Pollination by bumblebees (2006)

The bumblebees (*Bombus terrestris*) were supplied by Bioplanet S.P.A. (Cesena, Italy), in colonies of 4,000 individuals. Adult colonies already instructed for the pollination were chosen because they were less durable (two-three weeks) but more efficient in case of a concentrate flowering. The bumblebees were disposed inside the tents (*Fig.B1.3*) at the beginning of flowering (see *Table A2.1*) and supplied with water.

Fig. B1.3: Pollination by bumblebee in Tarquinia (Italy)

• Pollination by brushes (2006 and 2008)

The manual pollination was performed by means of brushes. The male sterile and male fertile flowers were isolated by an anti-aphid net, in 2006 and 2007 (*Fig. B1.4* A, B), and in tents, in 2008. The pollen was recollected early in the morning from the male flower and brushed back to the male sterile flowers. The operation was repeated every day (*Fig. B1.5* A) to maximize the efficiency of the pollination (Bernal *et al*., 2005).

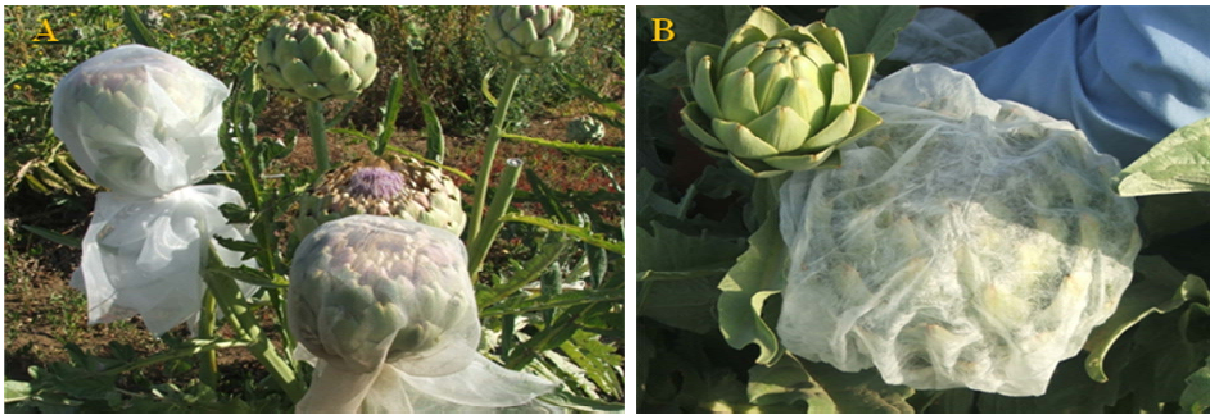

Fig. B1.4: Isolation of the single heads with an anti-aphid net

Fig. B1.5: Pollination by brushes (A) and honey bees pollinating purple and white flowers (B and C.

• Pollination by honey bees (2008)

In 2008, a local bee-keeper supplied beehives of 3,500 individuals plus a queen (*Fig. B1.5* B, C). The beehives were disposed inside the tents at the beginning of flowering (see *Table A3.1*) and supplied with water.

One month after the end of flowering (*Fig. B1.6 A*), the seeds were already mature enough to be collected (*Fig. B1.6 C* and *D*), so the heads were cut and stored in a shady dry place for other 10 days in order to allow drying completely.

The seeds were collected manually both in 2006 and 2007. During the last year of the experiments, the seeds were collected by combine harvester machine (*Fig. B1.6* B).

Fig. B1.6 Dried head ready for the seed collection. (A) collection by combine harvester machine, (B) globe artichoke seeds (C and D)

• Analysis system

The total number of seeds produced was evaluated and the average number of seeds per heads and per plant were calculated. If the number of seeds was higher than 150, the total number of seeds was estimated counting and weighting 100 seeds for three times and calculating an average weight of 100 seeds. Then, the estimated total number of seeds dividing the total weight for the average weight of 100 seeds has been calculated.

In 2006, in order to verify some possible significant differences between the different methods applied, an ANOVA analysis was performed.

In 2008, the density of insects that visited the flowers was evaluated in the tents of the first experiment and in one field of open pollination (F-1 F-19 Red x MS6). The evaluation was made during the two week of the highest level of flowering, counting the number of bees visiting the flowers of 6 male sterile and male fertile plants per tent and in open field.

B1.3 RESULTS

SEED PRODUCTION

The number of seeds/plant, obtained by the clone MS6 pollinated with the male fertile plants AM 1 and AM 2 using three different systems such as (i) pollination by bumblebee, (ii) manual pollination, and (iii) open pollination, is reported in *Figs B1.7 A* and *B*. Less than 60 seeds/plant were obtained by the female parent MS6 crossed with AM1 and AM2 in all the three conditions. Although bumblebees seem favor seed the sib-crosses among AM1plants, also in this case, seed production always remains low for each of the three pollination systems used. Moreover, a high level of variability in the single plants and the heads analyzed has been evidenced; a lot of empty heads has been found either for MS6 or for the genotypes AM 1 and AM 2.

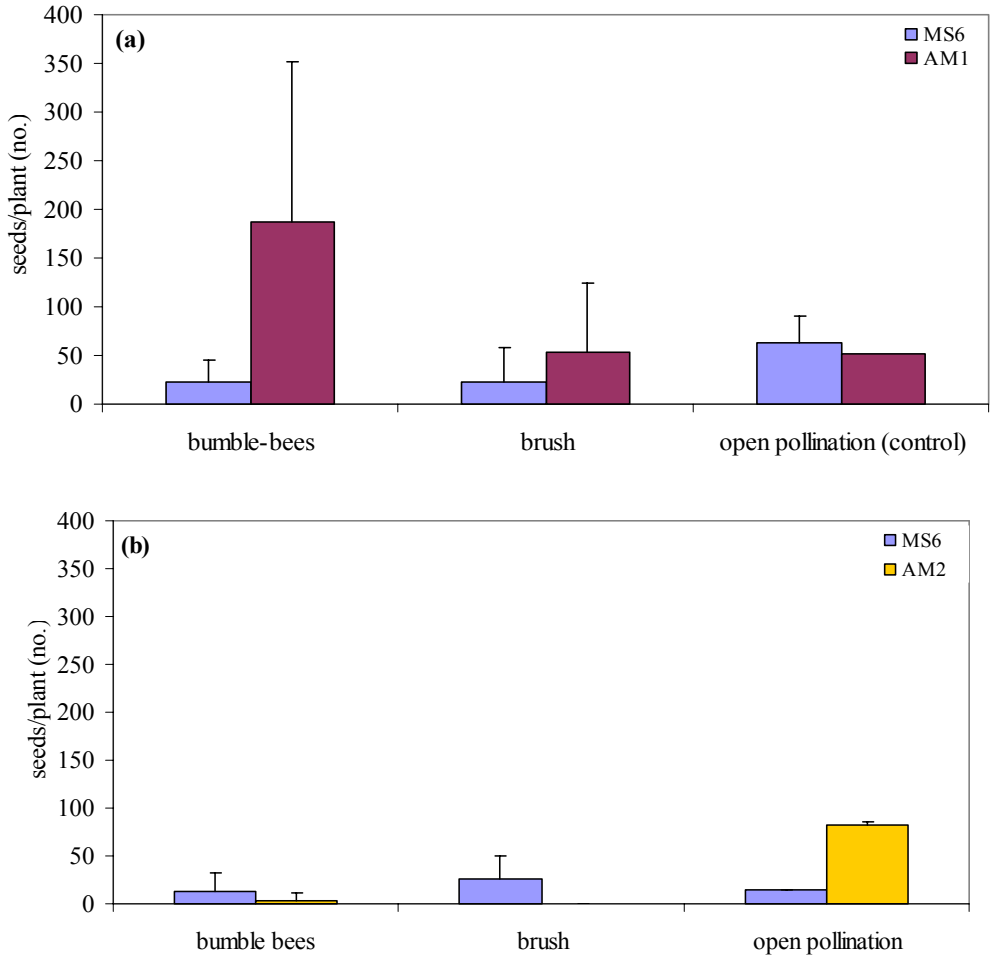

Fig. B1.7: Seed production obtained by the clones MS6, in the crosses, and AM1 (A) and AM 2 (B), in the sib-crosses, using different pollination systems.

The high variability among plants in the same conditions was responsible of the statistically no significant differences that were found among the different systems and the genotypes considered. In addition, during 2006, there was a consistent infestation of *Larinus cynarae*, a curculionid typical of the *Compositae*. Its grubs are parasites of the globe artichoke heads and they can compromise seed production. This infestation produced a high percentage of empty heads.

The number of seeds/plant obtained by both the male sterile (MS6) and male fertile (22BD) plants, when pollinated by honey bees (2008), is reported in *Fig. B1.8*. The number of seeds per plant obtained by the clone MS6 was very low. Less than ten seeds per plant were obtained in all the situations and also the manual control gave a low amount of seeds (only 13,5 seeds for plant).

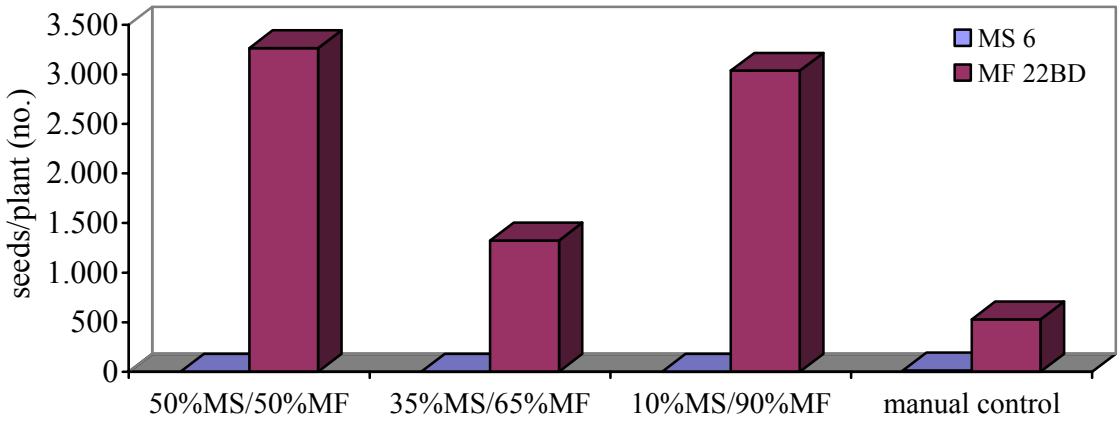

Fig. B1.8: Seed production after honey bee pollination using the same MF genotype per tent (US, 2008)

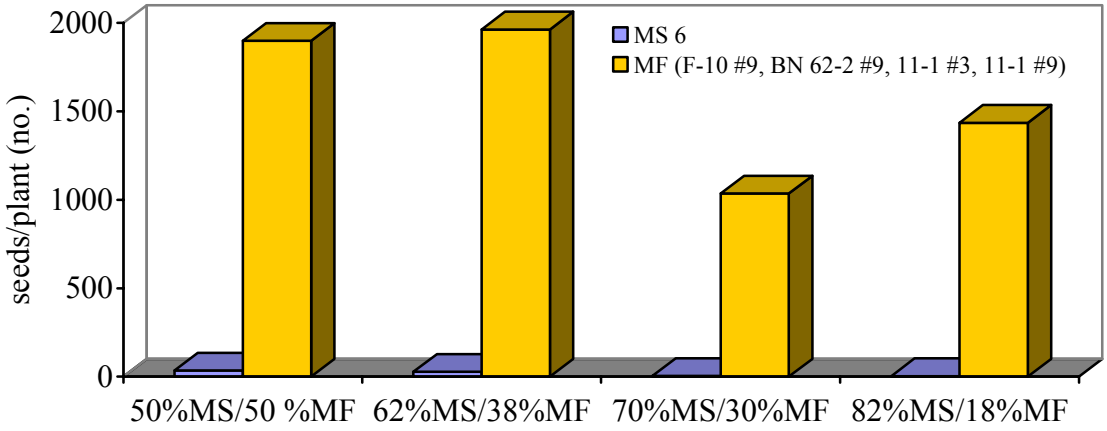

Fig. B1.10: Seed production after bee pollination using different MF genotypes per tent (US, 2008).

These results were confirmed by the other experiment in which different MF lines were evaluated. Also in this case, the amount of seeds produced was low; all cross-combinations produced less than 50 hybrid seeds per plant.

An interesting result was obtained evaluating the number of seeds produced under open field conditions. In all cross-combinations, the number of seeds per plant was higher than in isolated conditions. Only MS6 x F-19 CXL produced less than 20 seeds per plant. The highest levels of hybrid seed, was obtained by the combination cross MS6 x BN that gave almost 160 seeds per plant. However, also these values are consistently lower than those obtained by the MF clone in the other experiments.

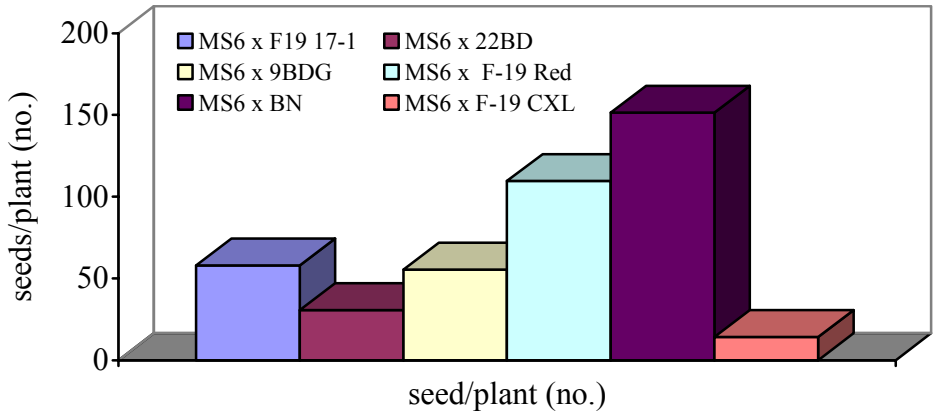

Fig. B1.11: Seed production under open pollination conditions for different cross-combinations (US, 2008)

In order to understand deeply the honey bee behavior, a study on the level of attractiveness by flowers was performed during the two weeks of more intense activity of the insects.

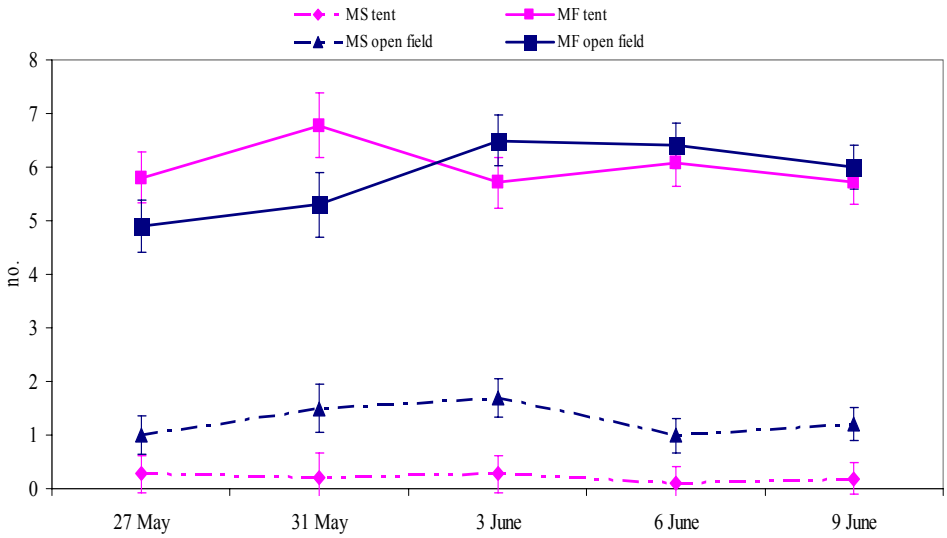

Fig. B1.12: Number of visits of honey bees on MS and MF flowers (USA, 2008)

The density of the insects that visited the various lines showed that, in both conditions (open pollination and in the tents), there were significant differences between the number of bees pollinating the male sterile and the male fertile flowers. In fact, in the male fertile flowers, the number of bees per head counted was higher in both conditions (open pollination and in the tents). An interesting result is that the number of bees counted in the male sterile flowers, under open field conditions, was higher than in the male sterile flowers in the tents, even if the density was consistently lower than that evaluated in the male fertile flowers.

B1.3 DISCUSSION

The results confirmed in part what was found in literature about the difficulties to produce globe artichoke hybrid seed in industry conditions (Chatelet, 2005).

To our knowledge, there are not data in literature on the possibility of using bumblebee for globe artichoke pollination. This insect is a natural globe artichoke pollinator (Pinzanuti *et al.*, 1981) and could represent an interesting alternative to the use of honeybees. Unfortunately, the results obtained in 2006 were very variable and could be interesting to repeat the experiment. Unfavorable environmental conditions and the infestation by *Larinus cynarae* determined further problems to seed production.

American varieties which are normally good seed producer, in 2006, produced a low amount of seed also under open pollination conditions.

The result of the first experiment in USA (*Fig. B1.9*), suggest that there was low compatibility between both the genotypes MS and MF chosen for the experiment in fact also the manual control gave an inconsistent amount of hybrid seed. It's possible to hypnotize that in this case two factors influenced the results: the low attractiveness of the bees of the MS flowers but also the low compatibility between the two genotypes. This is confirmed also from the results of the open pollination condition (*Fig. B1.11*). In fact, the cross-combination MS6 x 22BD gave less than 30 seeds per plant also in open field conditions.

The results of the two experiments performed in USA (*Fig. B1.9* and *Fig. B1.10*), confirmed what was reported in literature by Morison *et al.* (2000) and by Chatelet (2005): there is a different level of attractiveness of the honey bees among the different male fertile and male sterile lines and, in general, the male sterile lines are less attractive for the honey bees than the male fertile lines. However, also the male fertile lines could be less attractive, and could be possible find MS lines which are more attractive (see Chapter A2).

As already analyzed in Chapter A1, the attractiveness of the bees in part depends on the lack of pollen (an obvious deterrent to bees in their pollen-gathering period) but this cannot totally explain flower unattractiveness to nectar-gathering individuals. Moreover, the observations of the nectar-producing structures did not show any difference between the male sterile and male fertile flowers. Some differences in the nectar composition could probably explain these differences.

The results of the study on the density of the honey bees in the globe artichoke flowers could suggest an explication to the higher production of hybrid seeds in open field conditions. In fact, it is possible that the highest number of bees that visited the male sterile flowers in

open pollination condition plus the contribute of some others pollinators increased the number of hybrid seed produced in this condition.

Chapter B2

Development of new F_1 hybrids

B2.1 INTRODUCTION

To develop new F_1 hybrids, the parental lines described in the *Chapter A2* have been used in different cross-combinations. After an evaluation of the phenotypic traits, the most stable cross-combinations have been chosen. During the first two years, all the field experiments were carried out at the Regional Agency for the Development and Innovation of Agriculture (ARSIAL), in Tarquinia (Viterbo, Italy). In the third year, open field activities were carried out in the experimental fields of Big Heart Seed Company (BHSC), in Brawley, California (USA).

B2.2 MATERIALS AND METHODS

GENOTYPES

The hybrids already available in the field according to the cross-combinations indicated in *Table B2.1* of this chapter were analyzed in 2006. The parental clones SP1 and AM1 were already described in the Chapter A2, while 'C3' is a very common "Romanesco" clone (*see* General part), 'Terom' is an early selection belonging to Violetto di Toscana, '12' is a MF parent belonging to the INRA F_2 segregation population (*see* Chapter A2).

The genotypes used as male parents for the production of F_1 hybrids both in 2006 and 2007 were already described in the Chapter A2. The cross combinations and the number of heads covered per every cross-combination are summarized in the *Tables B2.8* and *B2.9* of this chapter, respectively.

Moreover, in 2007, pollen coming from BHSC plants collected in Brawley (CA) and then stored at 4°C was brought to Italy and here used in the crosses. The cross-combinations and the number of heads used per cross are indicated in the *Table B2.10* of this chapter.

In 2008, the self and OP-lines selected as the most stable lines of BHSC (*see Table A2.10*) were used as MF parents in the crosses. The crosses were performed inside the tents using bees as pollinators; the cross-combinations are indicated in *Table B2.13* of this chapter.

Moreover, some plants of these lines were selected and then used individually as male parents in the crosses with the clone MS6 (the cross combinations and the number of heads covered are indicated in the *Table B2.12* of this chapter). The objective of this experiment consisted in comparing, during 2009, the stability of the F_1 hybrids obtained with all the

inbred line as male pollinator with the stability of a hybrid produced with only one plant of this line as male pollinator.

In 2007, some of the mother plants selected by BHSC, which produced the inbred lines in the same year, were directly used as male parents (the cross-combinations and the number of heads covered are indicated in *Table B2.11* of this chapter). The objective of this experiment consisted in comparing seed production (2008) and the stability (2009) of the new F_1 hybrids developed with both the mother plants and the in inbreed generations obtained by those as male parents.

Some other stable lines already tested by BHSC and planted in isolated fields were crossed with MS6 under open pollination conditions (*see* Chapter B1)

The MS clones used during the three years were the clones MS6, MS16, and MS14 (*see* Chapter A2).

