

## **REGENERATION *IN VITRO* AND GENETIC TRANSFORMATION OF PEPPER (*Capsicum* spp.): THE CURRENT STATE OF THE ART**

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### **Abstract**

The review presents an update on pepper biotechnology of the recent years. The progress and difficulties of the following topics are discussed: regeneration via organogenesis and somatic embryogenesis, vegetative micropropagation, culture of anthers and isolated microspores, and genetic transformation as a tool for transgenic breeding of *Capsicum annuum* L..

### **Introduction**

About three decades ago tobacco and petunia became models for research of certain aspect of modern biotechnology and molecular biology. At the same time potato turned to be a prominent archetype for clonal micropropagation and virus elimination by meristem culture. Transgenic cultivars of these solanaceae, as well as of tomato and eggplant cultivars, have been released to the market, or are being at different stages of field trials. By contrast, pepper (*Capsicum annuum* L.) lags behind, and is only at the entrance to the era of advanced biotechnology and of transgenic breeding.

This review is not a comprehensive literature survey. Rather, it presents an update on pepper *in vitro* technologies and genetic transformation, based on information accumulated in recent years. Further details can be found in previous reviews (Fári & Andrásfalvy 1994, Regner 1996). The objectives of our presentation are: first, to display contemporary progress on regeneration *in vitro* and genetic transformation related to pepper breeding and horticulture; second, to highlight unresolved problems that merit further research and development.

### **Regeneration**

#### *Regeneration via organogenesis*

Effective pepper regeneration systems *in vitro* serve three main purposes: (a) Micropropagation of special value elite plants (e.g. male sterile plants, or F1 plants displaying heterosis (i.e. Gupta et al. 1998)). (b) The production of transgenic plants. (c) The generation of microspore- or pollen-derived dihaploid plants (Renger 1996).

The last years have seen numerous studies on regeneration via shoot and root organogenesis induced on explants prepared from seedlings at different times after sowing. In rare cases spontaneous shoot regeneration, from explants cultured on a medium devoid of growth regulators, has been observed (Ezura et al. 1993, Binzel et al. 1996a). Usually, as in all papers cited hereafter, regeneration had to be induced and accomplished by exogenous growth regulator supplements to the medium. The explants used were: "half seed explants" (Binzel et al., 1996a), cotyledons (Szász et al. 1995), hypocotyls and decapitated hypocotyls (Szász et al. 1995, Ramirez-Malagon & Ochoa-Alejo 1996, Ramage & Leung 1996), leaf tissue (Christopher & Rajam 1996, Zhu et al. 1996), or protoplasts derived from leaves taken from *in vitro* shoots (Prakash et al. 1997). Commonly, explants were placed onto an agar-solidified shoot induction medium supplemented with a cytokinin (benzyl adenine (BA), kinetin, zeatin or thidiazuron

(TDZ)), and often also an auxin (IAA, IBA or NAA). Subsequently, shoot elongation may, in some cases, take place after transplantation of small shoots *ex vitro* (Ebida & Hu 1993). However, in most instances shoot - or shoot bud - clusters were transferred to a shoot/stem elongation medium *in vitro*, because shoot elongation has repeatedly been found as a major obstacle in obtaining normal pepper plants. Gibberellic acid (GA<sub>3</sub>) is usually the plant growth regulator added to elongation medium. Franck-Duchenne et al. (1998) attempted increasing stimulation of stem elongation by planting shoot buds on a medium including 24-epi-brassinolide. Although this growth regulator did improve the rate of plantlet recovery, it did not provide a general alleviation of stem elongation difficulties. Adventitious shoots root either spontaneously on a hormone-free medium, or consequently to planting shoots onto a medium supplemented with a low auxin concentration.

The number of plants regenerated per explant stands usually in the range of 1-10. In some papers even lower regeneration rates have been indicated. We assume that there are genotypes for which serious attempts to develop regeneration procedures failed, and obviously these failures have not been published. Our group was also involved in developing regeneration and transformation technology for the last four years, and we gained similar experience: A screening for genotypes with regeneration capabilities was conducted. Explants prepared from young seedling organs were cultured according to numerous protocols published. Out of more than 40 genotypes tested, obtained from the breeding collection available at the Volcani Center and from a seed company, only 4 genotypes were finally identified to have consistent repeatable regeneration capabilities. Using our best genotypes and the choice protocols, we were able to generate an average of about 5 fully functional plants per (cotyledon or hypocotyl) explant (Wolf et al. 1998). Other genotypes had either lower regeneration rates or did not display any normal regeneration.

