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Garlic Breeding System Innovations

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ABSTRACT

This review outlines innovative methods for garlic breeding improvement and discusses the techniques used to increase variation like mutagenesis and *in vitro* techniques, as well as the current developments in florogenesis, sexual hybridization, genetic transformation and mass propagation. Sexual sterility of garlic reduces its potential for improvement of desired traits. Restoring fertility in this crop, which has been vegetatively propagated for millenia, provides new genetic possibilities for breeding purposes and/or genetic studies. In this context the recent developments on the manipulation of garlic florogenesis are discussed and it is shown that specific environmental conditions might allow for fertility restoration and seed production in bolting garlic. Furthermore the introduction of *Agrobacterium*-mediated and biolistic gene transfer systems in garlic, a species known for its recalcitrant behaviour in *in vitro* culture, are reviewed. Attention is paid to the development of a high quality callus year-round production method for transformation. Also the first garlic transgenics resistant to beet armyworm (*Spodoptera exigua*) and herbicides will be introduced. Garlic friable embryogenic calluses are of pivotal importance for the establishment of cell suspension cultures needed for rapid multiplication of elite garlic genotypes. The development of these suspension cultures are discussed in this review. It is shown that a large number of somatic embryos (potentially 8 x 10^9 to 10^{11}) can be produced annually, from a single clove, for each current variety and that the conversion into plantlets amounts approximately 50%.

Keywords: embryogenic suspension cultures, fertility restoration, florogenesis, genetic transformation, sexual hybridization
Abbreviations: **AFLP**, amplified fragment length polymorphism, **GFP**, green fluorescent protein; **GLUAlCS**, γ glutamyl allyl L cysteine; **GMO**, genetically modified organism; **GUS**, β-glucuronidase; **LYSV**, leek yellow stripe virus; **OYDV**, onion yellow dwarf virus; **PCV**, packed cell volume; **QTL**, quantitative trait locus; **RAPD**, randomly amplified polymorphic DNA; **SNP**, single nucleotide polymorphism; **SSR**, simple sequence repeat

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INTRODUCTION

Garlic is a diploid (2n=2x=16) predominantly cross-fertilizing species. Garlic however has lost its blooming potential and fertility already millenia ago and thus vegetative propagation is the sole production system used. Consequently garlic breeding has been limited to the selection of the pre-existing genetic variability and increase in garlic variability was attempted via mutation breeding and *in vitro* techniques, however with very limited successes. In recent years increase in garlic variability is sought via sexual hybridization and genetic transformation. In all cases, an efficient mass propagation system is needed for multiplication of selected elite material for commercial purposes. In this review, the current status of mutation breeding, somaclonal variation, sexual hybridization, genetic transformation and mass propagation in garlic will be discussed.

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Invited Review

MUTAGENESIS AND SOMACLONAL VARIATION

Natural occurring variation originates from two sources namely genetic recombination and mutation. Domestication of garlic involved the selection of genotypes with strong vegetative development and bulb production. This selection process decreased generative reproduction and resulted in the development of almost only asexually propagating garlic cultivars. How long this situation is already occuring is not known, but it can most probably safely be stated that genetic recombination did not contribute much to the increase of variation in the garlic genepool over the last thousands of years. No information is available on the effects of natural occuring mutations in garlic.

Reports dealing with mutation breeding research in garlic are very scarce. Al-Safadi *et al*. (2000) showed that via the use of gamma irradiation (4-7 Gray) the resistance to white rot (caused by *Sclerotium cepivorum*) could be improved. In cultivar 'Kisswany' the infection by white rot could be reduced to 3% as compared to 29% in the control, in cultivar Yabroudy these figures amounted less than 5% white rot infection for mutant lines versus 20% infection for the control. Recently Taner *et al*. (2005) determined the effective dosis for Cesium-137 around 4.5 Gray for mutation breeding in garlic.