POLLINATION TECHNIQUE

In 2006 and 2007, the pollination technique used for all the crosses consisted in rubbing the male head on the female one. Then, before the beginning of flowering, both MS and MF heads were covered by an anti-aphid net. When the highest percentage of florets was blossoming and the pollen grains were pushed upward out of the corolla, the entire male fertle heads were collected and then stored in the refrigerator (4˚C). When the female parent heads were ready, they were cross-pollinated with the male parent heads stored. The male head were used directly as a brush rubbing the female flowers. This operation was repeated every day early in the morning, until the end of flowering.

Only for the crosses performed, in 2007, between the MS clones and the pollen collected at BHSC and brought to Italy, the crosses were performed by brushes.

In 2008, the pollination technique used was that by brush (see chapter B1), when the male parents were the single plants, while by bees inside the tents, when the male parents were the entire lines selected. In fact, after the selection of the best lines (March 2008) and before the flowering, the tents were installed in order to isolate one or more rows of every male fertile line and one or two rows of the MS6 clone (*see* Chapter B1). In this way, inside the same tent, hybrid seed was obtained from the MS6 heads and sib-cross seed was obtained from the male fertile line heads.

The flowering periods are indicated in the *Table A2.1* of Chapter A2. When the flowering was finished and the head started to dry, anti-aphid net was removed to protect the development of seed from high temperatures.

One month after the end of flowering, the seeds were already mature enough to be collected, so the heads were cut and stored in a shady dry place for other 10 days in order to allow drying completely.

SOWING

Sowing was performed as indicated in Chapter A2. The dates of sowing are the same indicated for the parental lines. In general, F_1 hybrids were planted in plots of 24 plants (6 x 4), depending on the seed availability. In 2006, one plot per cross-combination was planted while, in 2007, 4 replications for every cross-combination were considered. Some of the hybrids indicated in the *Table B2.9* of this chapter (MS6 x SP2, MS6 x SP A2, MS 6 x AM A3 and MS6 x AM A7) were planted in USA during 2007; they were produced in Tarquinia and then sent to California. Moreover, in 2007, also the F_1 hybrid seeds obtained using the BHSC pollen as well as the male sterile clones were sent to USA and there planted (*Table.B2.10*). Only those appearing homogeneous were evaluated.

ANALYSIS SYSTEM

The morphological traits were evaluated on the basis of the ***Protocol for Globe Artichoke*** European Union CPVO (2004); the traits analyzed (*see Table B2.1*) both in 2006 and 2007 are included in those already described in the Chapter A2.

In 2008 evaluation of F_1 hybrids, some traits were added respect to the previous years because they appeared responsible of a consistent variability in the field. These traits were as follows: MHP (main head point), MHM (presence/absence of mucron in the bracts), MBC (presence of golden background color in the external bracts), and SS (number of lateral shoots) (*see* Chapter A2). PD (plant diameter) was removed from the 2008 hybrid analysis because it did not result responsible of a significance variance (*see* the results of 2005 F_1 hybrid PCA). The number of plants analyzed for every cross-combination is indicated in the *Table B2.1.*

The phenotypic data were analyzed using the software SPSS (Field, 2005). The first two years, phenotypic measurements were recorded for all plants of each hybrid while, in the third year, they were evaluated on six plants per the four replications; environmental variation was not estimated because the plants of the replications were not clones. Therefore, every single plant was considered as participating in all conditions to the research experiment (Wendorf, 1997) and the data were analyzed by one-way ANOVA and Duncan test. Data had to be evaluated separately for every year because, in order to find the most homogeneous F_1

hybrid, the majority of the cross-combinations have been changed every year. Moreover, as indicated in literature, a lot of morphological characters analyzed were highly affected by the environmental condition (Lòpez Anido *et al*, 1998; Crinó *et al* 2007). Therefore, for the aim of the project, it was more significant evaluating every group of hybrids in the environmental condition in which they have grown.

For the one-way ANOVA:

- TMH and TPH were transformed in countable variables, counting the number of days between the sowing and the harvesting;
- MHC, MHSp, and MHM, were normalized calculating Log_{10} for each value.

The qualitative traits were ordinated with the frequency analysis. Moreover, Discriminant and PCA analysis were performed for the data of every single year.

The total seed production was evaluated and the average seed production per head was calculated. If the number of seeds was higher than 150, the total number of seeds counting and weighting 100 seeds for three times were estimated, calculating an average weight of 100 seeds. Then, dividing the estimated total number of seeds for the total weight the average weight of 100 seeds has been calculated.

B2.3 RESULTS

2006, 2007, 2008 MORPHOLOGICAL DATA

During 2007, only one cross-combination grew up enough to be evaluated in time. Unfortunately, it was not possible to analyze statistically 2007 data.

In *Table B2.1,* only the results of the one–way ANOVA tests for 2006 and 2008 data have been summarized. In 2006, for almost all traits analyzed, significant differences at 1‰ level have been shown among the cross-combinations; only for PD (plant diameter) and PHD (diameter of the first order of heads number on the lateral shoots), any significant difference has been recorded. In 2008, for PHN (number of the first order of heads on the lateral shoots) and PHD (diameter of the first order of heads on the lateral shoots), statistically no significant differences have been noticed among the crosses, while PD (plant diameter) was already excluded from the analysis. Almost all the traits analyzed in 2008 showed differences significant at 1‰ level; only PHL (length of the first order of heads on the lateral shoots), SHN (number of the second order of heads on the lateral shoots), and MHSp (presence/absence of spines) showed differences significant at 1% level, while for MHD (diameter of the main head) and MHL (length of the main head) the differences were significant at 5% level. The standard error gave a first indication of the most stable hybrids; in fact, there were some cross-combinations that had standard errors lower than others for all the traits. For example, in 2006, the F_1 hybrid MS6 x SP1 05 showed the lowest standard errors for the majority of the traits analyzed while, in 2008, the lowest standard errors were evidenced for the Alyssa 07 F_1 hybrid. On the basis of the traits concerning the main head (MHT, MHD, MHL, MDL, MHS, MHC, MHSp, MCB, and MHM) and the total number of heads (THN), in 2006, hybrids showed a similar level of variability and only MS 6 x SP1 05 was more uniform. In 2008, lower standard errors values were obtained also for MS6 x SP A2 07 as well as for Alyssa 07. In 2007, only the evaluation of MS 6 x SP106, that showed values of standard error comparable with MS6 x SP1 05, has been reported. Moreover, the MS6 x SP1 05 mean values of the main head traits were similar to the mean values of MS6 x SP1 06; in fact, shape, diameter, length, and color of the main head were similar. Both hybrids had spineless and a similar shape index (MDL) of the heads; also the characteristics of the secondary heads and the total number of heads were similar. The Duncan test underlined that, in 2006, the majority of F_1 hybrids produced contemporaneously and, in particular, only the combination MS16 X C3 05 produced 8 days later than the others.

Table B2.1: Mean and standard errors of the phenotypic traits evaluated in 2008. One-way ANOVA and Duncan test results.

2006											
	Plants (no.)	PH (cm)	PD (cm)	SS (no.)	MHT (days)	MHD (cm)	MHL (cm)	MDL	MHS	MHC trasf.	MHSp trasf.
2006											
MS6xSP1 05	6	98.33±2.108 a	143.33± 9.189 a	3.17±0.167	186.00±0.000 a	11.00±0.258 de	8.92±0.200 ab	1.23±0.007 c	5.00±0.000 d	0.30±0.000 ab	0.00±0.000 a
MS16xSP1 05	14	127.15±4.016 b	171.43±4.430 b	3.50±0.174	189.29±1.050 a	10.82±0.353 de	9.79±0.408 bc	1.12±0.036 b	2.93±0.398 bc	0.27±0.033 a	0.00±0.000 a
MS6xAM1 05	11	90.27± 6.933 a	164.55±5.618 b	2.91±0.162	188.64±1.497a	11.60±0.485 e	8.91±0.542ab	1.33±0.063 c	1.54±0.281 a	0.32±0.040 ab	0.00±0.000 a
MS16xAM1 05	16	119.38±4.229 b	173.75±4.269 b	3.25±0.111	186.44±0.437a	10.09±0.271 cd	9.19±0.157 ac	1.10±0.020 b	3.00±0.341 bc	0.40±0.046 ac	0.17±0.038 b
MS6x12 05	14	90.71±3.625 a	167.14±0.176 b	3.17±0.112	190.50±0.930 a	8.86±0.242 ab	8.43±0.164 a	1.05±0.024 ab	3.28±0.322 a	0.42±0.038 bc	0.00±0.000 b
MS16 X 12 05	14	103.57±2.938 a	167.14±6.060 b	3.57±0.326	185.28±3.934 a	8.42±0.319 a	8.81±0.237 ab	0.96±0.034 a	1.43±0.291 bc	0.37±0.027 ac	0.04±0.029 a
MS16xC3 05	13	91.69±4.841 a	166.15±6.749 b	2.85±0.273	197.31±1.151 b	9.58±0.357 bc	10.23±0.250 c	0.94±0.035 a	2.69±0.364 b	0.50±0.029 cd	0.21±0.039 b
MS16 xTER 05	5	126.00±6.964 b	166.00±11.661 b	3.60±0.400	186.00±0.000 a	11.90±0.400 e	11.60±0.678 d	1.03±0.046 ab	4.00±0.000 cd	0.60±0.000 d	0.24±0.06 b
Total		105.53±2.200	166.88±2.156	3.24±0.082	189.03±0.764	10.04±0.166	9.33±0.138	1.08±0.017	2.76±0.154	0.38±0.016	0.08±0.013
F		11.541***	1.526 n.s.	1.586 n.s.	4.39***	11.610***	6.717***	11.891***	8.458***	5.86***	11.306***
2007											
MS6xSP	21	81.50±2.79	163.00±7.75	2.40±0.163	201.00±0.00	11.94±0.17	9.50±0.188	1.26±0.0	5.00±0.0	0.30±0.0	0.00±0.0

1 06		4	3		0	5		26	00	00	00
					2008						
		PH (cm)	SS (no.)	MHT (days)	MHD (cm)	MHL (cm)	MDL	MHS	MHC trasf.	MHSp trasf.	MHM trasf.
MS 6xSP A2 07	24	88.48±1.627a	2.56±0.105 ab	164.78±1.036 b	9.83±0.115 ab	8.96±0.112 a	1.10±0.022 c	3.61±0.46c	0.36±0.018 a	0.00±0.000 a	0.00±0.000 a
MS6xSP2 07	24	89.43±1.913a	2.78±0.087 b	163.43±0.708 b	9.61±0.147 ab	9.11±0.157 ab	1.06±0.023 bc	2.35±0.395 b	0.41±0.019 ab	0.00±0.000 a	0.10±0.030 b
MS6xAM 1 A7 07	24	87.87±1.829a	2.58±0.103 b	163±0.792 b	9.45±0.116 a	9.77±0.099 c	0.97±0.016 a	2.21±0.313 b	0.39±0.020 ab	0.00±0.000 a	0.23±0.027 c
MS 6xAM1 A3	25	96.917±1.625 b	2.33±0.115 a	162.75±0.958 b	9.54±0.195 a	9.47±0.143 bc	1.01±0.0189 ab	1.42±0.21 a	0.44±0.015 b	0.00±0.000 a	0.23±0.027 c
Alyssa 07	40	96.57±0.994b	2.325±0.075 a	151.35±0.362 a	10±0.126 b	10.275±0.5 d	0.98±0.010 a	1.15±0.084 a	0.41±0.014 ab	0.06±0.023 b	0.23±0.020 c
Total		92.46±0.759	2.49±0.044	159.86±0.582	9.72±0.066	9.60±0.077	1.02±0.009	2.01±0.14	0.40±0.009	0.02±0.007	0.16±0.012
F		8.957***	4.080***	72.754***	2.790*	15.757***	10.272***	12.868***	2.340*	3.757 **	16.954***

~ Table A2.1

	PHT (days)	PHN (no.)	PHD (cm)	PHL (cm)	SHN (no.)	TrHN (no.)	THN (no.)
				2006			
MS6 x SP1 05	193 ± 0 a	3.16 ±0.17	10 ±0.166	7.25 ± 0.214 a	5.16 ± 0.307c	0 ± 0	9.33 ± 0.422 a
MS16 x SP1 05	198.21 ± 1.46 bc	3.5 ± 0.17	8.71 ± 0.21	8.21 ± 0.28bc	2.43 ± 0.81bc	5.71 ± 0.42	12.64 ± 1.23b
MS6 x AM1 05	195.36 ± 1.23 ab	2.91 ± 0.16	8.68 ± 0.16	7.54 ± 0.24 ab	3.09 ± 0.74c	3.81 ± 0.96	10.82 ± 1.07ab
MS16 x AM1 05	194 ± 0.683 ab	3.25 ± 0.111	7.875 ± 0.133	7.78 ± 0.164 ab	0.87 ± 0.455ab	5.06 ± 0.381	10.19 ± 0.62ab

MS6 x 12 05	200.67 ± 1.068 cd	2.71 ± 0.321	7.40 ± 0.231	7.23 ± 0.194 a	0.29 ± 0.220 a	5.571 ± 0.441	9.57 ± 0.541 ab
MS16 x 12 05	195.64 ± 1.937 ab	3.57 ± 0.326	6.92 ± 0.264	7.5 ± 0.179 ab	0.14 ± 0.14 a	5.28 ± 0.285	10.00 ± 0.502 ab
MS16 x C3 05	202.64 ± 0.243 d	2.84 ± 0.273	7.83 ± 0.188	8.71 ± 0.241 c	1.69 ± 0.644 ac	3.54 ± 0.550	9.08 ± 1.046 a
MS16 x TER 05	193 ± 0 a	3.6 ± 0.4	7.9 ± 0.29	8.3 ± 0.51 bc	5.6 ± 0.67 c	7.2 ± 1.11	17.40 ± 1.94 c
Total	196.93 ± 0.55	3.17 ± 0.09	8.05 ± 0.114	7.83 ± 0.096	1.817 ± 0.258	4.7 ± 0.245	10.69 ± 0.374
F	6.98***	1.824 n.s.	14.292***	4.644***	9.384***	9.232***	5.036***
				2007			
MS6 X SP 06	213 ± 0	3.4 ± 0.163	10.75 ± 0.134	7.6 ± 0.194	4.4 ± 0.305	*0 ± 0*	8.8 ± 0.388
				20080			
	PHT (days)	PHN (no.)	PHD (cm)	PHL (cm)	SHN (no.)	TrHN (no.)	THN (no.)
MS 6 X SP A2 07	172.67± 1.010 b	2.5 ± 0.104	9.10 ± 0.147 bc	8.56 ± 0.101 a	3.83 ± 0.310 a	4.04 ± 0.348 a	11.37 ± 0.650 a
MS 6 X SP2 07	172.88 ± 0.623 b	2.71 ± 0.094	9.23 ± 0.141 c	8.77 ± 0.156 ab	4.08 ± 0.503 ab	4.21 ± 0.496 ab	12 ± 0.953 ab
MS6 X AM A7 07	171.37 ± 0.560 b	2.45 ± 0.103	8.65 ± 0.132 a	9.12 ± 0.121 b	5 ± 0.255 bc	5.29 ± 0.343 b	13.75 ± 0.522 bc
MS 6 X AM A3 07	172.25 ± 0.812 b	2.375 ± 0.117	8.77 ± 0.162 ab	9.06 ± 0.162 b	4.67 ± 0.286 ac	4.79 ± 0.403 ab	12.83 ± 0.636 ac
Alyssa 07	157.77 ± 0.595 a	2.4 ± 0.1	8.97 ± 0.082 ac	9.03 ± 0.073b	5.26 ± 0.231 c	6.67 ± 0.299 c	14.9 ± 0.611 c
Total	168.09 ± 0.611	2.478 ± 0.047	8.948 ± 0.058	8.92 ± 0.054	4.64 ± 0.148	5.18 ± 0.187	13.19 ± 0.324
F	103.815 ***	1.462ns	3.01ns	3.793**	3.774**	9.533***	4.654***

PH: plant height; PD: plant diameter; MHT: number of days between the sowing and the harvesting of the main heads; MHD: diameter of the main head; MHL: Length of the main head; MDL: MHD/MHL; MHC: main head color; MHS: main head shape; MHSp: main head spines; MHM: main head mucron; PHT: number of days between the sowing and the harvesting of the first order of heads on the lateral shoots; PHN: number of the first order of heads on the lateral shoots; PHD: diameter of the first order of heads on the lateral shoots; PHL: length of the first order of heads on the lateral shoots; SHN: number of the second order of heads on the lateral shoots; Tr HN: number of third order of heads on the lateral shoots; THN: total number of heads;

In 2008. Alyssa 07 produced 8 days earlier than the other hybrids and it was the least variable in MHT. In 2006 and 2008, the mean of earliness, expressed as number of days to the first harvesting, was different; this could be due to differences of the environmental conditions. In fact, in 2008 the plants were grown in a warmer weather than in 2006 and 2007; this could have allowed the plants to produce in 2008 the main heads 30 days earlier than in 2006.

In 2006, the most productive hybrid was MS16 x TER 05, which produced 17 heads per plant; the least productive were MS6 x SP1 05 and MS16 X C3, which produced 9 heads per plant. In 2008, the most productive hybrid was Alyssa 07 with 15 heads per plant, while the least productive was MS6 x SP A2 07 with 12 heads per plant.

Results on the frequency analysis of each main hybrids obtained for some primary ordinal traits such shape, color, and spines of the head are summarized in the following figures, where the main characteristics of the head are also reported.

F_1 hybrid MS6 X SP1 05

Only one frequency class for each trait analyzed was individuated (*Fig. B2.1 A, B, C*). The typical plant of the hybrid (*Fig. B2.1 D* and *E*) had trasverse eliptic, purple striped green and spinless heads.

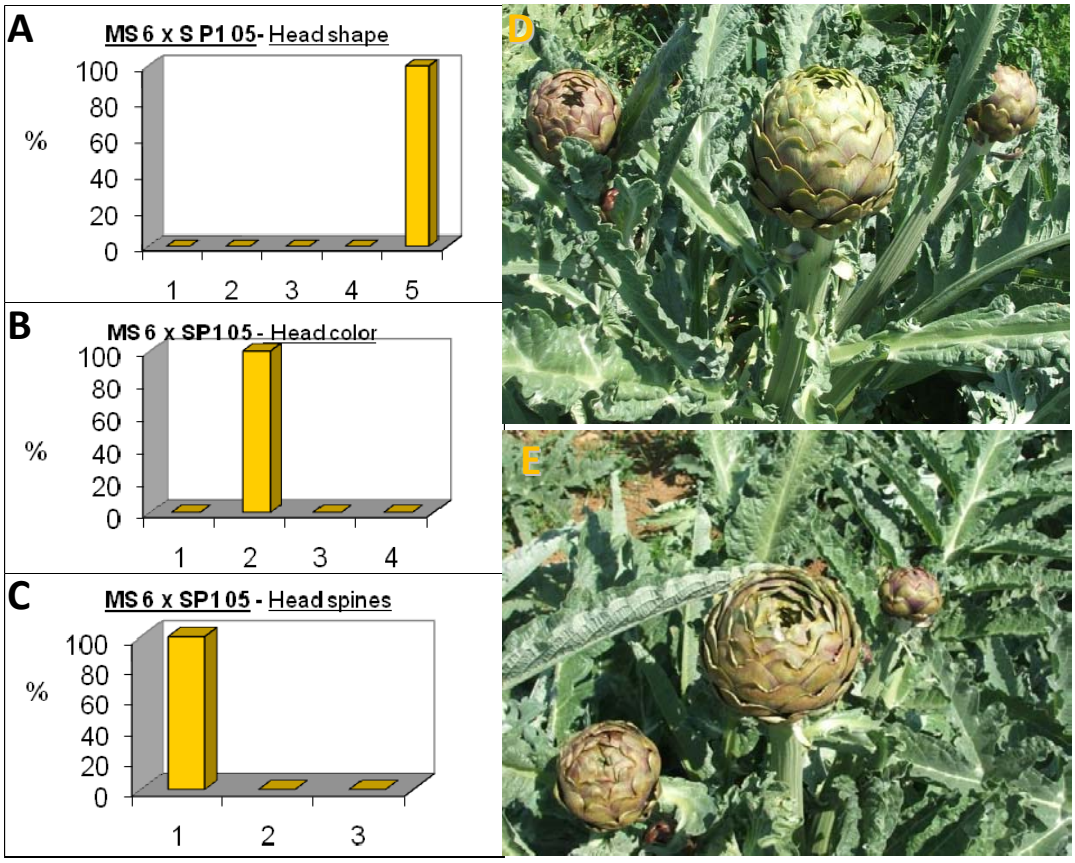

Fig. B2.1: F_1 hybrid MS6 X SP1 05. (A, B, C,) percentage frequency of ordinal traits analyzed in 2006; (D, E) plants of the hybrid.

F_1 hybrid MS6 X SP1 06

Results of the frequency analysis for some primary ordinal traits of the hybrid are summarized in (*Fig. B2.2*) Also in this case, only one frequency class for each trait analyzed was individuated (*Fig. B2.2 A, B, C*). The classes corresponded to those observed in the previous year for the same cross-combination. The typical MS6 x SP1 06 plant (*Fig. B2.2 D* and *E*) had eliptic, purple striped green, and spinless heads.

