Summary

Nowadays, paprika and chile peppers (*Capsicum* spp.) are profusely cultivated throughout the world and they are amongst the most important vegetables for both fresh and spice consumption. From the five cultivated species of this genus, *C. annuum* L., is the most popular, genetically diverse, and economically important species and, therefore, continuous and huge breeding efforts are made every year to develop new improved materials. In this sense, two strategies are of paramount importance for breeding programs: i) interspecific hybridization, to harness genetic variability of related species and, ii) to shorten breeding cycles, which are necessary to produce inbred (selfed) lines or for successive backcrosses. In both case, embryo culture must be considered useful tool. Thus, this technique allows overcoming the postzygotic barrier of embryo abortion, quite usual in interspecific hybridization. In addition, it enables the excision of young embryos from immature fruits and their *in vitro* germination, instead of waiting for full ripening and recovery of mature seeds.

Thereby, the first objective of this PhD Thesis was to increase the efficiency of *in vitro* germination of immature embryos from *Capsicum* genus, dealing with the following factors: i) genetic diversity in terms of species and, even, genotypes within species, ii) the main embryo immature stages (globular, heart, torpedo and early cotyledonary) and iii) *in vitro* media formulation and culture conditions (MS salts, sucrose, growth regulators, initial dark incubation). The second objective was to evaluate and clarify the interspecific barriers which can be found within the *annuum* complex (*C. annuum*, *C. chinense* and *C. frutescens*) and between this complex with *C. baccatum*, evaluating strategies like embryo rescue and, alternatively, the genetic bridge cross to achieve *C. annuum* × *C. baccatum* hybrids. Finally, the third objective was to evaluate the usefulness of embryo culture to shorten pepper (*C. annuum*) cycles and, consequently, to accelerate breeding programs of this crop.

In our study, *in vitro* germination and adult plants were achieved for immature embryos of the five cultivated species and at all embryo stages, with the only exception of globular embryos from *C. chinense*. Regarding embryo stage, the mean efficiency of *in vitro* culture increased gradually with the stage of development: globular (5%), heart (21%), torpedo (37%) and early cotyledonary (69%). This is consistent with the reports from other species. Thus, embryos at the earliest phases of development, are highly dependent in terms of nutrition and

draw upon the endosperm, the suspensor and the surrounding maternal tissues (heterotrophic phase), while, later, they are metabolically capable of synthesizing substances required for its growth (autotrophic phase).

In terms of the composition of *in vitro* media we found that sucrose levels had the highest contribution to culture efficiency, while MS showed a lower effect. In contrast to the sucrose levels recommended in literature for early embryos (8-12%), we found that 4% levels offered the best response at any embryo stage, with an average germination rate of 43%. In the case of mineral salts, $^{1}/_{2}$×MS levels provided average germination rates higher than 1×MS (48% *v*/*s* 38%). Therefore, sucrose at 4% and $^{1}/_{2}$×MS were chosen to carry on with the optimization assay.

Once established 4% sucrose and $^{1}/_{2}$×MS dose as the formulation of reference, the levels of effect of the growth regulators (indole-3-acetic acid, IAA and zeatin) were then studied for the four immature embryos stages in several *C. annuum* accessions. We found that growth regulators at a low dose (0.01 mg/L) showed on average the highest rates (33%), followed by the medium without growth regulators (24% efficiency), while high levels (0.2 mg/L) especially zeatin, had negative effects on *in vitro* germination rates, with extremely low rates, which were comprised between 0% and 6% for any genotype and embryo stage.

Finally, on the basis of the medium with 4% sucrose, ½×MS, and 0.01 mg/L of both IAA and zeatin, we found that five days of initial incubation in darkness had a favorable effect on the *in vitro* embryo germination at any embryo stage, highlighting heart stage, with a mean increase rate from 17% to 27% and, especially, in globular embryos, with an increase from 3% to 22%. Although these rates may appear relatively low, they must be considered very successful as this is the first report about the *in vitro* germination of these delicate and very early embryos in peppers. Moreover, the *in vitro* germination rates for the next stage (heart), was also considerably higher than those reported previously by other authors. This is of paramount importance for interspecific hybridizations as depending on parent genotypes, embryo abortion may occur in the earliest stages, and, therefore, breeders need protocols for embryo rescue suitable for any stage. As a whole, the best formulation for the factors under study was: sucrose 4%, ½×MS (alternatively 1×MS for globular embryos in some specifics genotypes), IAA and zeatin at 0.01 mg/L, combined with five days of initial dark incubation.

Regarding the hybridization between *C. baccatum* and *C. annuum*, we have validated the two strategies under study. Within the genetic bridge strategy, we have established that *C. chinense* works very well as bridge species, while *C. frutescens* shows a lower efficiency as bridge, mainly due to abnormal hybrids or a lower crossability with both *C. baccatum* and *C. annuum*. Thus, the largest number of plants and with a high pollen fertility was achieved with this cross scheme: [*C. baccatum*$_{(♀)}$ × *C. chinense*$_{(♂)}$]$_{(♀)}$ × *C. annuum*$_{(♂)}$. Considering the strategy of *C. annuum* × *C. baccatum* direct cross combined with embryo rescue (based on the optimized media formulation), we observed an early hardening of the endosperm, that made very difficult embryo excision and low efficiency in terms of the number of hybrids achieved. Consequently, after hundreds of crosses and cultured embryos, we consider that the most efficient cross scheme to achieve BC1 (back cross 1) is: [*C. annuum*$_{(♀)}$ × *C. baccatum*$_{(♂)}$]$_{(♀)}$ × *C. annuum*$_{(♂)}$, based on *in vitro* rescue of *C. annuum*$_{(♀)}$ × *C. baccatum*$_{(♂)}$ embryos.

Finally, our study showed that no more than two generations per year are possible in peppers following conventional growing procedures. Thus, the total length of conventional breeding cycle under Autumn-Winter (AW) and Spring-Summer (SS) growing season ranged between in 264 days and 321 days in Guindilla (Cayenne) and Bola types, respectively. By contrast, the *in vitro* strategy shortened these AW + SS estimates to 183-240 days of the mentioned accessions. Such findings showed that this strategy will allow *Capsicum* breeders to achieve at least three generations per year, including California Wonder peppers, and up to four generations in the case of cayenne-type peppers. Consequently, breeding programs which require F8-F10 lines could be achieved in two or three years by means of the *in vitro* germination of immature (torpedo and early cotyledonary) embryos.

Through this study we have optimized the embryo culture technique in *Capsicum* peppers and validated this technique for two important applications in breeding: interspecific hybridization and shortening cycle, thus useful information is available to peppers breeders. In addition, the transfer of genetic material from *C. baccatum* to *C. annuum* has been achieved by means of both strategies: genetic bridge cross (using *C. chinense*) and *in vitro* rescue of *C. annuum* × *C. baccatum* embryos. Both strategies provided fertile materials and, therefore, the information reported in this work will be very useful for *Capsicum* breeders. Moreover, it is

possible to accelerate breeding programs in peppers using *in vitro* germination of immature embryos.