Induction of somaclonal variation in garlic and its use in plant breeding was scarcely carried out. *In vitro* culture was primarily aimed at the rapid propagation of elite material (for review see Novak 1990). Novak (1990) concluded that direct organogenesis in the absence of a dedifferentiating callus stage in *in vitro* culture of various garlic tissues, like bulb plate, inflorescences and apical meristems, resulted in genetically uniform plants. A callus phase on the other hand often leads to somaclonal variation. In this context, Al-Zahim *et al*. (1999) showed that basal plate callus cultures generated significant somaclonal variation of five garlic cultivars as determined by RAPD and karyotype analyses. Via RAPD analyses it was found that the frequency of variant RAPD fragments was around 0.63%. Karyotype analyses indicated that 16% of the regenerants were abnormal (non-diploids). Comparing these data with data from other plant species indicated that stress due to *in vitro* conditions has a considerable impact on the genome constitution of garlic (Al-Zahim *et al*. 1999).

SEXUAL HYBRIDIZATION

Florogenesis

Similar to many geophytes (Flaishman and Kamenetsky 2006), garlic florogenesis consists of five distinct phases: meristem transition from vegetative to reproductive stage, scape elongation, inflorescence differentiation, completion of floral development, and anthesis (Kamenetsky *et al*. 2004b). In garlic, the apical meristem may undergo any of the following developmental processes: (1) initiation of an incomplete leaf, cessation of growth activity, and finally degeneration; (2) formation of a dormant clove; (3) initiation of a spathe (prophyll), followed by the formation of a floral meristem and inflorescence differentiation (Takagi 1990). The transition of the apical meristem from a vegetative to a reproductive state occurs in the field, during the active growth stage (Kamenetsky and Rabinowitch 2001).

In general, the garlic inflorescence can be described as an umbel-like flower arrangement, whose branches (flower clusters) arise from a common meristem. An initial elongation of the flower stalk precedes the spathe (prophyll) formation and the swelling of the floral meristem. The differentiation of floral initials begins only after the scape reaches 5-7 mm in length and the apex diameter exceeds 0.5 mm. Later, the apical meristem subdivides to form several swellings, each of which gives rise to a number of individual flower primordia. Concomitantly, leaf-like membranous bracts appear at the periphery of the inflorescence, and grow faster than the developing floral primordia. The differentiation of individual flowers begins when the inflorescence meristem reaches a diameter of about 2-3 mm (Kamenetsky and Rabinowitch 2001; **Fig. 1A-C**).

In the individual flower, each perianth lobe and the subtended stamen arise simultaneously from a single primordium, as in bulb onion (Jones and Emsweller 1936; Esau 1965; de Mason 1990), shallot (Krontal *et al*. 1998) and other *Allium* species (Kamenetsky and Rabinowitch 2002). Following differentiation of the outer perianth lobes and stamens, the inner whorl is differentiated, and the carpels are initiated when the outer perianth lobes overarch the stamens (Kamenetsky and Rabinowitch 2001). In some garlic cultivars, e.g., the Japanese 'Shanhai-wase', floral malformations and abnormal formation of embryo sacs may occur during flower differentiation (Etoh 1985).

When floral pedicels elongate and the inflorescence becomes spherical, new undifferentiated meristematic domes become visible at the periphery of the inflorescence. These quickly differentiate and develop into small inflorescence bulbs (topsets) (Kamenetsky and Rabinowitch 2001; **Fig. 1D**), which intermingle with the young flowers and physically squeeze the developing floral buds, thus causing their degeneration (**Fig. 1E**). Therefore, in some garlic clones, a perpetual removal of the developing topsets resulted in the development of a small number of normal flowers some of which produced viable pollen and seed (Konvicka 1984; Etoh *et al*. 1988; Pooler and Simon 1994; Jenderek 1998; Jenderek and Hannan 2000; Kamenetsky and Rabinowitch 2002; Simon and Jenderek 2004).

Gustafsson (1946, 1947; cited by Etoh 1985) assigned *Allium* species to the group of viviparous plants, in which topsets (bulbils) develop instead of flowers or intermingle with flowers in the inflorescence. Later sterility was as-