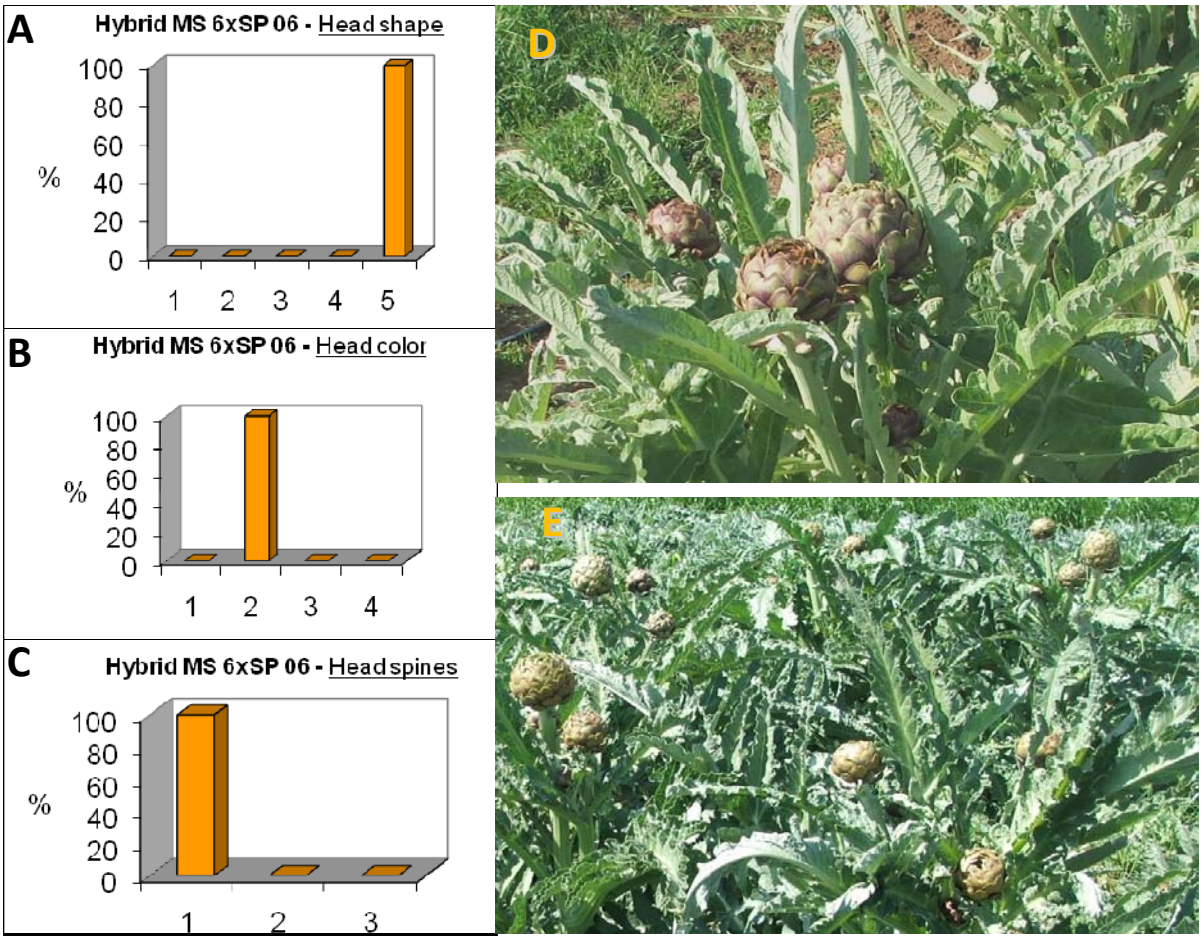

Fig. B2.2: F_1 hybrid MS6 X SP1 06. (A, B, C,) percentage frequency of ordinal traits analyzed in 2007; (D, E) plants of the F_1 hybrid.

F_1 hybrid MS6 X SP2 07

The results on the frequency analysis of the F_1 hybrid MS6 x SP2 07 (*Fig. B2.3 A, B, C, D, E, F*) showed more variability in the shape and color of the main head; in fact, 60% of the plants had circular heads while 36% had trasverse eliptic heads and only 4% triangular heads. The color of the external bracts was purple striped green for 40% of the plants, while 60% had green striped purple heads. In 2008, more qualitative traits were analyzed; all plants were spinless but 40 % of them had the mucron. The shape of the head point was variable with 65% of the plants having a round head point and 35% having a flat head point. Moreover, the majority of the plants (80%) presented a golden background color in the external bracts. On the basis of these morphological traits, it was not possible to individuate a typical MS6 x SP2 07 plant (*Fig. B2.3G, H*).

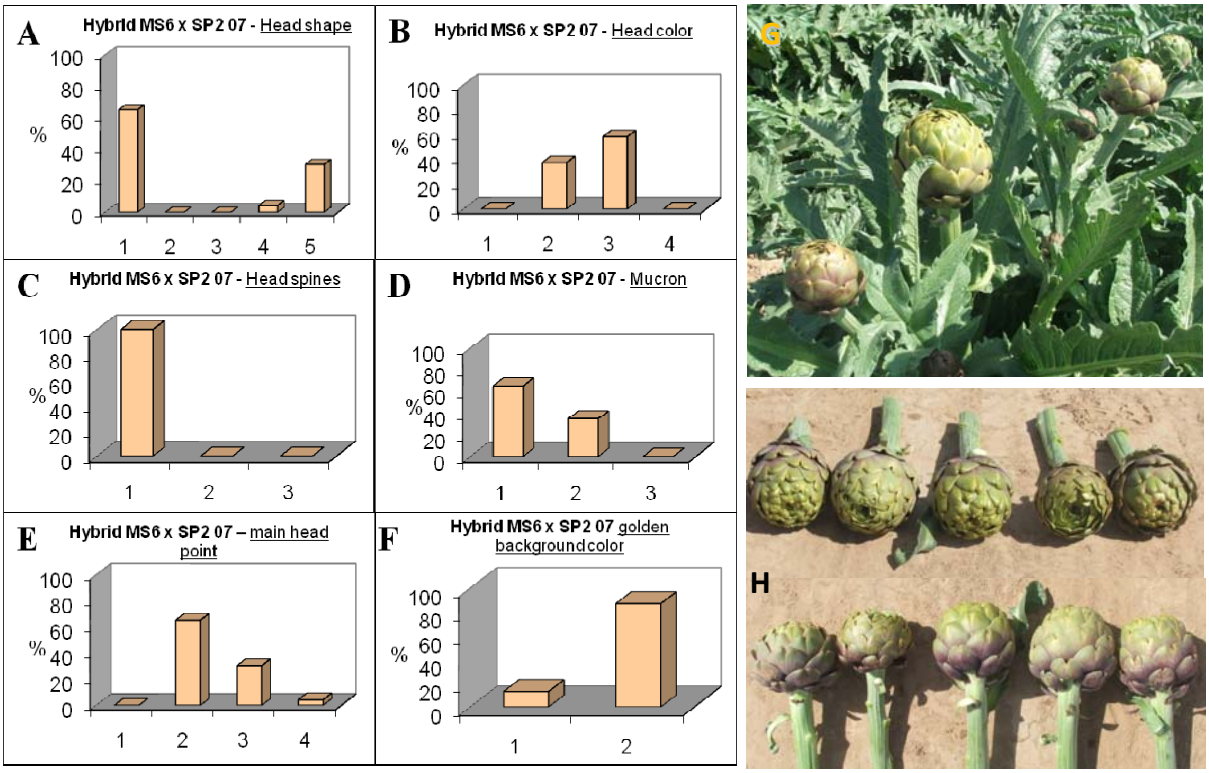

Fig. B2.3: F_1 hybrid MS6 X SP2 07. (A, B, C, D, E, F) percentage frequency of ordinal traits analyzed in 2008; (G, H) plant and heads of the hybrid. Background color

F1 hybrid MS6 x SP A2 07

The hybrid showed less variability in the shape and color of the main head than MS6 x SP2 07 (*Fig. B2.4 A, B, C, D, E, F.*); the 40% of the plants had circular heads while 60% of had trasverse elliptic heads. The external bracts were purple striped green for the 65% of the plants while the 35% had green striped purple heads. All plants were spinless and without mucron.The shape of the head point was variable: 40% of the plants had a round head point, 55% a flat point and 5% a depressed point. Golden background color in the external bracts was present in 70% of the plants. This hybrid showed a high variability.

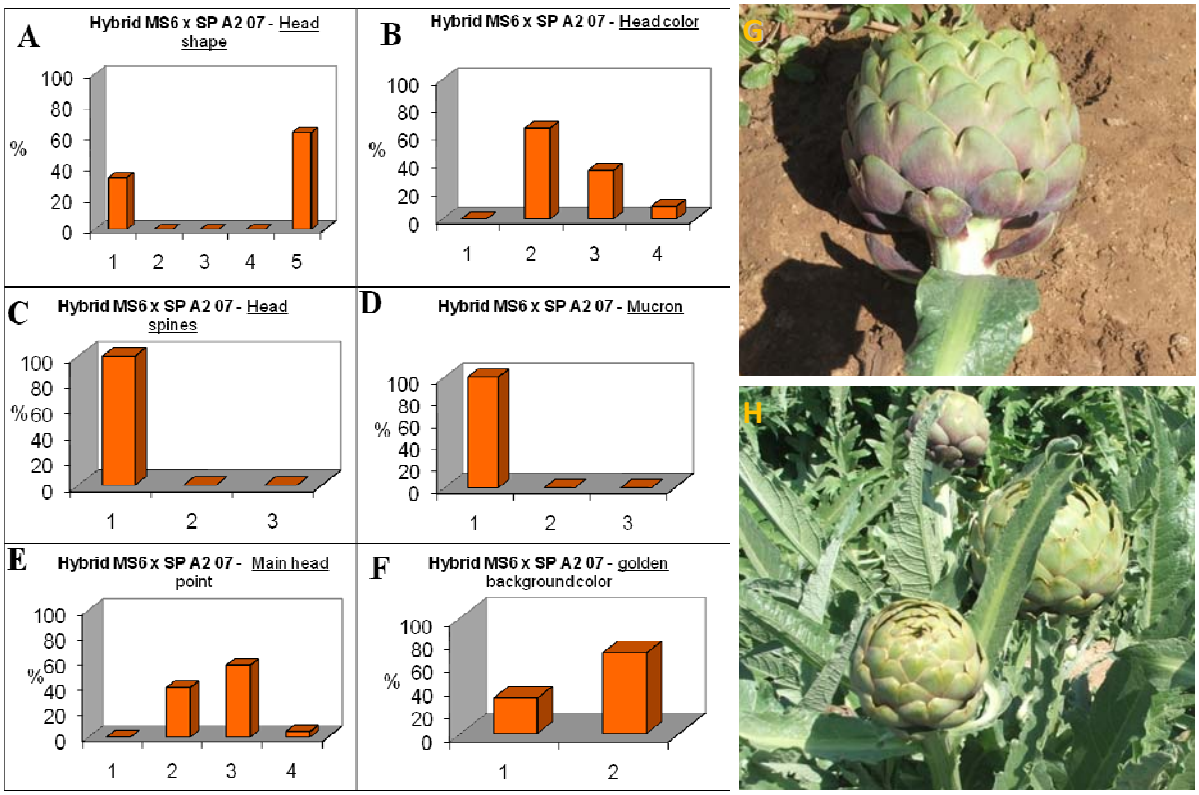

Fig. B2.4: F_1 hybrid MS6 X SP A2 07. (A, B, C, D, E, F) percentage frequency of ordinal traits analyzed in 2008; (G, H) plant and heads of the hybrid.

F_1 hybrid MS16 x SP1 05

The main head shape was circular in 35% of the plants and triangular in the 65% (*Fig. B2.5 A, B, C*). Green heads were present in 14% of the plants, purple striped green heads in 79%, and green striped purple heads only in 7%. A typical plant of the hybrid was not individuated (*Fig. B2.5 D, E*).

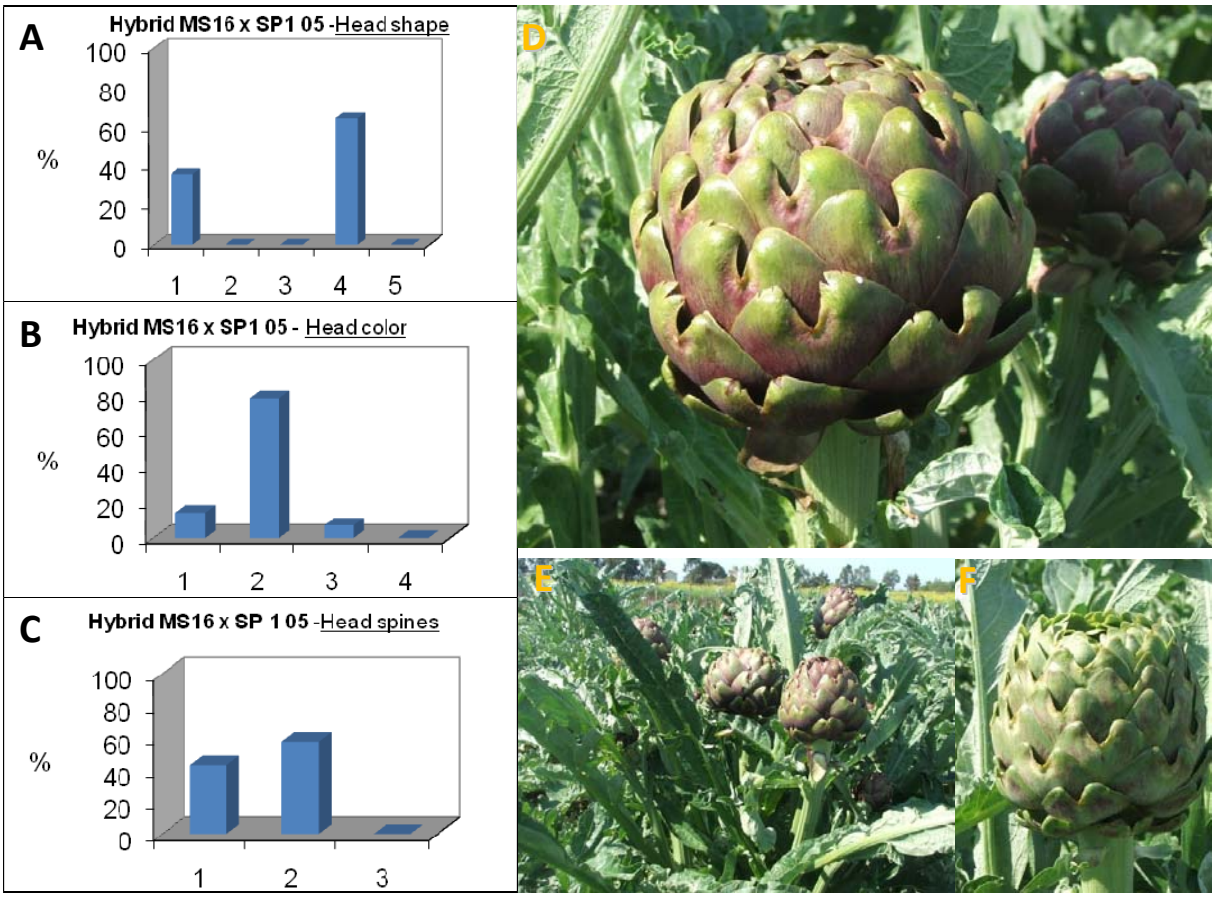

Fig. B2.5 F_1 hybrid MS16 X SP1 05. (A, B, C,) percentage frequency of ordinal traits analyzed in 2006; (D) heads of the F_1 hybrid.

F1 Hybrid MS6 x AM 1 05

The hybrid had a large range of variability in the traits analyzed (*Fig. B2.6 A, B, C)*: the main head shape was circular in 63% of the plants, elliptic in 27% of the plants and triangular in 9% of the plants. Also the color of the external bracts was not stable (*Fig. B2.6 D, E)*; in fact, 9% of the plants had green heads, 63% purple striped green heads, and 27,3 % green striped purple heads. Ninety per cent of the plants had spineless heads while 10% had spiny heads.

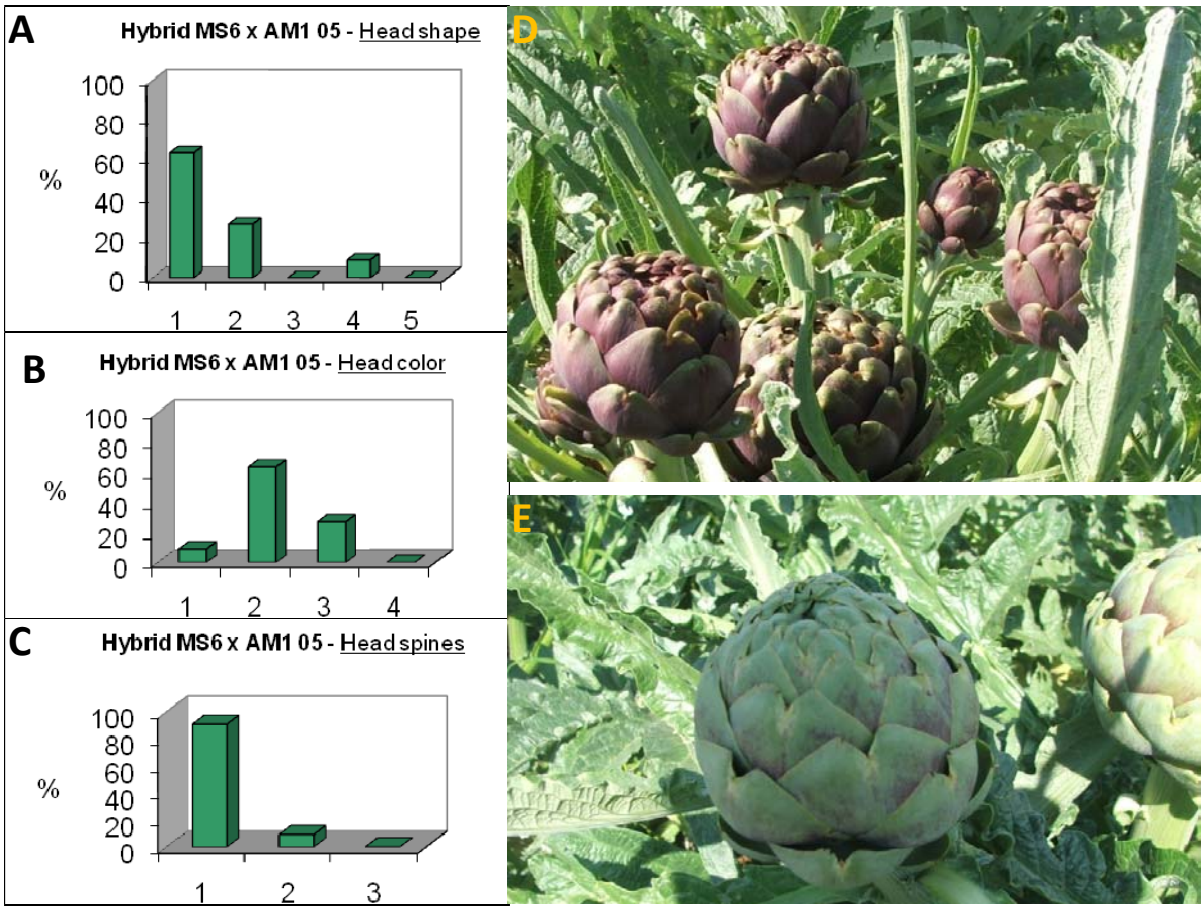

Fig. B2.6: F_1 hybrid MS6 X AM1 05. (A, B, C,) percentage frequency of ordinal traits analyzed in 2006; (E, F) heads of the hybrid.

F_1 hybrid MS6 x AM A3 07

The F_1 hybrid MS6 X AM A3 07 (*Fig. B2.7A, B, C, D, E, F*) had an almost stable main head shape, which segregated as circular, elliptic, ovate, and triangular in 79, 13, 4 and the remaining 4% of the plants, respectively. Also the color was variable with the individuals concentrated in the two central frequency classes: purple striped green and green striped purple. All the plants had spineless heads, but the 75% of them had the mucron in the external bracts. The shape of the main head point was round for 52% of the plants and flat for 48%; the golden background color was present in 45% of the plants.

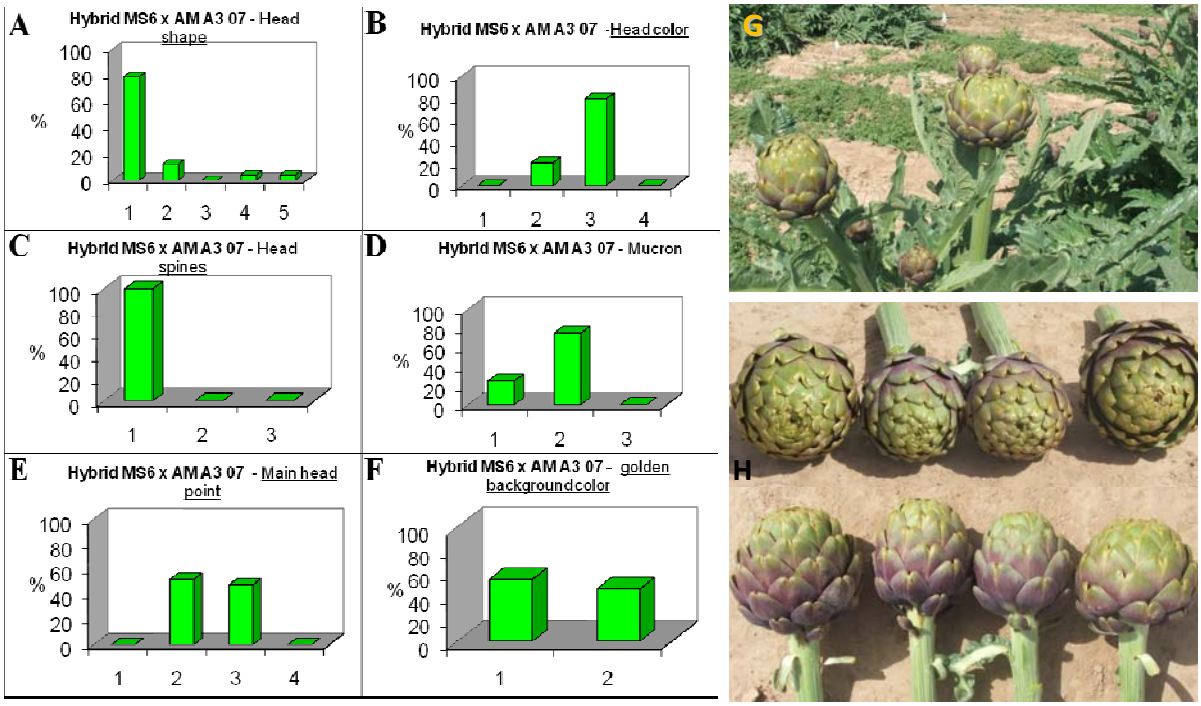

Fig. B2.7: F_1 hybrid MS6 X AM A3 07. (A, B, C, E, F) percentage frequency of ordinal traits analyzed in 2008; (G, H) plants and heads of the hybrid.