Several characteristics emerge from the studies reported by different laboratories: (a) The hormonal composition and the sequence of application of growth regulators has to be adapted to the genotype and to explant type. (b) Cytokinin, whether applied alone or in combination with an auxin, is the critical regulator for adventitious shoot induction. These two rules have been found to be valid in different *Capsicum* species (e.i. Wang et al. 1991, Christopher & Rajam 1994, Fári & Andrásfalvy 1994), and they are not unique to pepper species. (c) Invariably of pepper genotype or explant type, profuse bud formation is visible within 2-3 weeks from culture initiation. Unfortunately, a majority of these buds develop either leafy structures or stunted and otherwise aberrant shoots. Only after transfer of cultures from a shoot induction to a stem elongation medium, a minority of buds eventually develops into normal shoots. The major problems during shoot elongation are less severe in hot pepper (Ochoa-Alejo & Irenta-Moreno, 1990; Valera-Montero & Ochoa-Alejo, 1992) and in some experiments in *C. frutescens* (Wang et al., 1991) than in sweet pepper varieties. Defects in shoot meristem differentiation or primordia organization cause the recurrent low incidence of normal plant recovery. Conditions that permit abundant normal shoot development have generally not been detected.

From the viewpoint of micropropagation via adventitious shoot organogenesis, many pepper genotypes are still considered recalcitrant. In spite of the difficulties described, we find a few encouraging cases of success. For example, Ma et al. (1991) reported an annual production of 4.7 million plants by micropropagation based on a multiplication rate of 9:1. This propagation scenario, being laborious and performed manually, can be economically viable provided the culture-derived plants are cheaper than the seedlings.

#### *Regeneration via somatic embryogenesis*

Direct somatic embryogenesis was first described in chilli pepper by Harini & Sita (1993) and in sweet pepper by Binzel et al. (1996b). In both studies, immature zygotic embryos were inoculated on a medium including 2,4-D, kinetin or TDZ, coconut water and 6-10% sucrose.

Somatic embryos formed directly on the immature zygotic embryo without the formation of an intermediate phase of embryogenic callus. The entire process, from embryogenesis induction to somatic embryo maturation, was accomplished on the initial medium without subculture. Noteworthy, somatic embryogenesis occurred on 10-85% (Binzel et al. 1996b) or even on all (100%) zygotic embryos explanted (Harini & Sita 1993). The multiplication rate (somatic embryos:zygotic embryo) was 13 (Harini & Sita 1993), or up to 8 (Binzel et al. 1996b).

Somatic embryos were obtained also from mature zygotic embryos, however, through an intermediate stage of embryogenic callus. The callus was generated on a medium with 2,4-D, and then transferred through a sequence of subcultures in different media (Büyükalaca & Mavituna 1996). Moreover, a recurrent somatic embryogenesis process in liquid media was developed: all stages of embryogenesis, from growth of the embryogenic suspension cultures to embryo maturation, were performed in a bioreactor as a series of drain-and-fill batches, keeping the embryos in the bioreactor all the time (Mavituna & Büyükalaca 1996). The multiplication rates obtained by recurrent somatic embryogenesis in a bioreactor were significantly higher than the common multiplication rates reported for caulogenesis. The production of artificial seeds, consisting of somatic *C. annuum* embryos encapsulated in calcium alginate gel beads, has also been achieved (Büyükalaca et al. 1995). Although it is conceivable that some details of the culture conditions will require genotype-dependent adaptations, propagation in automated computer-controlled bioreactors could become the way to profitable large-scale micropropagation of elite material. However, since (somaclonal?) variations amongst culture-derived pepper plants were detected (Shen et al. 1994), future research and development of mass propagation technologies should include genetic stability and a follow-up in horticultural fidelity of the propagules.

#### *Anther and microspore culture*

Immature pepper pollen or microspores can switch from a gametophytic to a sporophytic developmental pathway. Microspore embryogenesis is usually triggered by the exposure of freshly excised anthers to a few days of elevated temperature stress of about 35°C in the dark, followed by incubation at 25°C in the light. Anthers are inoculated onto a solidified medium supplemented with sucrose, 2,4-D and kinetin (Dumas de Vaulx et al. 1981). The extent of androgenic response is clearly source genotype-dependent (Kristiansen & Andersen 1993, Mitykó et al. 1995, Dolcetsanjuan et al. 1997), and is influenced by the growth conditions of the plants from which anthers are harvested (Kristiansen & Andersen 1993). Recently, Dolcetsanjuan et al. (1997) considerably modified the protocol of Dumas de Vaulx et al. (1981): 35°C induction treatment was substituted by a 7°C treatment; sucrose in the medium was replaced by maltose; the culture vessel atmosphere was periodically ventilated with CO<sub>2</sub>-enriched air; finally, anthers were cultured on a growth regulator-less medium. The new procedures increased, in some genotypes, the incidence of embryogenesis induction to such an extent that the limiting factor in the number of plants eventually regenerated resided not in embryo formation but in embryo maturation and conversion to plants (Dolcetsanjuan et al. 1997).

Embryogenesis induction in cultures of isolated microspores rather than in anther cultures is another approach that could lead to an increase in the yield of microspore-derived embryos. Research in this direction was initiated several years ago by González-Melendi et al. (1995), Testillano et al. (1995), and González-Melendi et al. (1996). Multicellular, initial stages of embryo-like and callus-like structures were successfully induced in isolated microspore cultures, but the formation of fully developed embryos has not yet been achieved.

The regeneration potential of microspore embryos in anther culture, and the recovery of the homozygous trait through chromosome doubling, has been utilized for breeding of new cultivars as well as for genetic studies by research laboratories and seed companies (see Vagera 1990,

Regner 1996 and references therein). Hence, while the application of vegetative micropropagation in horticultural practice is still limited, androgenesis has already contributed to the establishment of commercial products worldwide.