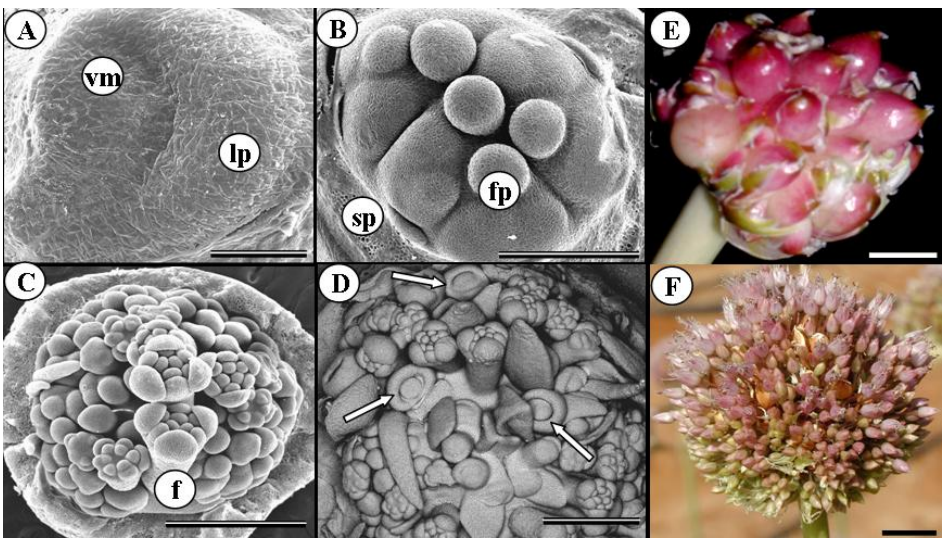

Fig. 1 Florogenesis of garlic. (**A**) Apical meristem at vegetative stage (vm); leaf primordium (lp) is visible; accession #3026. Bar = 0.1 mm; (**B**) Apical meristem at reproductive stage; first flower primordia (fp) are visible; spathe (sp) removed; accession #3026. Bar = 0.5 mm; (**C**) Differentiation of individual flowers (f); accession #3026. Bar = 1 mm; (**D**) Differentiation of topsets (arrows) following flower differentiation. Bar = 1 mm; (**E**) Topset formation in the inflorescence of accession #2509. Pre-planting storage at 4°C. Bar = 1 cm; (**F**) Fully differentiated inflorescence of accession #3026 after spathe opening. Pre-planting storage at 4°C. Bar = 2 cm. From: Rotem *et al*. (2007), with kind permission of Oxford University Press.

sumed to result from competition for nutrients, between floral and vegetative buds (topsets) in the developing inflorescence (Koul and Gohil 1970); degeneration of the tapetum (Novak 1972) or degenerative-like diseases, induced by organisms such as rickettsia, mycoplasma and/or viruses (Konvicka 1973).

In garlic, differentiation of topsets begins in the periphery of the apex. Size, number and pace of development vary with the genotype. Following initiation, topsets develop quickly and, in most cases, with consequent degradation and abortion of the developing flower buds. Different morphotypes vary in the ratio between flower and topset buds, and time of floral abortion.

In horticultural practice softneck and hardneck garlics are recognized, however from a physiological point of view the terminology bolters and non-bolters is more correct. Scape elongation and inflorescence development vary considerably among garlic varieties and were classified by Takagi (1990) as: (1) *Nonbolting* – with a few exceptions these plants do not form normally a flower stalk, but produce cloves inside an incomplete scape. They form a bulb made of cloves developing in the axils of the five or youngest foliage leaves, (2) *Incomplete bolting* – plants produce a thin, short flower stalk, bear only a few large topsets, and usually form no flowers; the stalk and topsets may remain enclosed by the pseudostem. These genotypes form a bulb made of cloves developing in the axils of the two or three youngest leaves and (3) *Complete bolting* – plants produce a long, thick flower stalk, with many flowers and topsets; these genotypes normally form bulbs made of cloves in the axils of the two youngest foliage leaves.

Scape development and blooming in garlic are determined by the genetic make-up of the individual plant and the environment (Takagi 1990) and thus variation can also be observed within a single clone (ERJ Keller, pers. comm., 2002). Long photoperiod is both obligatory and quantitative for the reproductive process of garlic. Long days are required for scape elongation beyond the leaf sheaths while continuous short photoperiods result in a dwarfed scape enclosed inside the false stem. The process is triggered by long photoperiods in post-transitional plants and permits the completion of florogenesis (Kamenetsky *et al*. 2004b).