F_1 hybrid MS6 x AM A7 07

The hybrid (*Fig. B2.8*) had a variable main head shape, with 58% of the plants having round head shape, 12% elliptic, 21% ovate, and 9% transverse elliptic head shape. The color was purple striped green for the heads of 50% of the plants, green striped purple for 45% of the plants and purple for 5% of the plants. All the plants were spineless and the 75% of the plants had mucron in the external bracts. Eight per cent of the plants had a sharp main head point, 71% of the plants had round main head point and 21% flat point. The golden background color was present in 75% of the plants.

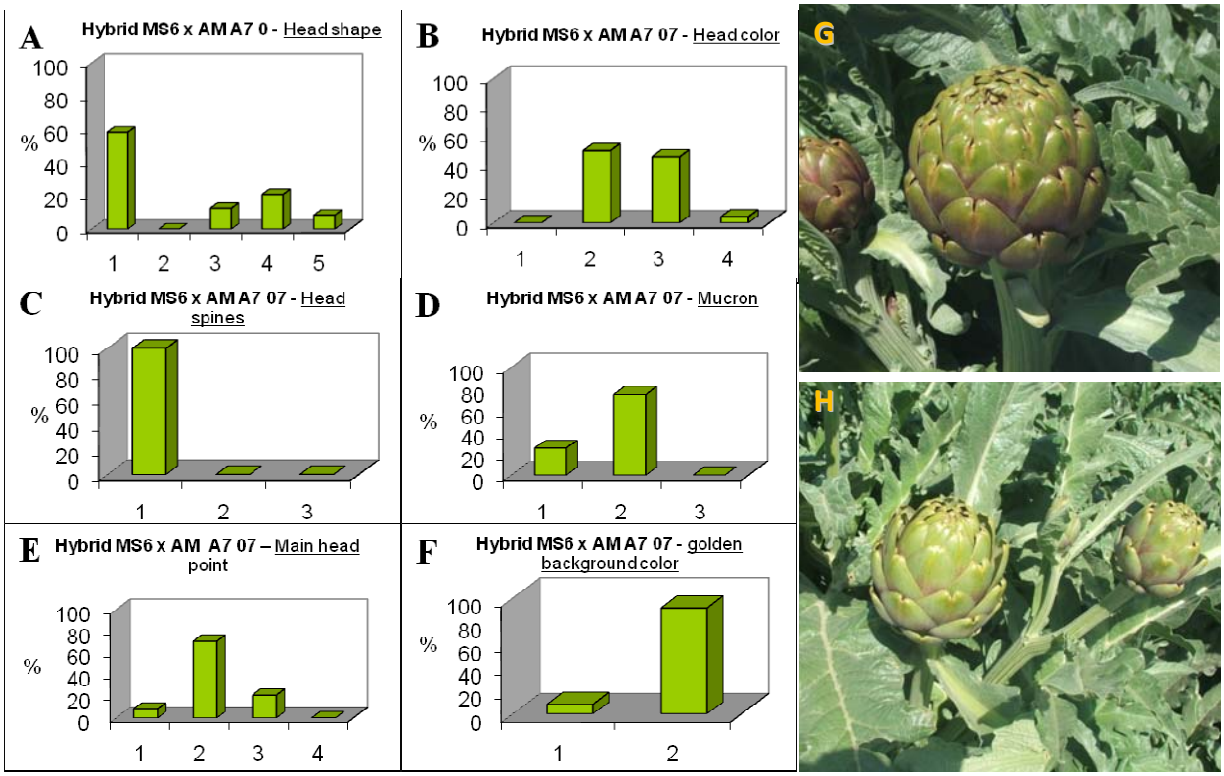

Fig. B2.8: F_1 hybrid MS6 X AM A7 07. (A, B, C, E, F) percentage frequency of ordinal traits analyzed in 2008; (G, H) plants and heads of the hybrid.

F_1 hybrid MS16 x AM 1 05

The hybrid (*Fig. B2.9 A, B, C*) had round heads in 19% of the plants, elliptic heads in 25%, triangular in 50% and transverse elliptic heads in 5%. Also the color was consistently variable and the plants felled in all the 4 frequency classes. Fifty-six per cent of the plants was spineless.

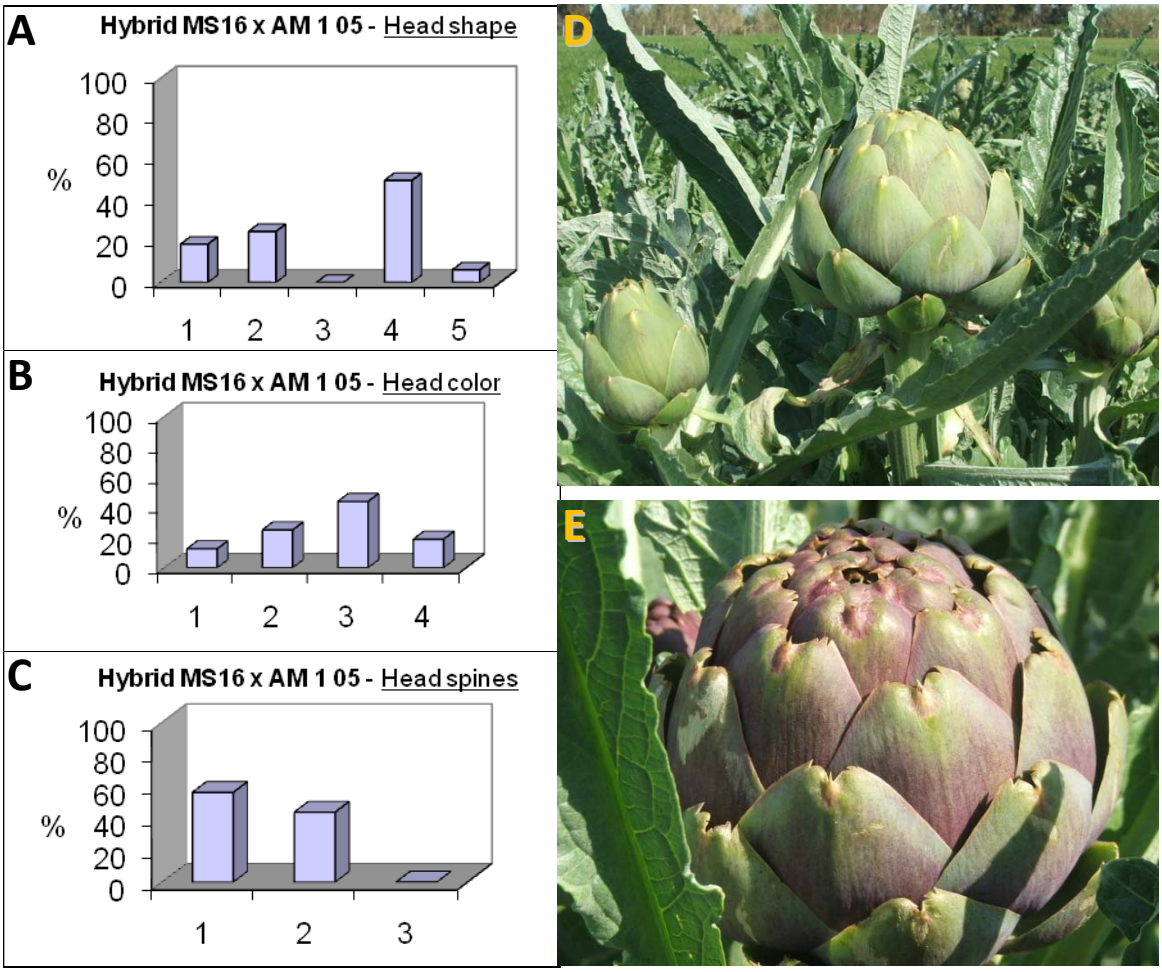

Fig. B2.9: F_1 hybrid MS16 X AM 1 05. (A, B, C, E, F) percentage frequency of ordinal traits analyzed in 2006; (G, H) the typical plant of the hybrid.

F_1 Hybrid Alyssa 07

More stable was the F_1 hybrid Alyssa 07 (*Fig. B2.10 A, B, C, E, F*). In fact, the majority (92%) of the heads had a round shape; the color of the heads was purple striped green for 40% of the heads while 60% had purple green striped heads. The majority of the plants (92%) were also spineless and mucronate. A higher level of variability was found in the head point; in fact, the majority of the plants had a round head point but 6% had sharp heads point, 8% flat, and 3% depressed head point. The golden background color was present in the heads of almost all (92%) the plants.

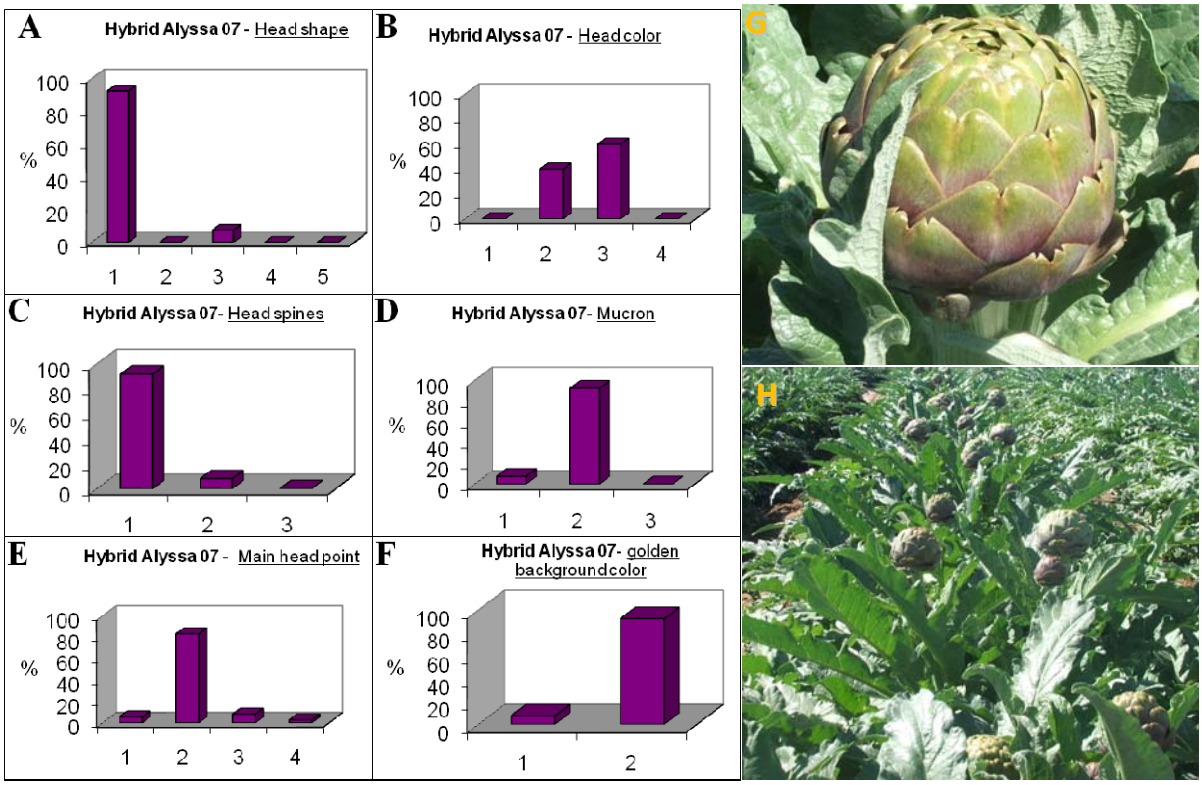

Fig. B2.10: F_1 Hybrid Alyssa 07. (A, B, C, E, F) percentage frequency of ordinal traits analyzed in 2008; (G, H) plants of the hybrid.

F_1 hybrid MS 6 X 12 05

The hybrid had a high range of variability (*Fig. B2.11 A, B, C*). Fourteen per cent of the plants had circular main heads, 14% elliptic, and 72% triangular. Also the color was variable, the majority of plants (72%) felling in the third class; there were also plants with green heads (7%), purple striped green heads (14%), and purple heads (7%), as represented by the *Figs B2.11 D* and *E*. Moreover, 71% of the plants were spineless.

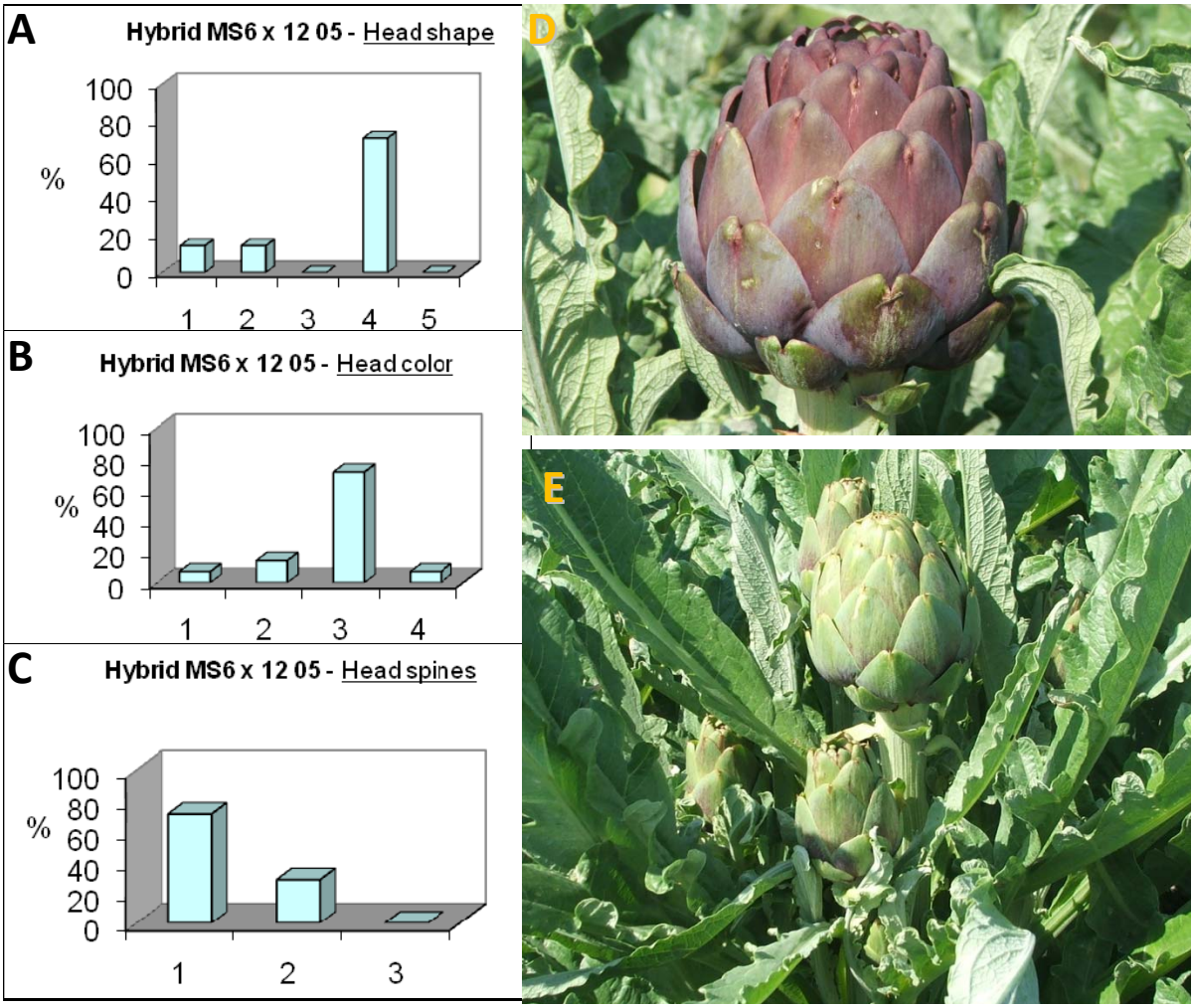

Fig. B2.11: F_1 hybrid MS 6 X 12 05. (A, B, C) percentage frequency of ordinal traits analyzed in 2006; (D, E) plants of the hybrid.

F_1 hybrid MS 16 x 12 05

The hybrid had 86% of the plants with circular and 14% with triangular main heads (*Fig. B2.12 A, B, C*). The color of the external bracts was purple striped green for 64% of the plants, green striped purple for 29%, and purple for 7%. All plants were spineless.

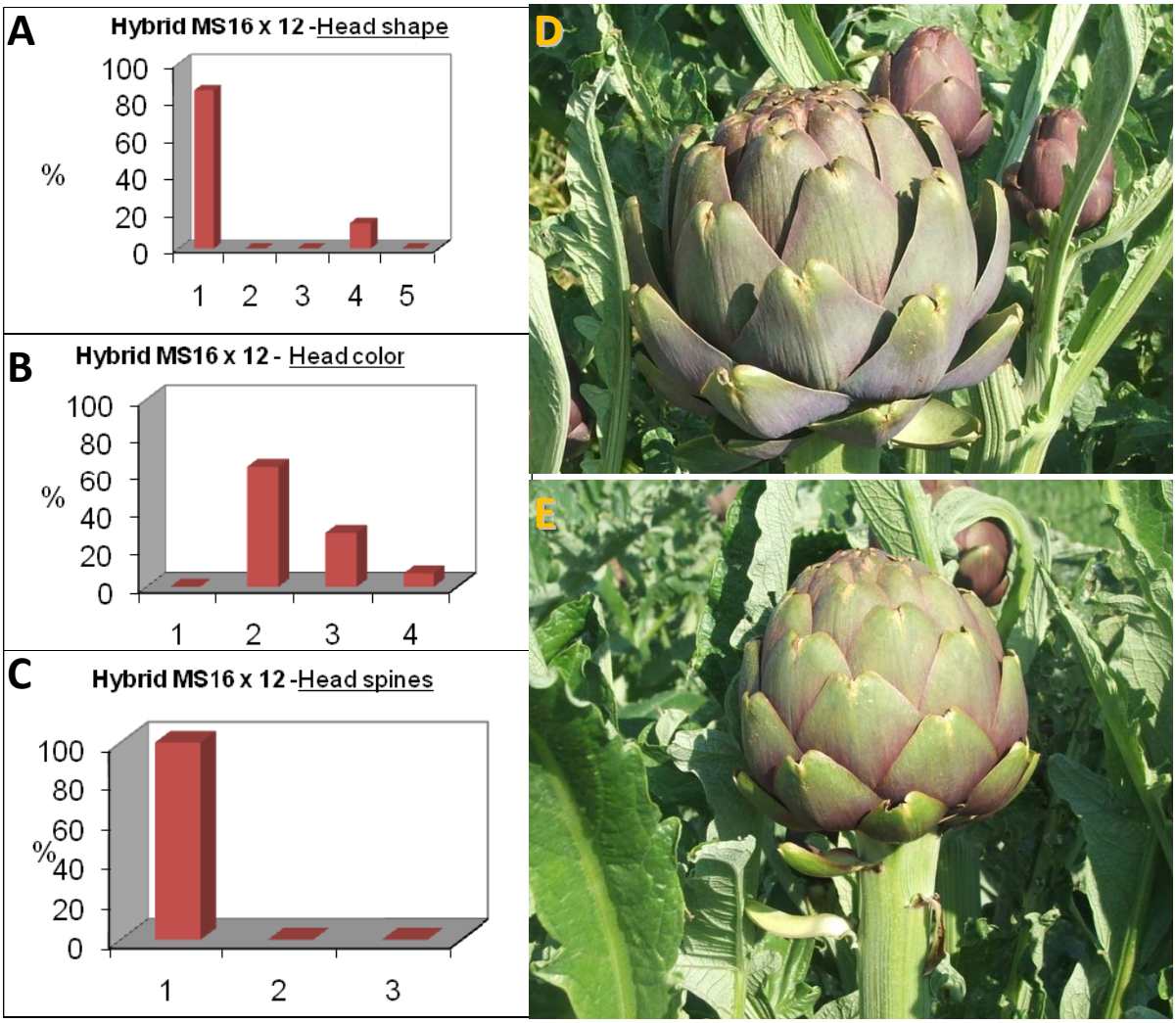

Fig. B2.12: F_1 Hybrid MS 16 X 12 05. (A, B, C) percentage frequency of ordinal traits analyzed in 2006; (D, E) plants of the hybrid.