### Transformation and transgenic breeding

The recent literature contains reports describing the generation of transgenic pepper plants. We discuss mostly full articles and refrain from expanding on short meeting reports that, unfortunately, lack experimental details. Transformed shoot buds were reported by Liu et al. (1990), but no functional transgenic plants could be secured. Since the early 90's, communications from laboratories in China have indicated successful transformation of *Capsicum frutescens* (Wang et al., 1991) and *Capsicum annuum* (Dong et al., 1992; Zhang et al., 1994; Zhu et al., 1996). In this later species, the creation of transgenic resistance to CMV, either by the use of cDNA from a viral satellite or the viral coat protein, was the main goal of the work. A similar work was reported by a Korean group, which described the transformation of hot pepper (*C. annuum*) with a CMV satellite construct (Lee et al., 1993; Kim et al., 1997). Partial attenuation of symptoms and a decrease of virus titer were observed. A recent report from India described the generation and characterization of transgenic hot chilli (*C. annuum*) (Manoharan et al., 1998).

In spite of this promising progress, tremendous efforts invested worldwide in the direction of transgenic pepper breeding has not yielded other successful results accompanied by scientific documentation. A critical evaluation of the published papers discloses: (a) A very low transformation efficiencies (Szász et al. 1995, Yu-Xian et al. 1996, Manoharan et al. 1998, Mihalka et al. 1998, Wolf et al. 1998). (b) The use of similar strategies by the different successful groups: *Agrobacterium* mediated transformation was exclusively used, cotyledons were generally preferred as the target explants, and NPT II (coding for resistance to kanamycin) was the selective tool in all cases. In most cases, each group concentrated on a specific *C. annuum* cultivar, and we therefore cannot exclude a strong genotype dependency for the described protocols.

The genetic transformation of pepper is also actively pursued in laboratories from the private sector. A patent on "genetically transformed pepper plants and methods for their production" was granted to DNA Plant Technology Co. (US 5262316) in 1993. The APHIS-USDA database for Environmental Releases (<http://www.aphis.usda.gov/bbep/bp/>) includes 9 individual field trials with transgenic peppers that were performed in US to the present (table 1).

Table 1: Genetically engineered and field tested pepper produced by the biotechnology industry in the US.

Company	Year	Introduced transgene	Selectable gene	Attempted modification
Seminis	1998	?	NPT II	Resistance to CMV
DNAP	1997	Hemicellulase	ALS	Prolonged shelflife
Seminis	1997	CMV and TEV coat protein	?	Resistance to CMV and TEV
Seminis	1997	CMV coat protein	NPT II	Resistance to CMV
PetoSeed	1996	CMV coat protein	NPT II	Resistance to CMV
Seminis	1996	CMV coat protein	NPT II	Resistance to CMV
DNAP	1996	?	ALS	Altered fruit ripening
DNAP	1995	$\beta$ -1,3-glucanase antisense	ALS	Altered fruit ripening
DNAP	1994	$\beta$ -1,4-endoglucanase antisense	ALS	$\beta$ -1,4-endoglucanase activity

Only partial information is provided on the composition of the transgenic material and no details are available on the genotypes utilized, the efficiency of the methodology and the induced phenotypes. However, some general statements can be offered: 1) NPT II and ALS (acetolactate synthase) are the preferred selectable genes. 2) Resistance to viruses and modification of the fruit ripening process seem to be major targets for transgenic improvement. It was mentioned elsewhere that sense suppression of endo-(1-4)- $\beta$ -D-glucanase in some of these transgenic lines resulted in modifications of the glycan cell wall fraction of ripening fruits, with a concomitant decrease of water loss and an improved shelflife (see Bedbrook, in Carpita et al. 1996). Hopefully, full reports will eventually shed light on these promising findings.

### Conclusions and future prospects

The repetitive search in laboratories worldwide - along decades - after the basics in pepper regeneration methods, reflects a persisting dissatisfaction from the state of the art amongst scientists, breeders and growers. The observation common to regeneration studies from somatic cells and from microspores, is the marked source-genotype dependency of the developmental process to a given set of culture conditions. This may indicate that an expectation to find protocols of validity and reproducibility across a wide range of *Capsicum* genotypes is, perhaps, unrealistic. Consequently, the regeneration hurdle will have to be overcome by adjustment of culture conditions for individual cases where transgenic breeding is being planned. Proliferation of somatic embryos in computer-controlled bioreactors will probably become the technology of preference for future mass micropropagation. Regarding androgenesis, the interesting new findings by Dolcetsanjuan et al. (1997) on improved rates of microspore embryogenesis indicates, on the one hand, that there is ample space for improvements of recovery of haploid and doubled haploid plants in anther culture. On the other hand, the isolated microspore culture technology can be described as still being in its "early embryonic" stage. Finally, the genetic transformation discipline is only at its infancy. Therefore, we have all the reasons to anticipate the field of pepper biotechnology to be very interesting and dynamic in the 21st century.

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