Under exclusively long day conditions, scape elongation is fast, but only a few plants reach maximum inflorescence size and spathe-break, probably because of hormonal effects and changes in distribution of assimilates between the developing bulb and inflorescence (Rosen and Tong 2001). Cold storage (-2 to 9°C) or low field temperatures and long photoperiod may promote scape elongation in non-bolters and incomplete bolters, whereas high storage or field temperatures, and short days might inhibit scape elongation in bolting plants (Kamenetsky *et al*. 2004b).

In the developing inflorescence, flower formation is followed by differentiation of topsets and is promoted by a long photoperiod. Sophisticated manipulation of the environment, both prior to and after planting, can lead to the development of viable flowers in a topset-free umbel (**Fig. 1F**). However, normal flowering cannot be achieved if one of the developmental stages is disturbed.

Fertility restoration and seed production

The sexual sterility of garlic inhibits or markedly reduces possibilities of improvement of economically important traits, including pest and disease resistance, yield, and quailty, through breeding. Restoration of fertility after millennia of vegetatively propagation opens for the first time new horizons for breeding and genetic studies in garlic. Many researchers attempted to restore fertility in garlic (Kononkov 1953; Novak and Havranek 1975; Katarzhin and Katarzhin 1978; Konvicka 1984; Etoh 1985; Etoh *et al*. 1988; Etoh 1997; Etoh and Simon 2002; Kamenetsky *et al*. 2004a, 2004b; Simon and Jenderek 2004).

Under the same environmental conditions, garlic accessions differ significantly in morphological and physiological traits, including leaf number prior to bolting, flowering date (date of spathe opening), final stem length, as well as in flower/topset ratio and pollen viability (Etoh 1985, 1986; Takagi 1990; Pooler and Simon 1993a, 1993b; Engeland 1995; Etoh and Simon 2002; Simon 2003; Kamenetsky *et al*. 2004b; Simon and Jenderek 2004; Kamenetsky *et al*. 2005). Marked variation among garlic clones with regard to flowering ability and flower to topset ratio led Etoh (1985) to propose that garlic undergoes a process of transition from sexual to asexual reproduction. Accordingly, ancestral garlic had normal meiosis, was fertile, and developed numerous flowers in the long-scaped umbel. Compared with modern clones, ancestral plants probably had greater adaptation to a variety of climatic conditions, a larger numbers of foliage leaves, and diverse maturation dates. The status of garlic fertility prior to domestication is at present unclear. However, fertility restoration by decapitation, removal of topsets and/or environmental manipulation clearly indicates that the genetic cascade coding for flowering remained intact and is not impaired. In some garlic genotypes gradual accumulation of chromosomal mutations during millennia of vegetative propagation resulted in complete sterility, shorter scapes and fewer flower buds and topsets. Domestication and subsequent cultivation and sterility of garlic has probably been accelerated by selection for larger bulbs.

Central Asia, the centre of origin for many *Allium* species, is a valuable source for *Allium* diversity, and a good potential for solving the enigma of its sterility (Hanelt 1990; Simon 2003). In the early 1980s, expeditions to Central Asia, collected a number of garlic accessions in Uzbekistan, Tadjikistan, Kirgizistan, and Kazakhstan (Etoh *et al*. 1988). The collected plants were grown at Kagoshima, Japan, and following topset excision, 17 clones developed fertile flowers with over 3,000 viable seeds. One of the clones was male sterile. Later, more fertile garlic plants were found in Armenia, Georgia, and Sin-Kiang (Etoh *et al*. 1991). Pooler and Simon (1994) improved floral production and seed set by scape decapitation and removal of topsets, but seed germination was low and ranged between 10 and 12%. Later, Inaba *et al*. (1995) and Jenderek (1998) obtained 50,000 and 1.2 million garlic seeds, respectively. They identified 27 clones as highly fertile, producing over 400 seeds per umbel, with seed germination of 67 to 93%. Removal of topsets was necessary only in the early generations, as the strong selection pressure for blooming and seed production resulted in improved seed set. Recently, fertile accessions were identified in the USDA garlic collections (Jenderek and Hannan 2000, 2004), producing 0-85 seeds per umbel in the first propagation cycle. Major blooming traits, including flower stem appearance, spathe opening, umbel shape, and the number of flowers per umbel were stable and similar across populations evaluated.