F_1 hybrid MS 16 X Terom 05

All plants of the F_1 hybrid (*Fig. B2.13)* had triangular and green striped purple heads; the majority of the plants (80%) were spiny. This cross-combination was quite uniform.

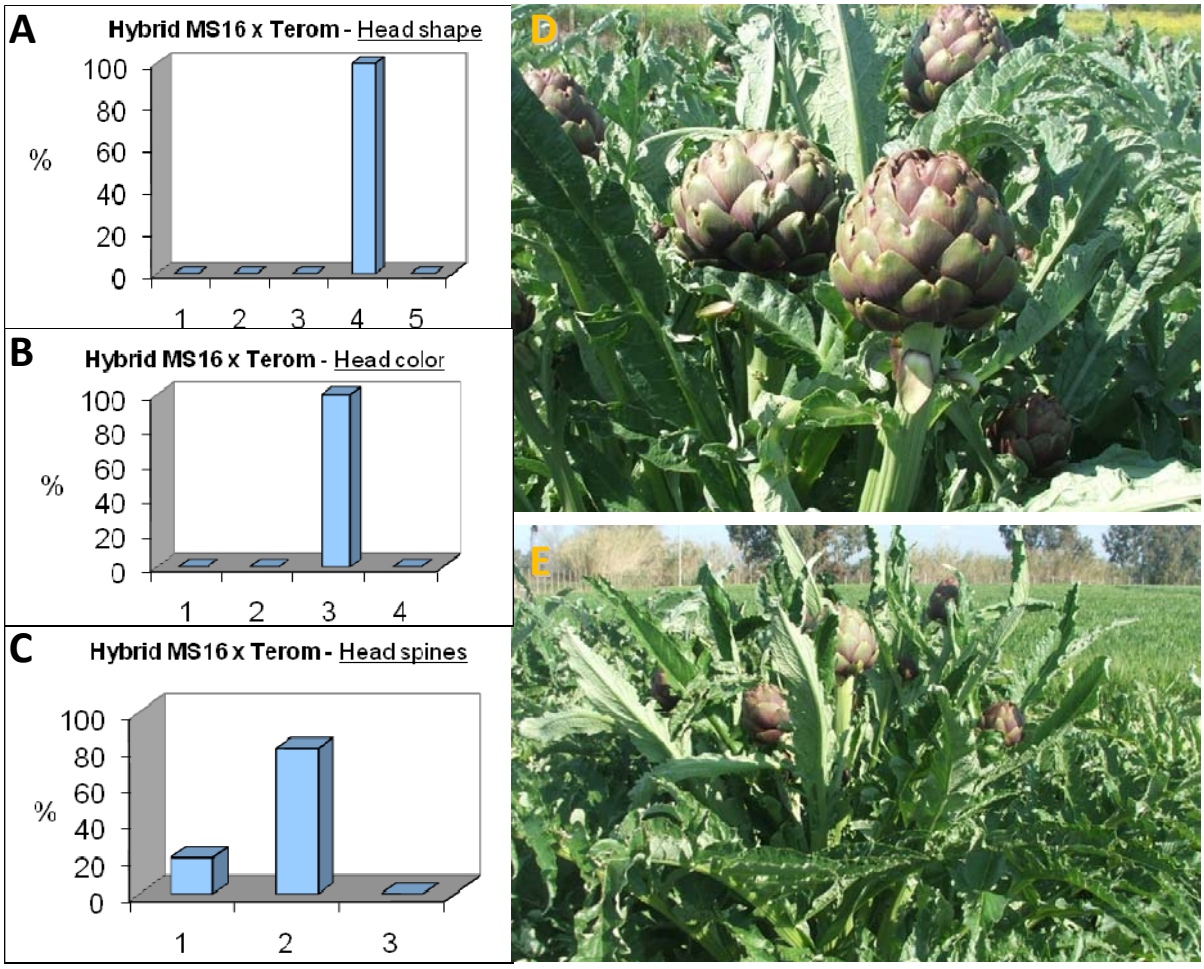

Fig. B2.13: F_1 hybrid MS 16 X Terom 05. (A, B, C) percentage frequency of ordinal traits analyzed in 2006; (D, E) plants of the hybrid.

F_1 hybrid MS 16 x C3 05

The hybrid had shape variable in three classes (*Fig. B2.14 A, B, C*). Twenty-three per cent of the plants had circular head shape, 31% elliptic and 46% triangular. The color of the external bracts was also variable: 15% of the heads had purple striped green heads, 46% green striped purple, and 39% purple. All the plants were spiny.

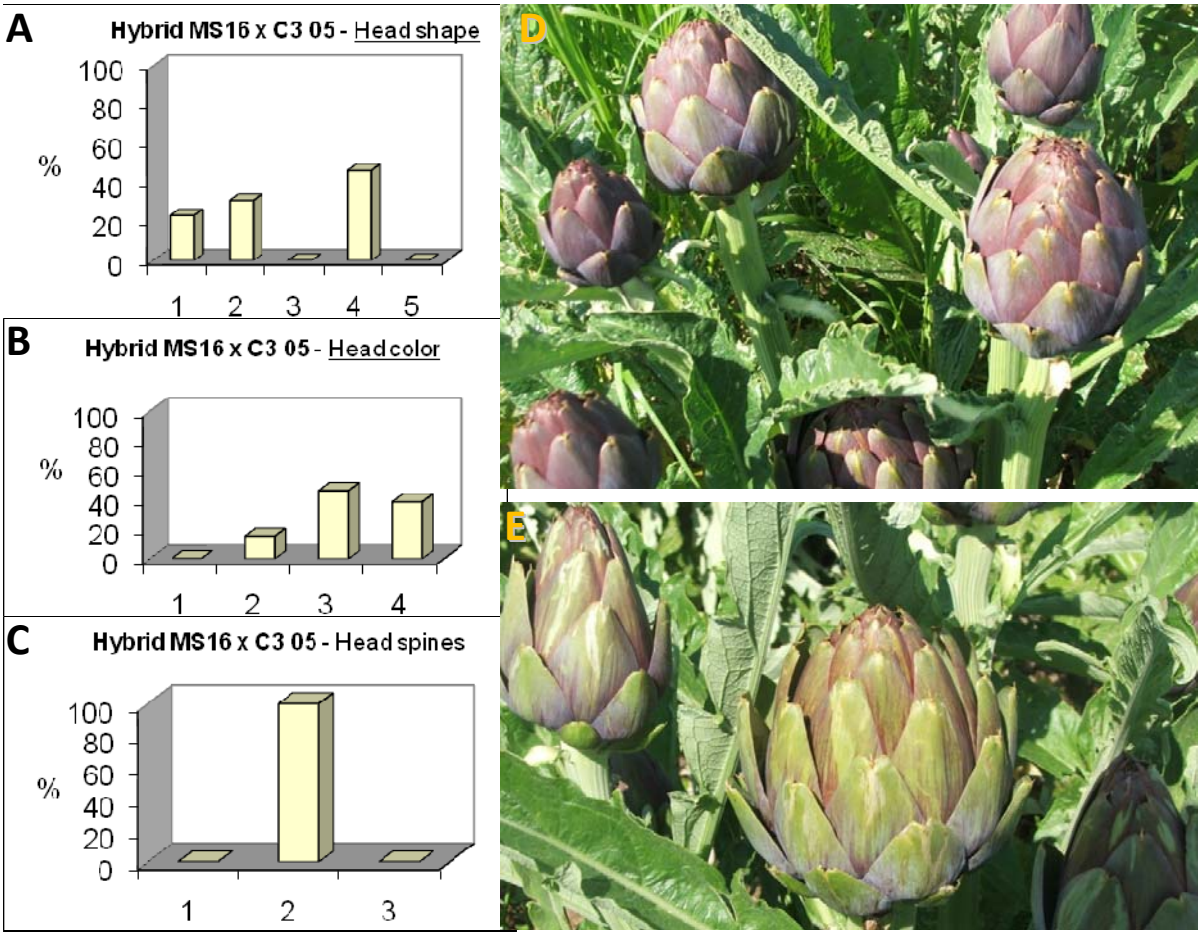

Fig.B2.14: F_1 hybrid MS 16 x C3 05. (A, B, C) percentage frequency of ordinal traits analyzed in 2006; (D, E) plants of the hybrid.

PCA (2006)

The morphological variables used in the first year were, in some cases, significantly (P< 0.01) correlated and the resulting PCA (*Table B2.2*) suggested that 15 components were necessary to explain all the variance, while the first nine factors accounted for > 90% of the variance. From the first six components extracted in the analysis, the first PC factor explained only the 19% of the variance, they including contributions from the traits MHD (diameter of the main head), SHN (number of second order of heads on the lateral shoots), and TNH (total number of heads). The second factor (17% of the variance) involved SS (number of lateral shoots), PHN (first order of heads on the lateral shoots), and TrHN (number of the third order of heads on the lateral shoots). The third factor (14% of the variation) involved MHT (time of harvesting of the main head), MHL (length of the main head), and PHL (length of the first order of heads on the lateral shoots).The fourth factor (10% of the variation), involved PHT (time of harvesting of the first order head on the lateral shoots). The fifth one involved MHS (main head shape) and MHC (main head color) while the sixth included only PD (plant diameter).

Table B2.2: 2006 PCA: trait correlations with the first 6 components extracted.

Morphologic trait	**Component**					
	1	2	3	4	5	6
PH	0,380	0,273	0,037	-0,447	-0,275	-0,008
PD	0,307	0,292	0,130	-0,056	0,028	0,672
SS	0,367	0,607	-0,537	0,228	-0,203	-0,207
MHT	0,038	-0,047	0,658	0,620	-0,082	-0,092
MHS	0,309	-0,229	0,036	0,014	0,494	-0,431
MHD	0,771	-0,451	0,147	-0,124	-0,013	0,182
MHL	0,549	0,204	0,564	-0,367	-0,087	-0,179
MDL	0,258	-0,674	-0,380	0,221	0,059	0,387
MHC	-0,073	0,176	0,225	0,040	0,709	-0,034
MHSp	-0,014	0,320	0,416	-0,476	0,191	-0,058
PHT	-0,021	0,178	0,563	0,725	-0,104	-0,058
PHN	0,367	0,607	-0,537	0,228	-0,203	-0,207
PHD	0,498	-0,649	0,087	0,126	-0,237	-0,028
PHL	0,209	0,114	0,578	-0,098	-0,420	-0,003
SHN	0,735	-0,382	-0,103	-0,013	0,114	-0,277
TrHN	0,354	0,623	0,077	0,194	0,288	0,382
THN	0,851	0,263	-0,119	0,167	0,247	0,026
variance explained (%)	19	17	14	10	8	7

DISCRIMINANT ANALYSIS (2006)

Seven discriminant functions were required to explain all the variance (*Table B2.3*). The first three functions explained 76% of the variance and each individual was plotted against the first two canonical functions (*Fig. B2.15*). Function 1 is linked mainly with MHD (diameter of main head), MDL (shape index, MHD/MHL), PHD (diameter of the first order of heads on the lateral shoots), SHN (number of the second order of heads on the lateral shoots), function 2 with MHSp (presence/absence of spines) and MHL (length of the main head), and function 3 with PH (plant height) and TrHN (number of the third order of heads on the lateral shoots). The 4th function was mainly linked with the MHS (shape of the main head).

The distribution of the single plants against the first two discriminant functions (*Fig. B2.15*) showed that all the cross combinations were grouped together but with the centroids well separated. Only the F1 hybrids MS16 x Terom and MS6 x SP 1 were more isolated.

Table B2.3: Discriminant analysis on 2006 data.Variance explained by each discriminant function and and its link with the main traits.

Function	Eigen-value	Variance explained (%)	Cumulative variance (%)	Larger correlation with the main traits
1	4,218	37,54	37,54	MHD, MDL, PHD, SHN
2	2,414	21,48	59,02	MHL, MHSp
3	1,920	17,08	76,10	PH, TrHN
4	1,184	10,54	86,64	MHS
5	0,733	6,52	93,16	PHT
6	0,422	3,75	96,92	SS, PHN
7	0,347	3,08	100,00	PHL, MHT, MHC, TPH, PD

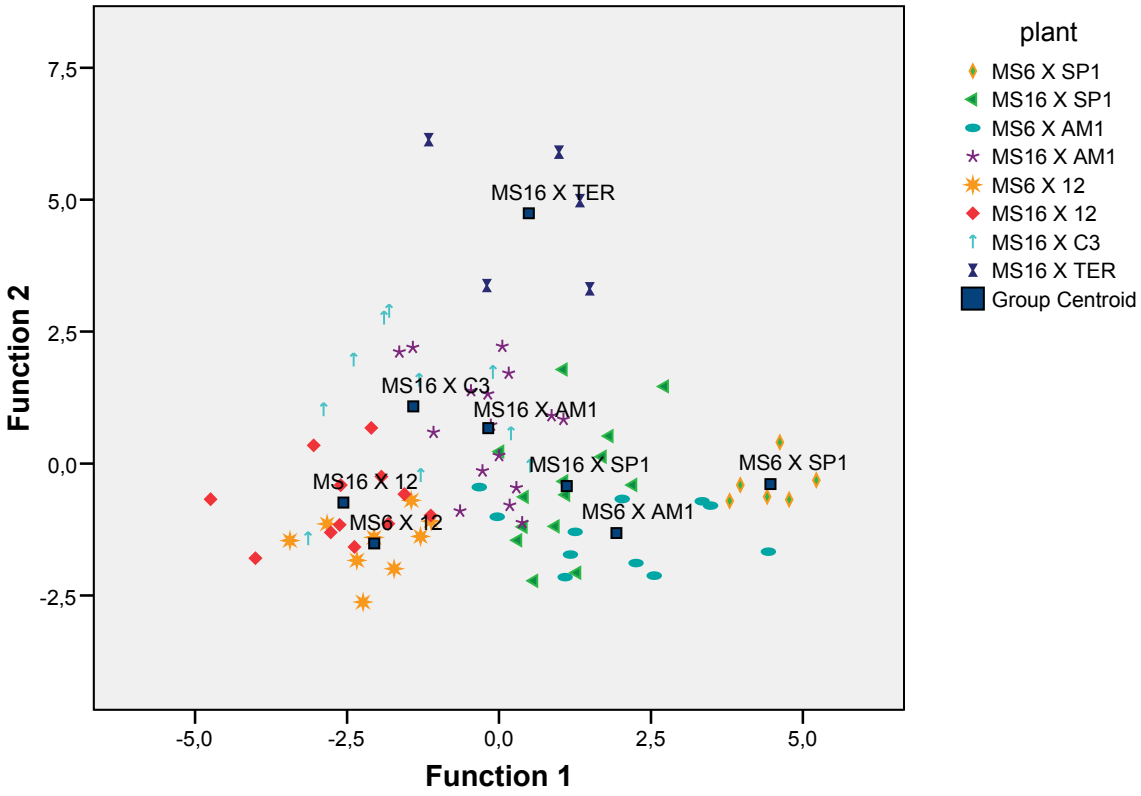

Fig. B2.15: Discriminant analysis applied on 2005 F_1 hybrids evaluated for morphological traits.

From the summary classification table (*Table B2.4*), it resulted that over all data, 88.8% of the original grouped cases were correctly classified. Moreover, the two hybrids MS16 x Terom 05 and MS6 x SP1 05 had 100% of the plants correctly classified while the others hybrids showed a percentage of cases misclassified. MS16 X 12 05 and MS16 X C3 05 had only the 75% and the 80% of the cases classified in the correct group, respectively.

Table B2.4: Summary classification table of the Discriminant Analysis on 2006 data

Cross-combination	MS6 x SP1 05	MS16 x SP1 05	MS6 x AM1 05	MS16 x AM 05	MS6 x 12 05	MS16 x 12 05	MS16 x C3 05	MS16 x TER 05	Plants (no.)
MS6 x SP 1 05	100,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	6,00
MS16 x SP 1 05	0,0	92,9	0,0	7,1	0,0	0,0	0,0	0,0	14,00
MS6 x AM 1 05	0,0	18,2	81,8	0,0	0,0	0,0	0,0	0,0	11,00
MS16 x AM 1 05	0,0	0,0	6,3	93,8	0,0	0,0	0,0	0,0	16,00
MS6 x 12 05	0,0	0,0	0,0	0,0	88,9	11,1	0,0	0,0	9,00
MS16 x 12	0,0	0,0	0,0	8,3	16,7	75,0	0,0	0,0	12,00

05									
MS16 x C3 05	0,0	10,0	0,0	0,0	0,0	10,0	80,0	0,0	10,00
MS16 x TER 05	0,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0	5,00

PCA (2008)

The correlation matrix of the morphological variables analyzed revealed, in 2008, some significant (P< 0.01) correlations and the resulting PCA (*Table B2.5*) suggested that 18 components were necessary to explain all the variance, while the first eleven factors accounted for 90% of the variance. From the first seven components, the first PC factor explained only the 26% of the variance and included contributions from the following traits: MHD (diameter of the main head), MHL (length of the main head), MHM (presence/absence of mucron), THN (total number of heads), PHN (number of the first order of heads on the lateral shoots), PHL (length of the first order of heads on the lateral shoots), SHN (number of second order of heads on the lateral shoots), TrHN (number third order of heads on the lateral shoots). The second factor (11% of the variance) involved SS (number of lateral shoots) and MDL (MHD/MHL). The third factor (9% of the variation) involved MHT (time of harvesting of the main head). The fourth factor (8% of the variation) involved PHD (diameter of the first order of heads) while the fifth involved only MHC (color of the main head).

Table B2.5: PCA on 2008 data. Trait correlations with the first 7 components extracted

Morphological traits	**Components**						
	1	2	3	4	5	6	7
PH	0,298	-0,066	-0,472	0,054	-0,225	-0,190	0,264
SS	-0,064	0,527	0,319	0,313	-0,089	0,037	-0,396
MHT	-0,834	0,005	0,648	0,018	-0,160	0,073	0,313
MHS	-0,514	0,356	0,087	0,100	0,100	0,116	-0,130
MHD	0,666	0,356	-0,678	0,376	0,042	0,351	-0,019
MHL	0,712	-0,322	-0,161	0,294	0,012	0,101	-0,227
MDL	-0,535	0,590	-0,393	0,032	0,024	0,196	0,172
MCB	0,111	0,286	-0,210	-0,122	-0,495	-0,100	-0,194
MHP	-0,494	0,011	-0,260	-0,197	0,372	0,171	-0,017
MHSp	0,212	0,171	-0,257	-0,594	0,179	-0,554	-0,083
MHM	0,528	-0,410	0,250	0,178	-0,042	0,372	-0,026

MHC	0,029	-0,004	0,194	-0,235	0,685	0,202	-0,074
PHT	-0,793	0,040	0,287	0,062	-0,151	0,018	0,374
PHN	0,015	0,531	0,392	0,151	-0,027	-0,264	-0,328
PHD	0,131	0,346	-0,096	0,534	0,425	-0,324	0,205
PHL	0,497	-0,076	0,208	0,466	0,136	-0,373	0,359
SHN	0,654	0,438	0,196	-0,231	-0,121	0,155	0,263
TrHN	0,762	0,311	0,124	-0,226	0,050	0,221	0,212
THN	0,769	0,470	0,223	-0,223	-0,031	0,168	0,204
Variance explained (%)	26	11	9	8	6	6	6

DISCRIMINANT ANALYSIS (2008)

Four discriminant functions were required to explain all the variance (Table *B2.6*). The first two functions explained 90% of the variance, and each individual was plotted against these two functions (*Fig. B2.16*). Function 1 was linked mainly with MHT (time of harvesting of the main head), MHS (shape of the main head), THN (total number of heads), PHT(time of harvesting of the first order of heads), TrHN (number of the third order of heads on the lateral shoots) function 2 is linked mainly with MHC (color of the main head), MHM (presence/absence of the mucron), MHSp (presence/absence of spines), PHL(length of the first order of heads), SHN(number of the second order of heads on the lateral shoots)

The distribution of the single cases (plants) against the first two discriminant functions (*Fig. B2.2*) showed that all cross combinations were grouped together but with the centroids separated. Only Alyssa 07 was more isolated.

Table B2.6: Discriminant analysis on 2008 data. Variance explained by each discriminant function and its link with the main traits.

Function	**Eigen-value**	**Variance explained (%)**	**Cumulative variance (%)**	**Correlation with the main traits**
1	6,238	77,2	77,2	MHT, MHS, THN, PHT, TrHN
2	1,085	13,4	90,6	MHC, MHM, MHSp, PHL, SHN.
3	0,565	7,0	97,6	PH, MHL, MDL, MHP MCB.
4	0,193	2,4	100,0	SS, PHN, PHD.

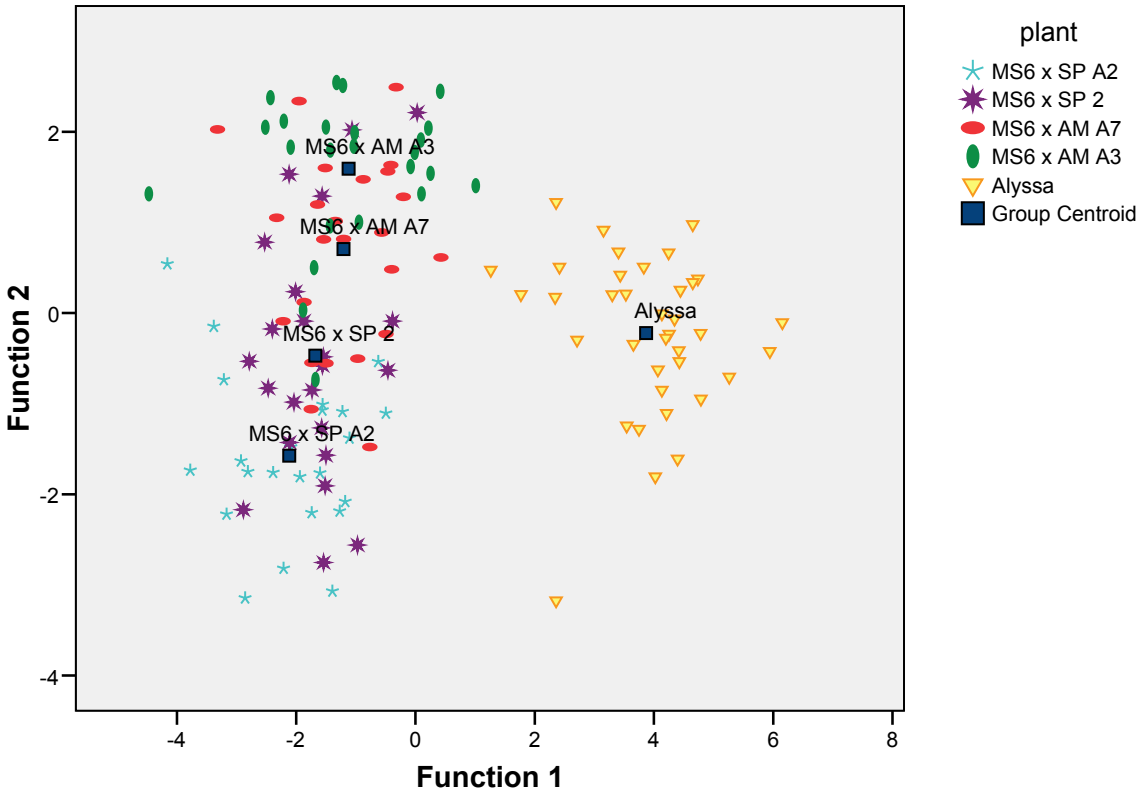

Fig. B2.16: Discriminant analysis of the 2008 F_1 hybrids on the basis of morphological characters considered

From the summary table (*Table B2.7*), it results that over all data 78.6% of original grouped cases were correctly classified. The hybrid Alyssa 07 had the highest percentage of cases (plants) correctly classified (97,3%), while MS6 x SP 2 07 had the lowest percentage of cases correctly classified (43,5%)

Table B2.7: Summary classification table of the discriminant analysis (2008)

	Predicted group membership				
Cross combinations	MS6 x SP A2 07	MS6 x SP 2 07	MS6 x AM A7 07	MS6 x AM A3 07	Alyssa 07
MS6 x SP A2 07	73,9	21,7	0,0	4,3	0,0
MS6 x SP 2 07	30,4	43,5	17,4	8,7	0,0
MS6 x AM A7 07	4,2	8,3	83,3	4,2	0,0
MS6 x AM A3 07	0,0	16,7	0,0	83,3	0,0
Alyssa 07	0,0	0,0	0,0	2,7	97,3

SEED PRODUCTION

In *Table B2.8,* the 2006 hybrid seed production per each cross-combination, the average number of seeds obtained per head and their percentage of germination are

summarized. Seed production per head was high for some cross combinations like MS 16 x Spinoso Sardo (547 seeds/head), MS 16 x Terom (440 seeds/head), and MS 16 x S. Erasmo (222 seeds/head); some other combination-crosses produced a low amount of seeds per head: MS 6 x SP1, MS 16 x AM 1, and MS 16 x AM 2 with only 28, 9.12 and 6.5 seeds/head, respectively. Some other combination-crosses such as MS 16 x Apollo and MS 16 x Brindisino did not produce any seed.

Table B2.8: Hybrid seed production and percentage of germination obtained during 2006 in Tarquinia.

Cross-combination ♀		♂	**Total seeds (no.)**	**Heads (no.)**	**Seeds/ head**	**Germination (%)**
MS6	x	AM1	219	24	9.12	70
MS6	x	AM2	143	22	6.5	72
MS 6	x	SP1	28	1	28.0	80
MS 16	x	San Erasmo	444	2	222.0	60
MS 16	x	Exploter	5	2	2.5	-
MS 16	x	Apollo	0	3	0.0	-
MS 16	x	Terom	881	2	440.5	75
MS 16	x	Brindisino	0	1	0.0	-
MS 16	x	Spinoso Sardo	547	1	547.0	50

Table B2.9 reports the hybrid seed production obtained in 2007 by the cross-combinations with the MS clones and the single plants selected in Tarquinia (*see* the Chapter B2). Seed production was high for some combination crosses such as MS6 x SP B1 that produced 280 seeds/head, MS6 x SP A2 with 164 seeds/head, and MS 14 x AM B2 with 160 seeds/head. Other hybrids produced less than 100 seeds per head; they were MS 6 x AM A3 (91.2 seeds/head), MS6 x AM B2 (76.2 seeds/head), MS6 x SP1 (67.5 seeds/head), and MS6 x AM A7 (47 seeds/head). Some cross-combinations (MS6 x AM C4, MS6 x AM D2, MS6 x SP E4) did not produce any seed.

Table B2.9: Hybrid seed production and percentage of germination obtained with the selected plants during 2007 in Tarquinia

Cross-combination ♀		♂	**Total seeds (no.)**	**Heads (no.)**	**Seeds/head (no.)**	**Germination (%)**
MS 6	x	AM A7	94	2	47,0	51,5
MS 6	x	AM A3	456	5	91,2	26,1
MS6	x	AM C4	0	4	0,0	-
MS 6	x	AM B2	381	5	76,2	46,9
MS 6	x	AM D2	0	2	0,0	-
MS 14	x	AM B1	160	1	160,0	-

MS 6	x	SP2	540	8	67,5	42,8
MS 6	x	SP A2	988	6	164,7	28,3
MS 6	x	SP B1	280	1	280,0	56,7
MS 6	x	SP E4	0	2	0,0	-

Moreover, in 2007, some cross-combinations gave a consistent amount of seed using as male parents the pollen brought stored from California. In fact, See Smoke 07 (=F-19 11-1 #10 x MS16) produced 303 seeds, using only one head of MS16, Alyssa 07 (=F-19 26-2 # 9 x MS16) produced 205 seeds with one head of MS6, Rusty 07(=F-19 11-1 #4 x MS16) produced 170 seeds with one head of MS16 (*Table B2.10*).

Table B2.10: Hybrid seed production obtained during 2007 in Tarquinia with pollen brought from California as male parent

Name	**Cross combination** ♀		♂	**Total seeds (no.)**	**Heads (no.)**	**Seeds/head (no.)**
Rusty 07	MS16	x	F-19 11-1 #4	170	1	170
	MS16	x	F-19 11-1 #5	0	1	0
	MS16	x	F-19 11-1 #9	4	1	4
Sea Smoke 07	MS16	x	F-19 11-1 #10	303	1	303
	MS16	x	CYL 39-2	2	1	2
	MS16	x	BN 263	1	1	1
	MS16	x	BN 263	6	2	3
	MS16	x	F-19 26-2 32	0	1	0
Chiara 07	MS16	x	BN 2-2	119	1	119
Connye 07	MS6	x	BN 32-2 12-5	96	1	96
	MS6	x	F-19 26-2 #2	0	1	0
Alyssa 07	MS6	x	F-19 26-2 # 9	205	1	205
	MS6	x	F-19 1-2 #4	0	1	0
	MS6	x	F-19 1-2 #1	2	1	2
	MS6	x	F-19 17-1	0	1	0
	MS6	x	F-19 17-1	0	1	0

The *Table B2.11* reports the hybrid seed production obtained, in California during 2008, by cross-combinations among MS6 and some of the mother plants selected in BHSC during 2007; from these last lines, new self-pollinated and open-pollinated lines were obtained. The number of seed/head obtained was in general lower than those produced in Italy. The highest amount of seed per head (86.5 seeds/head) was obtained by cross combinations between MS6 and F 19 26-2 #9 (Alyssa 08), which was the same male parent of Alyssa 07. All others combinations gave less than 50 seeds per head (32 seeds/head from MS6 x F- 19 11-1 #9,; 26.6 seeds/head from MS6 x WB#5,; 25 seeds/head from MS6 x WB#1; 23.5 seeds/head from MS6 x BN 62-2 #9; 19.6 seeds/head from MS6 x BN 62-2

#8,) and three of them less than one seed per head (0.8 seed/head from MS6 x F 19 11-1 #6; 0.3 seeds/head from MS6 x BN tent#2; 0 seeds/head from MS6 x F- 19 11-1 #3; 0 seeds/head from MS6 x F 19 26-2 #4).

Table B2.11: Hybrid seed obtained by crosses among MS6 and some mother plants selected in BHSC in 2007 (from these lines, at BHSC some of the 2008 parental lines were obtained)

Cross combination ♀		♂	Total seeds (no.)	Heads (no.)	Seeds/head (no.)
MS6	x	BN tent#2	1,0	4	0,3
MS6	x	BN 62-2 #9	281,9	12	23,5
MS6	x	BN 62-2 #8	215,2	11	19,6
MS6	x	F- 19 11-1 #3	0,0	9	0,0
MS6	x	F- 19 11-1 #11	79,0	12	6,6
MS6	x	F- 19 11-1 #9	383,9	12	32,0
MS6	x	W B#1	224,9	9	25,0
MS6	x	WB#5	266,5	10	26,6
MS6	x	F 19 11-1 #6	7,0	9	0,8
MS6	x	F 19 26-2 #9 (Alyssa 08)	1037,5	12	86,5
MS6	x	F 19 26-2 #4	0,0	2	0,0

In *Table B2.12*, it is reported the hybrid seed production obtained by the cross-combinations among the clone MS6 and some plants selected within the male parental I_1 and OP lines. The majority of these plants derive from the inbred progeny of the plants mentioned above; for example, BN 62-2 #8 I_1 was selected from the inbred progeny of BN 62-2 #9BN, 62-2 #9 I_1 was selected from the inbred progeny of BN 62-2 #9, WB #1 I_1 and WB #5 I_1 were selected from the inbred progenies of WB #1 and WB #5, respectively. The number of seeds per head obtained from this group of plants was significantly lower than the others above mentioned. All plants produced less than 30 seeds per head and a lot of cross-combinations gave less than one seed per head.

Table B2.12: Hybrid seeds obtained by the crosses among the clone MS6 and some plants selected from the male parent I_1 and OP lines in 2008.

Cross combination ♀		♂ (single plant)	Total seeds (no.)	Heads (no.)	Seeds/head (no.)
MS6	x	CYL #1 I_1	3,0	6,0	0,5
MS6	x	CYL #9 I_1	58,0	4,0	14,5
MS6	x	BN 62-2 #9 I_1	2,0	6,0	0,3
MS6	x	F19 #1 I_1	0,0	2,0	0,0
MS6	x	F-19 11-1 #3 I_1	18,0	4,0	4,5
MS6	x	F-19 11-1 #11 I_1	3,0	5,0	0,6

MS6	x	BN 62-2 gr#9	3,0	6,0	0,5
MS6	x	BN 62-2 #6 I_1	14,0	3,0	4,7
MS6	x	F-19 26-1 #9 I_1	0,0	3,0	0,0
MS6	x	CYL #6 I_1	30,0	5,0	6,0
MS6	x	WB #1 I_1	15,0	4,0	3,8
MS6	x	WB#5 I_1	0,0	4,0	0,0
MS6	x	F-19 #4 I_1	65,0	3,0	21,7
MS6	x	BN 62-2 #8 I_1	0,0	6,0	0,0

Table B2.13 summarizes the results of the hybrid seed collected in tents by the crosses among the clone MS6 and the inbred lines selected. Also in this case, seed production was low and comparable with the seeds obtained when a single plant selected from the same lines was used as a male parent. In fact, the number of seeds per head obtained by the lines WB#1 I_1 and WB#5 I_1 were 5.4 and 1.1 seeds per head, respectively; these values are similar to those recovered when one single plant selected from the inbred lines was used as male parent (*Table B.2.12*). All the lines produced less than 30 seeds for head.

Table B2.13: Hybrid seeds obtained in the tents by the crosses among the clone MS6 and the male parental lines selected in 2008

Cross combination ♀		♂ **(line)**	**Total seeds (no.)**	**Heads (no.)**	**Seeds/head (no.)**
MS6	x	BN 62-2 #9 I_1	357,3	72	5,0
MS6	x	F19 #1 I_1	223,5	79	2,8
MS6	x	F-10 #9 I_1	704,1	73	9,6
MS6	x	F -19 11-1 #3 I_1	101	81	1,2
MS6	x	F-19 11-1 #11 I_1	12	72	0,2
MS6	x	BN 62-2 gr #9	70	72	1,0
MS6	x	BN tent #4 I_1	40	49	0,8
MS6	x	BN 62-2 #6 I_1	1570,6	84	18,7
MS6	x	BN 62-2 gr #6	137	132	1,0
MS6	x	F -19 26-2 #9 I_1	94	108	0,9
MS6	x	WB #1 I_1	250	46	5,4
MS6	x	WB #5 I_1	69	61	1,1
MS6	x	F-19 #4 I_1	2454,7	85	28,9
MS6	x	F -19 11-1 #6 I_1	134	143	0,9
MS6	x	BN 62-2 #8 I_1	117	122	1,0
MS6	x	BN tent#2 I_1	56	68	0,8

B2.4 DISCUSSION

To develop a method aimed at comparing the stability of different hybrids, an evaluation of the most important morphological traits used in the selection of globe artichoke was made during the three years of the PhD project. The traits evaluated were a combination of the CPOV descriptors with those described by Lopez Anido *et al.*(1998) and Crinò *et al*. (2007). A first indication on the homogeneity of the different hybrid progenies was obtained by the analysis of the means and standard errors of the parameters considered. A frequency analysis of some qualitative traits was made to have a description of the characteristics of the hybrids. The evaluation of the standard errors as well as of the one-way ANOVA substantially confirmed the description of the frequency analysis. Even if based only on few traits, this analysis is able to give a quick description of the observations made empirically in the fields.

In 2005, the most stable hybrids were MS6 x SP 1 and MS6 x Terom. Nevertheless, both hybrids had a too low number of plants analyzed for concluding that they were stables. For this reason, these crosses were repeated in 2006.

Unfortunately, the hybrid MS6 x Terom, analyzed in the second year, was less stable and later respect to the previous year (unshown data), so it was not evaluated. A probable reason for that could be attributed to different genetic backgrounds of the plants used as male parent (Terom) in the crosses of 2006 and 2005.

Only the evaluation of MS6 x SP 1 was repeated both in 2005 and 2006, it showing high homogeneity and interesting phenotypic characteristics for the market; the 2006 results confirmed the characteristics and the good quality of this hybrid. Therefore, the same cross-combination was repeated in 2007 in order to produce a higher amount of seeds and describe once more and better the characteristics of this hybrid. Unfortunately, the SP1 plant was lost during the 2006 summer, so it was substituted by another plant coming from the same selection (SP2) and showing phenotypic characteristics similar to SP1; the genotype SP I_1 A2, which derived from SP1 selfed, was used as male parent in the new crosses. Even though the hybrids obtained conserved some characteristics similar to MS6 x SP1, the stability was significantly lower and some defects like the depressed head point, which were inconsistent in the previous hybrid, were accentuated in the latter one. This is understandable because, as all vegetative propagated crops, globe artichoke is a plant that conserves a high heterozygosis (Pecault 1992). Therefore, even the selection made for commercial traits during the inbreed generations can help to improve the stability of the

parental lines and the lines could appear sufficiently stable, with plants apparently similar from a morphological point of view, the single plants used in the crosses can segregate for unexpected traits. In 2008, another hybrid that showed morphological characters and homogeneity was Alyssa 07. This hybrid had the least values of standard errors and, from the frequency analysis, also good head qualities. The results obtained both by the one-way ANOVA and the frequency analysis were confirmed also by the discriminant analysis. In fact, as already indicated in the Chapter A2, a common result that can be observed in evaluating the homogeneity of one group of individuals consists in the classification matrix of the discriminant analysis. In this case, hybrids that had the highest percentage of individuals classified correctly were MS6 x SP1 05, MS6 x Terom 05, in 2006, and Alyssa 07, in 2008. From the evaluation made, Alyssa 07 could be enough uniform to be already commercialized. For this reason, this cross-combination was repeated to obtain a good amount of seeds and confirm its morphological evaluation in 2009. The seeds of this cross-combination were planted in 2008 both in California (*Fig. B2.17*) and in Tarquinia (Italy). The plants will be evaluated in USA and in Italy, either in the Micozzi's Farm (Tarquinia, Viterbo) or in Lo Bianco's organic Farm (Sicily) and in in the SEMIORTO Sementi Farm (Salerno).

*Fig. B2.17*Experimental field in Brawley CA (USA) as view on January19th, 2009.

From the PCA analysis of the two years (2006 and 2008), it resulted that all the traits analyzed were important and all contributed to the variance expressed. In fact, in the first year, 15 functions were necessary to explain the 100% of the variance and only few traits were eliminated in the 2008 analysis. These results were confirmed from the PCA of 2008 in which 18 components were all necessary to explain the 100% of variance; this is in contrast with the results obtained by Crinò *et al.* (2007) who found a high correlation among the traits analyzed and only two principal components were enough to explain the

90% of the variance. Nevertheless, in this case, the material analyzed was different by the hybrids; it consisted in clones all deriving from "Romanesco" traditional populations and already selected for the production. In this experiment of the PhD program, the material derived from crosses and the variance among individuals could be high also for the effect of the segregation. Therefore, it is necessary, a higher amount of traits to explain the total variance. In fact, the differences among the cross-combinations are hidden by the differences inside the group.

However, all the results obtained suggested us that the male sterile clone MS6 gives lots of stability in the crosses and that if crossed with a stable male fertile parent can produce a very homogeneous hybrid. Moreover, the hybrids obtained with this male sterile clone, showed commercial traits very interesting for the Italian market and in particular for the market of "Romanesco" type.

Another important problem to make the hybrid seed production rentable consists in obtaining a good amount of seeds. For this reason, in the PhD program, an experiment aimed at comparing the different pollination techniques was performed (*see* Chapter B1). However, an important result on the tendencies to produce seed by the different cross-combinations was obtained by the results of the hybrid seed production.

In 2008, the number of seeds produced per head by all the cross-combinations was lower in California than in Italy, although the number of male sterile heads used for every cross-combination was higher. For example, seed production obtained, in 2007, in Italy brushing pollen brought from USA gave few cases an high amount of seeds per head while, in 2008, using plants coming from the same selections, only from the same plant and with the same pollination system (pollination by brushes), the seed production was lower. This is in contrast with what reported in the Chapter A2 on seed production by the selfs of the male parents.

An explication could be that the MS6 plants used in California were plants less developed than the plants used in Tarquinia, probably stressed for the transplantations and not adapted to the environmental conditions. Also the irrigation system could have influenced the performance of the male sterile parents; in fact, with the furrow irrigation system, globe artichoke plants risk to suffer more the effects of the hydric stress (Giardini, 2001) and have less resources to use for seed production. In all the evaluations, there were a lot of empty heads that made difficult to evaluate statistically the data. A possible solution could have been to exclude from the evaluation the empty heads (Morrison *et al.*, 2000).

Another interesting result may be underlined comparing the *Tables B2.11* and *B2.12*. The number of hybrid seeds per head obtained using as male parent the single plants from which were derived the self- and open pollinated lines was higher than the number of hybrid seeds per head obtained using as male parents the plants selected from the correspondent inbred lines. The pollination system was the same and the conditions were the same; therefore, the experiments were comparable. This experience gave a confirmation that for every inbred generation (I_1, I_2, I_3), in the genetic improvement of globe artichoke, the increase of the stability is paid with a reduction of the performance of the male parent to produce pollen. Therefore, artichoke inbred lines will be more uniform but also less good pollinators. For this reason, in breeding activity of this vegetable, it is important to alternate generations of self pollination with generation of sib

Chapter B3

Molecular analyses

B3.1 INTRODUCTION

A molecular analysis by ISSR markers was performed in order to characterize six globe artichoke hybrids and to compare them with both the male and the female parents used for the same crosses.

Moreover, the hybrids were characterized either morphologically or molecularly in order to compare the results of both the analyses and observe if they were concordant or not. All the work was realized in collaboration with the Dpto. Ciencia y Tecnologia Agraria of Universidad Politecnica de Cartagena (UPCT), Spain

BACKGROUND ON ISSR MARKERS

The markers are alleles of *loci* where there is sequence variation – or polymorphism - in DNA that is neutral in terms of phenotype (Jones *et al.*, 1999). Some advantages of DNA markers are that they are not influenced by the environment, are expressed in all tissues, and can be scored at all stages of plant growth. A large number of makers have been developed and two big groups of them can be distinguished as follow(Barcaccia *et al.*, 2005): (I) markers based on the *Southern Blot Hybridization* (SBH) that are the minisatellites RFLP (Restriction Fragment Length Polymorphism) and VNTR (Variable Number Tandem Repeat), (II) markers based on the *Polymerase Chain Reaction* (PCR). In this group the most used markers for globe artichoke and cardoon such as RAPD (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeat), I-SSR (Inter Simple Sequence Repeat), AFLP (Amplified Fragment Length Polymorphism) are included.