The first step towards marker-assisted selection of fertile garlic clones was made by Etoh and Hong (2001) using RAPDs. They screened twelve pollen fertile and sterile clones using 60 10-mer primers and found two RAPD markers, which amplified only in the pollen fertile clones. These two markers were further tested on 60 clones, and found to be correlated to pollen fertility.

During 1995-2001, collection missions to Central Asia have gathered over 300 garlic land races and plants from natural populations (Baitulin *et al*. 2000; Kamenetsky *et al*. 2004b). The collection was evaluated in Israel for fertility and many other traits, and 30 accessions produced seeds. In Israel, the seven most productive accessions produced 400-500 seeds per umbel, with germination rates around 90%, and normal seedlings development. The young plants formed two to five leaves, prior to bulbing and ripening. However in the Netherlands, under field and greenhouse conditions, the aforementioned clones produced only small number of seed with less than 5% germination. It is speculated that field conditions in the Netherlands are less conducive to quality seed production than those in Israel (Kamenetsky *et al*. 2005).

A search for genes involved in the control of flowering

in garlic, resulted in identification of garlic *LEAFY*/*FLO* homologue, *gaLFY* (Rotem *et al*. 2007). *gaLFY* is expressed in both flowering and non-flowering genotypes, however further comparative analyses of gene expression revealed two *gaLFY* transcripts, differing in 64 nucleotides, with clear splicing borders. The short one was common in both genotypes throughout their development, whereas the long variant appears in the flowering genotype only during the reproductive phase. Thus, the phenotypic differences in garlic, with regard to flowering, may be associated with the efficacy of the splicing process.

Open-pollinated families of garlic seedlings populations exhibit a large variation in many traits including single-clove bulbs with white, purple, gray and brown skins differed in bulbing ability and ripening dates (Kamenetsky *et al*. 2004b), survival ability (7.8-83.3%), bulb maturity, bulb weight (0.1-53.3 g), number of cloves (2.9-10.4), and the ability to mature in one growing season (76.5-100%) (Jenderek 2004; Jenderek and Zewdie 2005). Seedlings populations also possess important traits like disease resistance, for example, tolerance to rust, *Puccinia allii* (Jenderek and Hannan 2004). This partial list of variable traits indicates the potential present in sexually derived progenies for the improvement of garlic. The discovery of fertile plants on one hand and the development of a sophisticated environmental system (Kamenetsky *et al*. 2004b) for fertility restoration, thus enable in depth studies on the genetics of garlic, on flowering and fertility in plants, as well as classic and molecular breeding work crop improvement.

Zewdie *et al*. (2005) constructed the first linkage map of garlic on the basis of a population of 84 individuals, originating from a selfing of a single plant, using SNP, SSR, and RAPD markers. Thirty-seven genetic markers formed nine linkage groups covering 414.5 cM and many of the markers proved to have a skewed segregation. A male fertility locus was also included in the map. Ipek *et al*. (2005) constructed, on the basis of progenies originating from selfing two unrelated garlic plants, two low-density genetic maps, using AFLP and gene-specific markers. A total of 360 and 321 markers segregated in the two populations respectively, indicated a high level of heterozygosity in the nuclear genome. 94.7% of the segregating traits fit a 15:1 ratio for two loci, suggesting extensive levels of duplication in the garlic genome and supporting similar observations for onion.

One option for breeding new garlic varieties is the development of a system similar to the one used for potato, namely annual cross-fertilization of a large number of garlic clones and the subsequent selection between and within progenies over the years originating from these hybridizations (Kik 2002). An alternative option is the selection of highly fertile parental lines and the production of hybrid seed for direct seeding. The latter case would save the need for vegetative propagation and virus elemination (Salomon 2002).

GENETIC TRANSFORMATION

Regeneration systems

A successful garlic genetic transformation system depends on two key factors: refined of methods for the introduction of preferred target genes into garlic, and the development of sophisticated recovery methods of intact plants, either from fully dedifferentiated or from organized tissues.