I-SSR are generated from single-primer PCR reactions, where the primer is designed from di- or trinucleotide repeat motifs with a 5' or 3' anchoring sequence of one to three nucleotides (Gupta *et al*., 1994; Zietkiewicz *et al*., 1994). Anchoring sequences are generally random and may include redundant bases (Wolfe *et al.,* 1998). Different anchoring sequences used on a common microsatellite motif produce unique banding profiles. The amplified regions represent the nucleotide sequence between two SSR priming sites orientated on opposite DNA strands. The premise is that SSR regions are scattered evenly throughout the genome (Hamada and Kakunaga, 1982; Tautz and Renz, 1984; Condit and Hubbell, 1991) and the chances of amplifying between two adjacent regions within the limits of *Taq* polymerase processivity is high enough that a large number of polymorphic bands should be generated.

ISSR markers are inherited in a dominant Mendelian way (Gupta *et al*., 1994; Tsumura *et al*., 1996). They are interpreted as dominant markers similar to RAPD data and are scored as diallelic with "band present" or "band absent" (*Fig. B3.1*). Absence of a band is interpreted as primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement (Wolfe and Liston, 1998).

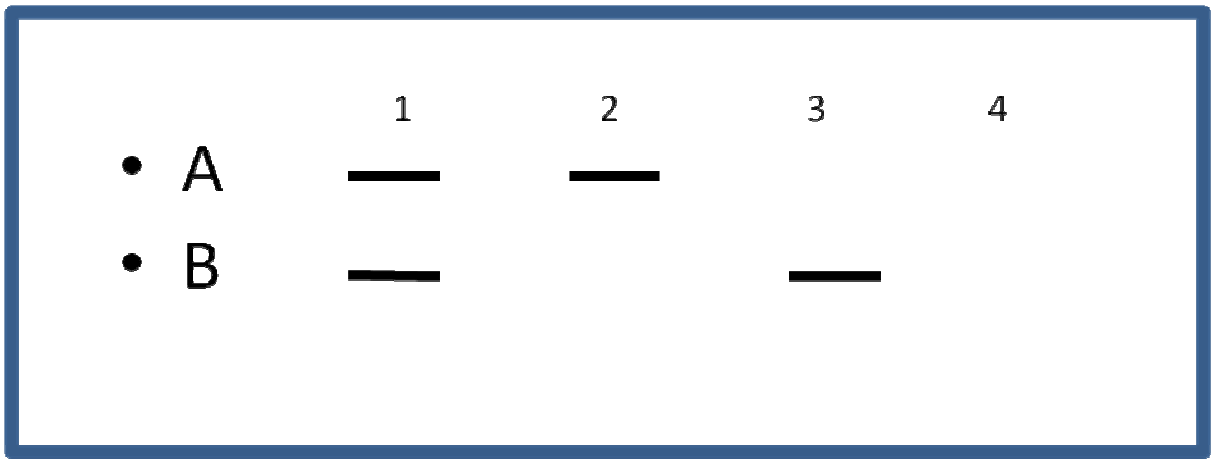

Fig. B3.1: Diagram of ISSR primer possible banding patterns.
A and B refer to inter-simple sequence repeat regions that are amplified if primer sequences anchored on the 5' end of microsatellite regions are intact. If all primer sites are present, two bands will result as in lane 1. If one or more primer sites are absent, one or both bands may be absents in lanes 2-4.

B3.2 MATERIAL AND METHODS

Five different hybrids and their parents plus a commercial F_1 hybrid used as control (F_1 Opal commercialized by Nunhems) were analyzed from a morphological and molecular point of view. In March 2007, young leaves from the parental lines were collected in Tarquinia (Italy) and then frozen into liquid nitrogen for DNA extraction in the laboratories of the Department of Agrobiologia and Agrochimica (DABAC) of the University of Tuscia, in Viterbo. In August 2007, the F_1 hybrid seeds collected in Tarquinia were shipped to Spain, where they were sown on September 19th, 2007 in the nursery of the Universidad Politecnica de Cartagena, Spain. Here, plant cultivation was conducted in the "Tomás Ferro" Experimental Agro-Food Station of the UPCT of Cartagena (Spain),, on the Mediterranean coast of Spain (lat. 37º 36' 52" N, long. 0º 58' 07" W). The cross-combinations studied and their percentage of germination obtained for each of them are summarized in *Table B3.1.* From ten plants for every cross-combination, the DNA was extracted and the molecular analyses were conducted.

TableB3.1: Cross-ombinations of the hybrids morphologically and molecularly analyzed

Cross-combination			Seed germination (%)	
♀ parent		♂ parent	Tarquinia (Italy)	Cartagena (Spain)
MS 6	x	AM A7	51.54	84.00
MS 6	x	AM A3	26.15	60.00
MS 6	x	AM B1	46.92	72.00
MS 6	x	SP2	42.78	46.00
MS 6	x	SP A2	28.33	74.00
	Opal F_1		40.00	76.67

MORPHOLOGICAL ANALYSES

During 2008 growing season, morpho-physiological data were twice per week recorded on ten plants per F_1 hybrid. Each single plant was phenotyped using 17 of the descriptors indicated in Chapter A2 and B2. (*Table B3.3* of this chapter). Moreover, the weight of the main head (MHW), as well as that of the primary heads in the secondary shoots (PHW), and the total yield per plant (Y) were evaluated.

DNA EXTRACTION AND DILUTION

The total genomic DNA was extracted individually from young leaves of ten plants per hybrid using a DNA extraction kit PureLink™ Invitrogen according to the extraction protocol indicated by the manufacturer. Sample concentration was assessed both spectrophotometrically and by 1% agarose gel electrophoresis. The DNA samples were diluted to 10 ng/µl prior for PCR amplification.

MARKER ANALYSES

A total of 17 ISSR primers (*Table B3.2*), obtained from the University of Columbia, in Canada were tested for DNA amplification. Ten primers were chosen for ISSR analyses of genetic diversity based on their reproducibly producing bands.

PCR reaction was carried out using a single primer at a time in 20 µl reaction mixture containing 20 ng of DNA, 1x buffer, 150 µM of each of the four dNTPs, 1 U of Taq DNA polymerase (ECOGEN, S.R.L.Spain), 1.5 mM $MgCl_2$, and 0.3 µm of primer. The amplification reaction occurred into a thermocycler (Bio-Rad, California, USA) was programmed for an initial denaturation step of 5 min at 94 ºC followed by 35 cycles of 1min at 94 ºC, 1 min at the specific annealing temperature and 2 min at 72 ºC, ending with a final extension step of 5 min at 72 ºC.

PCR products were separated in 1.5% agarose gels in 1xTBE buffer by electrophoresis at 90 V for 2 hours using a horizontal gel electrophoresis system. Gels were stained in etidium bromide. The amplified fragments were visualized and photographed under UV light using a gel documentation and image analyzer (Vilber Loirmat, Germany).

Table B3.2: ISSR primers screened and their annealing temperature

Primer	Sequence 5'-3'	T_a (˚C)	Primer	Sequence 5'-3'	T_a (˚C)
801	$(AT)_8T$	24.2	855	$(AC)_8CTT$	57.1
811	$(GA)_8C$	43.3	856	$(AC)_8CTA$	55.3
812	$(GA)_8A$	44.3	857C	$(AC)_8CTGC$	63.5
818	$(CA)_8G$	52.1	857G	$(AC)_8CTG$	58.9
826	$(AC)_8C$	53.3	861	$(ACC)_6$	67.4
827	$(AC)_8G$	54.9	867	$(GGC)_6$	88.5
841	$(GA)_8CTC$	48.5	872	$(GATA)_4$	28.9
847	$(CA)_8AGC$	59.8	878	$(GGAT)_4$	55.9
848	$(CA)_8AGG$	59.6			

STATISTICAL ANALYSES

Each band was considered an ISSR *locus*. The ISSR *loci* were scored as present (1) or absent (0) for all samples. Fragments showing the same electrophoretic mobility were treated as identical. In order to study how the ISSR *loci* were inherited by the F_1 progenies, the bands common to the F_1 hybrids and only to one parent were considered as ISSR discriminative *loci*. For every cross-combination, the total number of ISSR *loci* amplified by every primer (T), the total number of discriminative *loci* (D), the number of discriminative *loci* for the female parent as well as the progeny and the number of discriminative *loci* for the male parent and the progeny were calculated.

Nei's genetic distances index was calculated in all possible pair-wise comparisons in order to estimate genetic dissimilarities among pairs of plants. The Nei's dissimilarity matrix was used to generate a dendrogram using the unweighted pair-group arithmetic average (UPGMA) clustering procedure in a sequential agglomerative hierarchical combination (SAHN) strategy. These calculations and analyses were conducted using the appropriate routines of the Numerical Taxomony and Mulivariate Analysis package in NTSYSpc version 2.02 (Rohlf, 1998). For phenotypic traits, one-way ANOVA and discriminant analysis was carried out by the statistics program SPSS version 16.0 (Field, 2005). Separation of means was done by Duncan test. Mantel Test was performed to compare both the matrixes of morphological and molecular traits using he Pearson product-moment correlation coefficient. Mantel test was performed by XLstat program.

B3.3RESULTS

MORPHOLOGICAL EVALUATION

- One-way ANOVA

Results of on the one-way ANOVA applied on morphological data are reported in *Table B3.3,*. Some traits showed no significative differences among the different cross-combinations relatively to the plant height (PH) and diameter (PD), the time of harvesting both of the main head (MHT) and of the first order heads in the primary shoots (PHT), the number and weight of second order heads in the primary shoots (SHN and SHW). Differences related to the weight of the main head (MHW) and to the total yield (Y) resulted significant (P=0,05) and highly significant (P=0,001). No significant differences were found for the total number of heads (THN), whereas highly significant differences were found for the shape traits of the main and the primary heads: the two ratios MDL (main head diameter) and PDL (primary head diameter) showed differences significant per P=0,001%. On the basis of Duncan test, the shape of the main head was well differentiated (*Fig*. *B3.2*). In fact, for the trait MDL, the Duncan test classified the 6 hybrids in four classes: (a) MDL=1, in which Opal F1 fell; (b) MDL=1.11, in which MS6 x AM A3 fell; (c) MDL=1.26, in which both the hybrids MS6 x AM A7 and MS6 x SP2 fell; (d) MDL=1.35, in which MS6 x AM B1 fell; the hybrid MS6 x SP A2 fell in both the classes (the Duncan test classified it as "cd") . In this shape index, the length of the main head (MHL) seems to weight on this difference more than the diameter (MHD). In fact, almost all hybrids fell in only one class of diameter, while they were all well differentiated for the main head length. A similar result was given also by the ratio PDL (the shape index of the heads in the first order of shoots PHD/PHL).

Fig. B3.2: Shape of the heads in the different cross-combinations analyzed

Table B3.3: Mean and standard errors of the phenotypic traits evaluated in Cartagena (Spain) during 2008. One-way ANOVA and Duncan test results.

	PH (cm)	PD (cm)	MHT	MHD (cm)	MHL (cm)	MHW (g)	MDL	PHT	PHN (no.)	PHD (cm)	PHL (cm)	PDL	PHW (g)	SHN (no.)	SHW (g)	THN (no.)	Y (g)
MS6xAMA3	74.50 ±2.41	148.00 ±2.49 ab	170.30 ±1.67	10.00 ±0.24 a	9.00 ±0.20 b	440.00 ±14.83 bc	1.11 ±0.03 b	177.60 ±1.19	2.40 ±0.16	8.80 ±0.17a	7.85 ±0.20 a	1.12 ±0.01 b	231.00 ±10.90	2.80 ±0.20	180.00 ±0.00	6.20 ±0.20	1519.00 ±33.15 a
MS6xSP2	77.00 ±1.70	148.00 ±2.49 ab	170.90 ±1.92	11.15 ±0.15 b	8.85 ±0.11 b	450.00 ±8.56c	1.26 ±0.02 c	178.40 ±1.55	2.50 ±0.17	9.70 ±0.19 b	7.75 ±0.11 a	1.25 ±0.03 d	234.00 ±8.72	2.80 ±0.29	180.00 ±0.00	6.30 ±0.21	1520.00 ±29.17 b
Opal	76.50 ±2.79	140.00 ±3.16 a	170.80 ±1.69	10.80 ±0.21 b	10.50 ±0.15 c	402.00 ±8.00 a	1.03 ±0.01 a	177.80 ±1.53	2.50 ±0.17	8.80 ±0.13 a	9.35 ±0.13 b	0.94 ±0.01 a	209.00 ±8.72	2.80 ±0.20	140.00 ±0.00	6.30 ±0.26	1323.00 ±40.33 b
MS6xAMB1	76.00 ±3.23	146.00 ±3.86 ab	169.40 ±1.54	11.15 ±0.21 b	8.30 ± 0.19 a	417.00 ±11.93 ab	1.35 ±0.04 d	176.20 ±1.24	2.50 ±0.17	9.15 ±0.18 a	7.60 ±0.13 a	1.21 ±0.02 cd	213.00 ±9.43	2.80 ±0.25	180.00 ±0.00	6.30 ±0.21	1453.00 ±30.11 b
MS6xAMA7	78.00 ±2.13	147.50 ±3.18 ab	170.00 ±1.89	10.75 ±0.20 b	8.80 ±0.25 ab	410.00 ±9.43 ab	1.23 ±0.04 c	178.20 ±1.24	2.50 ±0.17	8.90 ±0.19 a	7.65 ±0.17 a	1.16 ±0.02 bc	233.00 ±9.32	3.00 ±0.26	180.00 ±0.00	6.50 ±0.22	1518.00 ±44.22 b
MS6xSPA2	72.00 ±2.13	152.50 ±2.81 b	170.90 ±1.92	10.95 ±0.12 b	8.65 ±0.13 ab	422.00 ±8.54ac	1.27 ±0.02 cd	179.40 ±1.49	2.50 ±0.17	9.75 ±0.21 a	7.75 ±0.17 b	1.26 ±0.01 d	229.00 ±7.22	2.70 ±0.21	180.00 ±0.00	6.20 ±0.200	1472.00 ±44.84 b
Total	75.67 ±0.91	147.00 ±1.28	170.38 ±0.69	10.80 ±0.09b	9.02 ±0.11	423.50 ±4.64	1.21 ±0.02	177.93 ±0.55	2.48 ±0.07	9.18 ±0.09	7.99 ±0.10	1.16 ±0.02	224.83 ±3.69	2.82 ±0.09	173.33± 1.941	6.30 ±0.09	1467.50 ±17.23
F	0.760 ns	1.787 ns	0.118 ns	4.914** *	18.848** *	3.017*	16.301* **	0.585 ns	0.060 ns	5.828***	19.113***	32.023***	1.529 ns	0.171 ns	-	0.249 ns	4.128 ***

PH: plant height; PD: plant diameter; MHT: number of days between the sowing and the harvesting of the main heads; MHD: diameter of the main head; MHL: Length of the main head; MHW: weight of the main head MDL: MHD/MHL; PHT: number of days between the sowing and the harvesting of the first order of heads on the lateral shoots; PHN: number of the first order of heads on the lateral shoots; PHD: diameter of the first order of heads on the lateral shoots; PHL: length of the first order of heads on the lateral shoots; PDL: (PHD/PHL); PHW: weight of the first order of heads on the lateral shoots; SHN: number of the second order of heads on the lateral shoots; SHW: weight of the second order of heads on the lateral shoots; THN: total number of heads; Y: total production.

The discriminant analysis showed that five discriminant functions are necessary to explain the 100% of the variance (*Table B3.4*), the first two functions explaining 96% of the variance and each individual was plotted against these two functions (*Fig. B3.3*). Function 1 is linked mainly with MHD (diameter of main head), PDL (shape index related to PHD/PHL), PHD (diameter of the primary heads on the lateral shoots); function 2 is mainly linked with MDL (shape index, MHD/MHL), MHW (weight of the main head), and time of harvesting of the main heads (MHT).

Table B3.4: 2008 Discriminant analysis of the morphological traits. Variance explained by each discriminant function and its link with the main traits.

Function	Eigen-value	% of variance explained	% of cumulative variance	Larger correlation with main traits
1	26,471	88,2	88,2	MHD, PDL, PHD;
2	2,337	7,8	96,0	MDL, MHW, MHL;
3	0,612	2,0	98,0	PHT, PD, PHN;
4	0,319	1,1	99,1	MHL, PH, PHW;
5	0,272	0,9	100,0	Y, SHN, PHL.

The distribution of the single plants against the first two canonical discriminant functions (*Fig. B3.3*) showed that all the cross-combinations were grouped together with the centroids well separated (the three hybrids MS6 x SP2, MS 6 xAM B1 MS6 x SP A2 have the centroids partially overlapped). Only Opal F_1 resulted differentiated respect to the others..

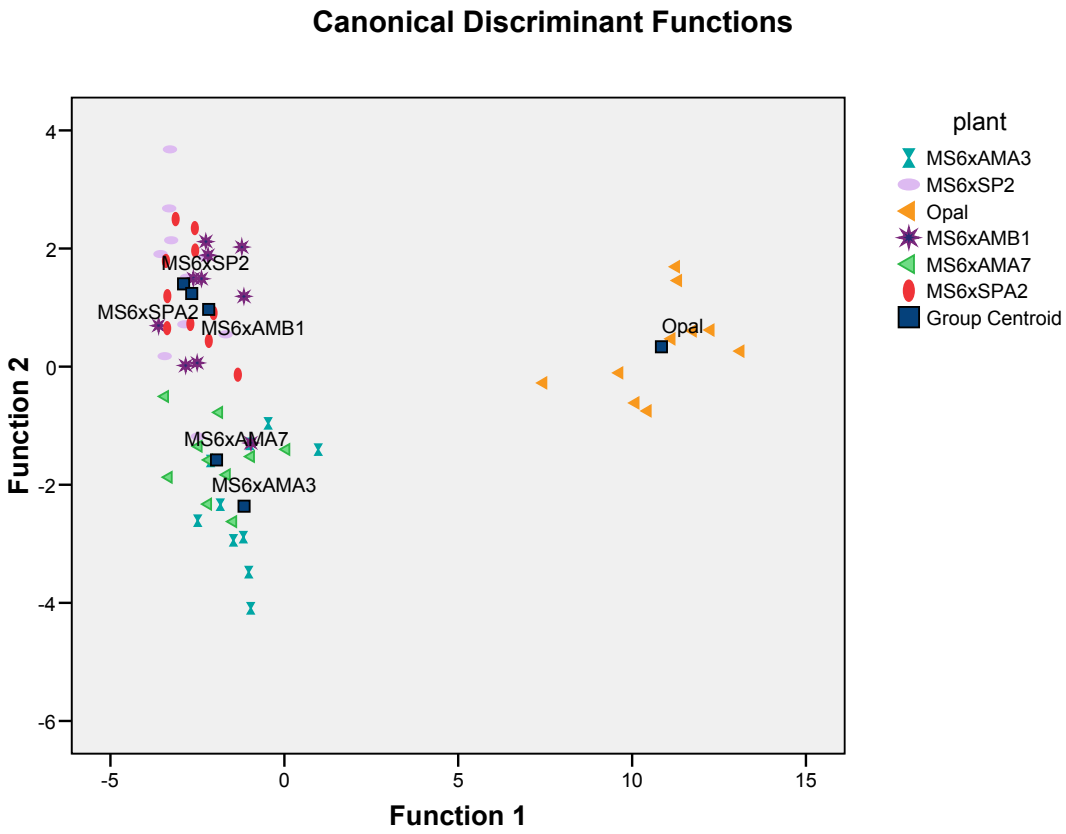

Fig.B3.3: Discriminant analysis among the 2008 F_1 hybrids on the basis of morphological traits

MOLECULAR ANALYSIS

The 10 primers utilized *(Table B3.5)* produced a total of 82 *loci,* 56 of which polymorphic; the proportion of polymorphic loci was 68.3%.*T*the number of ISSR *loci* amplified per primer and the percentage of polymorphism per primer have been also reported. The primers showing the highest percentage of polymorphism were 827 and 878, while the primer 811 produced four bands that resulted monomorphic for all the cross-combinations and it was excluded from the analysis. In *Fig.B3.4*, the electrophoretic patterns of the primer 848 for both the parents and all progenies are represented as an example of the polymorphism found.

Table B3.5: Number of ISSR *loci* amplified per primer and percentage of polymorphism per primer.

ISSR primer	ISSR *loci (*no.) total	polymorphic	Polymorphism (%)
811	4	0	0.0
827	8	8	100.0
841	7	3	42.9
848	9	8	88.9
855	11	7	63.6
856	10	4	40.0
857C	10	9	90.0
857G	8	5	62.5
872	7	4	57.1
878	8	8	100.0
Total	82	56	68.3

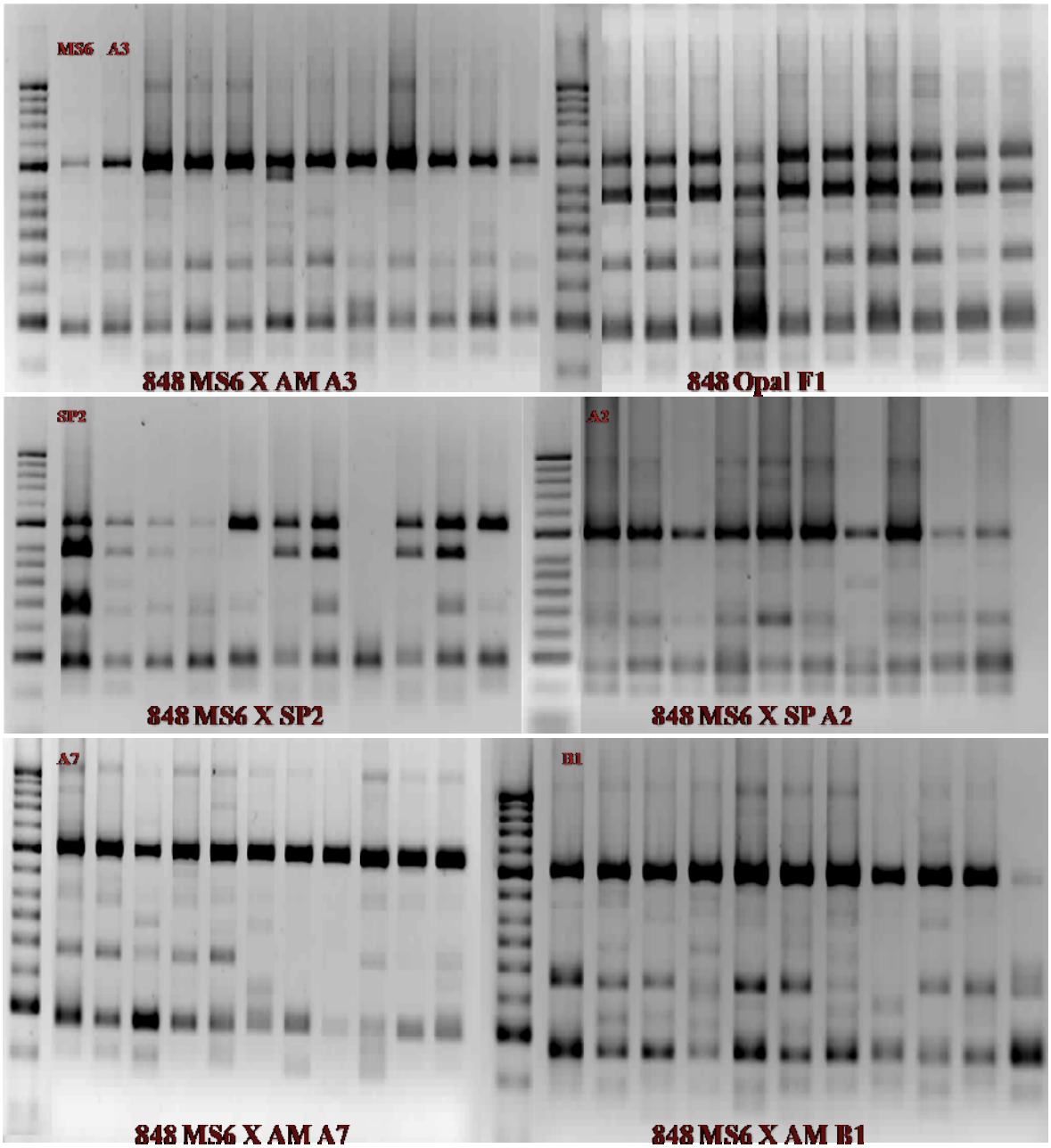

Fig. B3.4: Electrophoretic patterns of the primer 848 for the parents MS6, AM A3 (=A3), SP2, SP A2 (=A2), AM A7 (=A7), AM B1 (B1) and for the hybrid progeny

The percentage of polymorphism within every cross-combination is reported in *Table B3.6.* The cross-combination showing the highest level of polymorphism were MS6 x SP A2 and MS 6 x AM A7, while the lowest percentage of polymorphism was detected in Opal F_1 (18.29%), MS6 X AM A3 (20.73%) and MS6 X AM B1 (21.95%).

Table B3.6: Number of total *loci* and percentage of polymorphism within every cross-combination

Cross-combination	Total loci (no.)	%
MS6 X AM A3	624	20.73
MS6 X SP2	600	28.05
Opal F_1	606	18.29
MS6 X AM B1	565	21.95
MS 6 X AM A7	559	30.49
MS 6 X SP A2	532	30.49
Mean	581	25.00

The values of Nei's genetic distances (GD) and the similarities, calculated among the six F_1 hybrids, are reported in *Table B3.7*. The GD values were not high: the highest value of GD has been indicated between Opal F_1 and MS6 x AM A3, while the lowest one was found between MS6 x SP A2 and MS6 x AM A7.

Table B3.7: Pairwise comparison of Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the 5 hybrid progenies

Cross-combination	**MS6xAM A3**	**MS6 x SP2**	**Opal**	**MS6xAM B1**	**MS6xAM A7**	**MS6xSPA2**
MS6xAM A3	-	0.909	0.832	0.860	0.881	0.870
MS6xSP2	0.096	-	0.853	0.847	0.894	0.857
Opal F_1	0.184	0.159	-	0.910	0.846	0.802
MS6xAM B1	0.151	0.166	0.095	-	0.919	0.890
MS6xAM A7	0.127	0.112	0.167	0.085	-	0.919
MS6x SP A2	0.139	0.154	0.221	0.116	0.084	-

The results of the discriminant analysis of the molecular data are summarized in *Table B3.8*; five discriminant functions are required to explain 100% of the variance. The first two functions explained 85% of all the variance and each individual was plotted against these two functions (*Fig. B3.5*). In this plot the different groups are well separated with the centroids well distant and not overlapping. All the individuals of the same progenies resulted grouped together.

Table B3.8: 2008 discriminant analysis of the molecular data. Variance explained by each discriminant function and its canonical correlation.

Function	Eigen-value	% of variance explained	% of cumulative variance	Canonical Correlation
1	390.893	48.371	48.371	0.999
2	297.3403	36.794	85.165	0.9989
3	61.5296	7.614	92.779	0.992
4	43.705	5.408	98.187	0.989
5	14.649	1.813	100.00	0.968

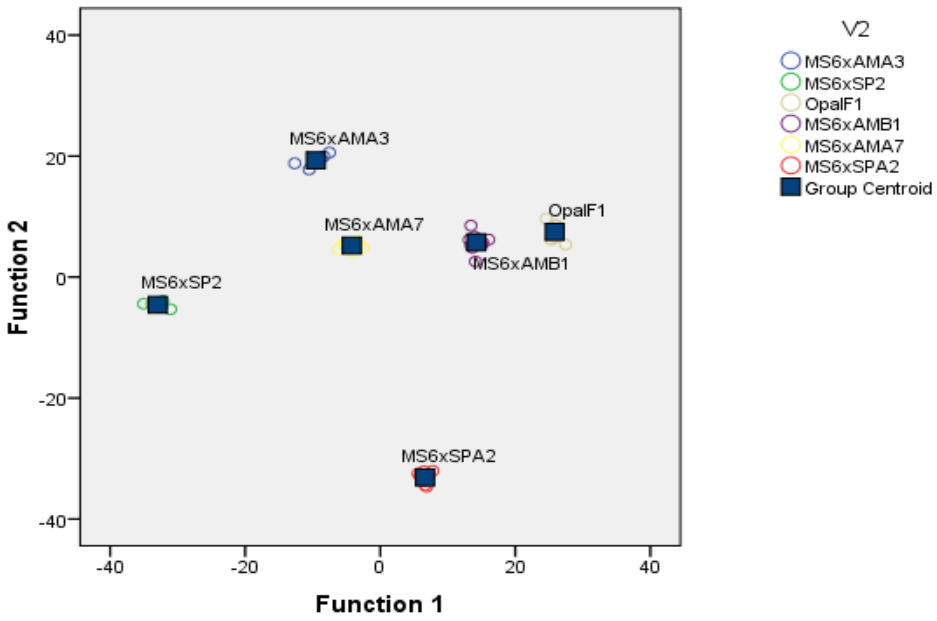

Fig. B3.5: Discriminant analysis among the 2008 F_1 hybrids molecularly analyzed.

On the basis of the Neis's genetic distances matrix, the UPGMA dendrogram was obtained (*Fig. B3.6*). The ISSR loci were able to distinguish almost all the individuals analyzed; only in Opal F_1, the plant 2 had an ISSR pattern corresponding exactly to the plants no. 7, 4, and 8. Two big groups are distinguishable, one including the two cross-combination MS6 x SP A2 and MS6 x AM A7 and the other including MS6 x AM B1, Opal F_1, MS6 x SP2 MS6 x AM A3. The two groups included hybrids that have the same female parent but different male parents.

Moreover, in the first group, the F_1 MS6 x SP A2 progeny is separated from F_1 MS6 x AM A7. The female parent (M) appears genetically more similar to both F_1 hybrids MS6 x SP2 and MS6 x AM A3, while it appears more genetically distant from the other cross-combination Opal F_1, which is excluded because it is a commercial hybrid. The male parents

are genetically similar to all the progenies. This fact, suggests that the progenies inherit more fragments from the male parent than from the female one (the MS6 in all the crosses). Therefore, in order to understand deeply this phenomenon, an analysis of the inheritance of the single *locus* for each cross-combination was performed. The bands that were common to the F_1 hybrid and only to one parent were defined as discriminative ISSR *loci*. The percentages of discriminative ISSR *loci* were calculated for both the male and the female parent. The results are summarized in *Table B3.9* showing a variable percentage of discriminative loci; the highest values were found for the F_1 hybrids MS6 x SP A2 (33%) and MS6 x AM B1 (29.2%). The lowest percentage of discriminative ISSR *loci* was obtained by the hybrid MS6 X AM A3.

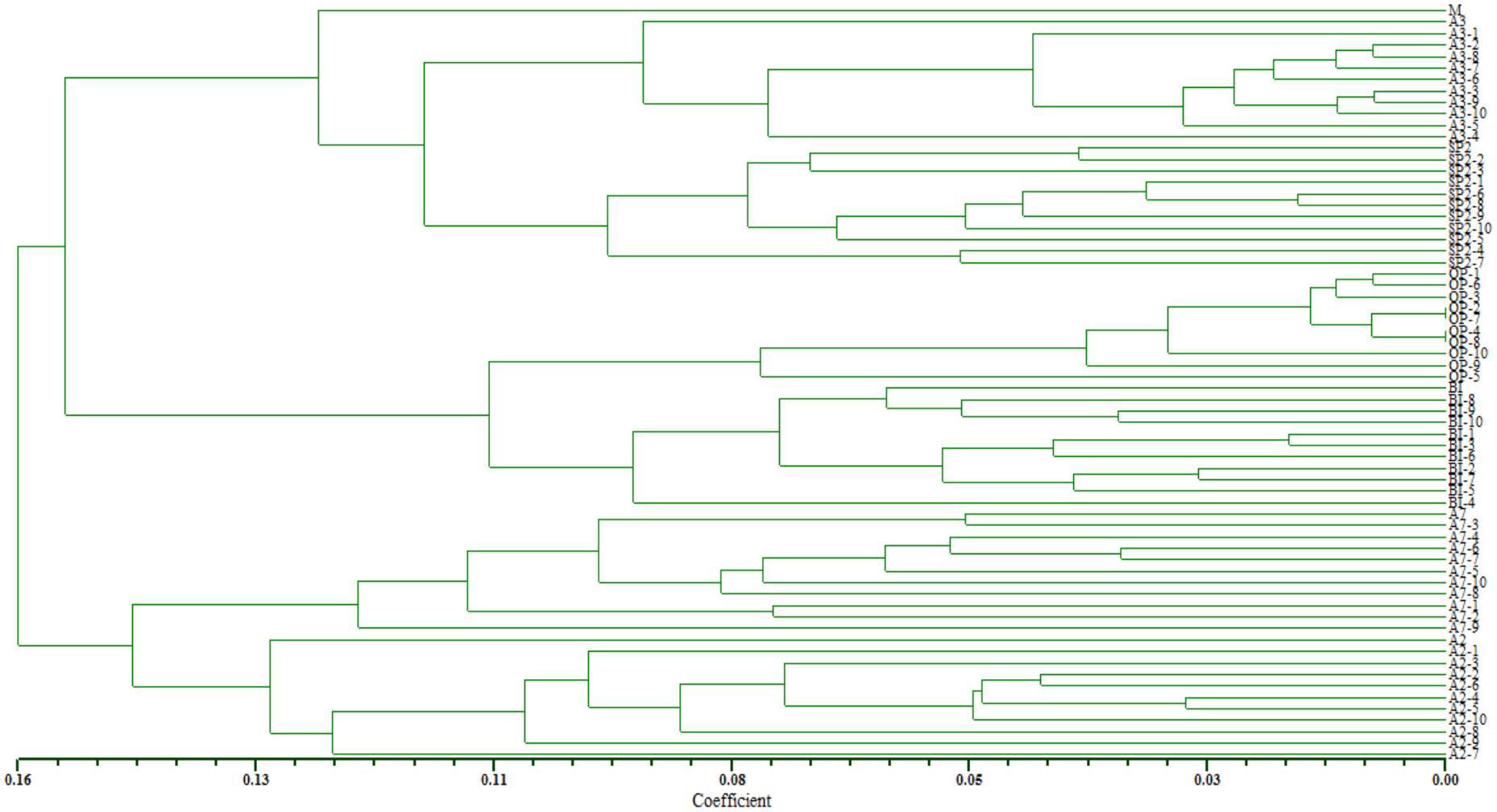

Fig. B3.6: UPGMA dendrogram of the F_1 progenies, male and female parents based on Nei's genetic distances. A3(1-10) = MS6 x AM A3, A3= male parent AM A3; SP2(1-10) = MS6 x SP2, SP2 = male parent; OP(1-10) = commercial F_1 hybrid Opal F_1; B1(1-10) = MS6 x AM B1, B1=male parent AM B1; A7(1-10) = MS6 x AM A7, A7=male parent AM A7; SP A2(1-10) = MS6 x SPAA2, A2 = male parent.

Table B3.9: Primers (Pr), total number of ISSR loci amplified (N), number of discriminative ISSR *loci* (D) [total, common to F_1 hybrid and female parent (F1♀), or to the male parent (F1♂) percentage of discriminative ISSR loci (%D) obtained in the F1 MS6 x AM A3, MS6 x AM B1, MS6 x SP2, MS6 x AM A7, MS6 x SP A2 and respective parents]

MS6 X AM A3					
Primer		D			
	N	F1♀	(F1♂)	total	(%D)
827	8	0	1	1	12.5
841	6	0	0	0	0.0
848	9	0	1	1	11.1
855	11	1	2	3	27.3
856	8	0	2	2	25.0
857c	8	1	2	3	37.5
857g	7	1	2	3	42.9
872	7	1	1	2	28.6
878	4	0	2	2	50.0
Total	68	4	13	17	25.0

MS6 x AM B1					
Primer		D			
	N	F1♀	(F1♂)	total	(%D)
827	8	1	1	2	25.0
841	6	0	0	0	0.0
848	7	0	1	1	14.3
855	8	2	0	2	25.0
856	7	0	0	0	0.0
857c	10	1	3	4	40.0
857g	8	2	3	5	62.5
872	6	1	0	1	16.7
878	5	1	3	4	80.0
Total	65	8	11	19	29.2

MS6 x SP2					
Primer		D			
	N	F1♀	(F1♂)	total	(%D)
827	8	2	1	3	37.5
841	7	0	0	0	0.0
848	8	1	1	2	25.0
855	8	1	0	1	12.5
856	8	0	2	2	25.0
857c	8	0	2	2	25.0
857g	7	0	2	2	28.6
872	7	1	1	2	28.6
878	6	0	4	4	66.7
Total	67	5	13	18	26.9

MS6 X AM A7					
Primer		D			
	N	F1♀	(F1♂)	total	(%D)
827	8	1	1	2	25.0
841	6	0	0	0	0.0
848	7	1	1	2	28.6
855	8	1	0	1	12.5
856	7	0	0	0	0.0
857c	9	2	2	4	44.4
857g	7	1	1	2	28.6
872	7	1	1	2	28.6
878	8	1	5	6	75.0
Total	67	8	11	19	28.4

MS6x SP A2					
Primer		D			
	N	F1♀	(F1♂)	total	%D
827	8	2	1	3	37.5
841	6	0	0	0	0.0
848	6	0	1	1	16.7
855	8	3	0	3	37.5
856	8	0	2	2	25.0

857c	8	3	2	5	62.5
857g	7	1	2	3	42.9
872	7	1	1	2	28.6
878	7	0	3	3	42.9
Total	65	10	12	22	33.8

For all cross-combinations, the percentage of discriminative loci for the female parent was lower than for the male parent; for example, in the F_1 hybrid MS6 X AM A3, the number of discriminative ISSR *loci* for the male parent was 76% and only the 24% of the discriminative *loci* were discriminative for the female parent.

From this analysis, an inconsistent percentage of bands resulted present in the hybrids but not in the parents. These recombinant bands were always obtained with the same primers, which were 848 and 857C. These bands are a consequence of recombination and rearrangements phenomena that can happen during the crossing-over phases.

Mantel test (r2 = 0.095*) showed a significative correlation (alpha=0.05) between both the matrix based on the morphological characters and that derived on the molecular analysis.

B3.4 DISCUSSION

Six hybrids were evaluated from the phenotypic and genotypic point of view in order to compare the results on these two typologies of analysis and to find any correlation between them. ISSR results were used to estimate the genetic relationship within and among samples of 10 individuals per each the six F_1 hybrids and their parents.

The UPGMA dendrogram obtained from the Nei's genetic distances matrix showed that all individuals of the hybrids have been clearly defined (*Fig. B3.6*) and individual of the same hybrid progenies were grouped together. Therefore, all individuals sampled from all the hybrid progenies were distinguishable by ISSR markers used in this study. Estimates of genetic relationships among individuals within a hybrid progeny would be improved increasing the number of polymorphic primers.

Bands specific to female and male parents were present in all hybrids, this ascertaining the hybrid nature of the F1 offspring.

The analysis on the fragment frequencies and on the discriminative loci is a direct method to evaluate the inheritance of the fragments and the uniformity of the hybrid progeny because it does not mask the intra-population variability. Imbalance between the percentage of fragments inherited by the hybrid progenies from the male parents respect to the percentage of fragments inherited from the female parent (MS6) could have an explication in the nature of the ISSR markers and in the genetic characteristics of MS6. In fact, ISSR markers, as others dominant markers (i.e. presence against absence of the band), cannot differentiateheterozygous individuals from the homozygous dominant ones (Kumar, 1999) and this is a disadvantage of this type of markers. In our case, the female parent is common in all crosses; therefore, the lower inheritance of the female parent fragments could be due to a high recessive homozygous condition of MS6. In fact, if the male parents are homozygous dominant for the majority of the ISSR loci, the correspondent hybrid progenies will be all heterozygous and will have an ISSR pattern similar to that of the male parent (*Fig. B3.1*). For this reason, it should be hoped to support the ISSR analysis of hybrids with other considering co-dominant markers, like SSR to distinguish the heterozygous individuals from the homozygous dominant ones. In order to find a correspondence between the genotypic and the phenotypic characteristics, a morphological analysis was carried out on the 6 hybrid offsprings. The results of the discriminant analysis based on the morphological traits

confirmed the one-way ANOVA previously applied in both cases, the shape of the head was an important trait for discriminating the hybrids.

The Mantel test showed a significant correlation between both the matrixes of molecular and morphological analyses. This means that the clustering of clones based on the morphological characters is consistent with that derived from the molecular analysis. Moreover, it could be possible to individuate if some *loci* result more correlated with some morphological traits in order to predict the characters expected by a F_1 hybrid offspring just analyzing them by ISSR markers.

Marker assisted selection (MAS), a pre-eminent breeding application for DNA markers, requires the definition of linkage between marker *loci* and traits. In globe artichoke, this approach remained untested since the first map in the species has only recently been elaborated (Lanteri *et al.,* 2006). The establishment of genetic correlations between a morphological trait and a molecular marker from the analysis of germoplasm collection could be a valid alternative to genetic mapping (Crinó *et al.,* 2007) and could be useful for the characterization and identification of F_1 offsprings at an early stage. This approach could consistently help the breeders in the genetic improvement of the globe artichoke for a first screening of the hybrids and to predict, at the beginning of the season, which will be the traits and hopefully the level of homogeneity within the hybrids that the different cross-combinations will show.

CONCLUSIONS

- Some male and female stable lines have been developed. It was demonstrated that the clone MS6 is a stable male sterile clone that gives homogeneity to the F_1 offsprings. Some male lines.

- More knowledge was achieved on the differences in the floral biology of male sterile and male fertile genotypes. An efficient staging of the flowers showed differences in the development of the female organs both the parents. Moreover, from the analysis of the pollen, it was confirmed a post-meiotic block in the pollen development, as previously described by Stamigna *et al.* (2002). A probable connection between the scarce viability of the male sterile pollen and a less development of the external exine structure of the pollen grains has been observed.

- Some homogeneous F_1 hybrids were constituted. They will be again tested, characterize deeply and released in order to start their commercial production.

- A system for the evaluation of the homogeneity of the different F_1 hybrids offsprings was developed. It resulted consistent and able to differentiate some quite homogeneous F_1 hybrids from those less homogeneous.

- All hybrid progenies have been well differentiated by both the molecular and morphological analyses. The clustering of clones based on the morphological characters resulted consistent with that derived from the molecular analysis.

- Data on the pollination technique showed the importance to deep the possibility of using the bumblebees as a valid alternative in the pollination system. Moreover, it was confirmed the scarce attractiveness of the honey bees for the male sterile flowers already reported in literature.

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