Regeneration via somatic embryogenesis in garlic was first reported from calli of bulb leaf discs and stem tips by Abo El-Nil (1977). Subsequently, Fereol *et al*. (2002, 2005a, 2005b) used young leaf explants from four European garlic cultivars to produce embryogenic calli and obtained efficient regeneration via somatic embryos. Regeneration in garlic was also reported from basal plate (Al-Zahim *et al*. 1999), leaf (Wang *et al*. 1994; Zheng *et al*. 1998a), receptacle (Xue *et al*. 1991), and flower buds (Suh and Park 1988). Suh and Park (1995) used abnormal garlic roots derived from anthers, pedicels, and bulbils as explants to regenerate plantlets. Other reports on organogenesis or embryogenesis in regenerants of garlic plants were mainly based on shoot tip or stem disc explants (Kehr and Schaeffer 1976; Nagasawa and Finer 1988; Choi *et al*. 1993; Ayabe *et al*. 1995; Ayabe and Sumi 1998; Myers and Simon 1999; Kondo *et al*. 2000; Hasegawa *et al*. 2002). Haque *et al*. (1997, 1999) obtained up to 75% shoot regeneration from garlic root tips without an intervening callus phase. It is thus evident that root tip explants are commonly used for the development of a garlic regeneration system with or without a callus phase (Haque *et al*. 1997, 1998; Barandiaran *et al*. 1999a, 1999b; Robledo *et al*. 2000). However, this type of explant is in limited supply per plant. Myers and Simon (1998) reported on a continuous callus production and regeneration system from garlic root segments including root tips, but the time from tissue sampling to regeneration required 10 months. Using both apical and non-apical garlic root segments from the four most widely cultivated European garlic cultivars, namely 'Messidrome', 'Morado de Cuenca', 'Morasol', and 'Printanor', Zheng *et al*. (2003) developed an efficient procedure for callus induction and regeneration procedure thus requiring 2 months for callus induction followed by 2 months of regeneration, with the same efficiency as the systems developed by Zheng *et al*. (1998b, 1999) for other *Allium* crops. The use of both apical and non-apical root segments for callus production provides ample root segments that can be harvested every 4–6 weeks from *in vitro* plantlets. Such young and actively dividing callus is uniquely suitable for genetic transformation year-around.

Transformation systems

Despite recent progress in restoration of garlic fertility, the availability of a reliable genetic transformation system remains highly valuable due to lack of variability in desired traits, or because of insurmountable species barriers. Transformation of recalcitrant monocots like rice, wheat, barley and maize has been achieved by direct gene transfer systems: chemical methods, electroporation, particle bombardment and silicon carbide fibres (Curtis 2004). Recently, *Agrobacterium*-mediated transformation of monocots has gained favour and many transgenic plants have been obtained using specific *Agrobacterium* strains (Hiei *et al*. 1994; Rashid *et al*. 1996; Cheng *et al*. 1997; Tingay *et al*. 1997; Ishida *et al*. 1996; Arencibia *et al*. 1998). *Agrobacterium*-mediated transformation offers several advantages over the other systems, the most important being the ability to deliver a single or low number of intact copies of relatively large segments of foreign DNA.

Recent reports also show that genetic transformation has become possible in *Allium* (for review: Eady 2002). Klein *et al*. (1987) were the first to develop a high-velocity microprojectile method and demonstrated that epidermal tissue of onion could take up foreign DNA sequences. Wang (1996) tried to develop transgenic nuclear male sterile leek (*Allium porrum* L.) as a first step to introduce F_1 hybrid breeding into leek. Particle bombardment was used to integrate *barnase* and *barstar* genes into the leek genome. It was observed that both genes were present in the genome, however no data on pollen fertility were presented. Transient expression was shown with particle bombardment in garlic (Barandiaran *et al*. 1998; Ferrer *et al*. 2000) and Park *et al*. (2002) and Sawahel (2002) reported that transgenic garlic plants could be generated by this method. With respect to *Agrobacterium*-mediated gene transfer, Dommisse *et al*. (1990) demonstrated that onion is also a host for *Agrobacterium* as evidenced by tumorigenic responses and opine production inside these tumours. Using *Agrobacterium tumefaciens*, Eady *et al*. (2000, 2003a, 2003b, 2005) developed a stable transformation protocol using immature embryos of *A. cepa*, *A. porrum* and *A. sativum*. Kondo *et al*. (2000) used highly regenerative calli from shoot primordial-like tissues to produce transgenic garlic plants by

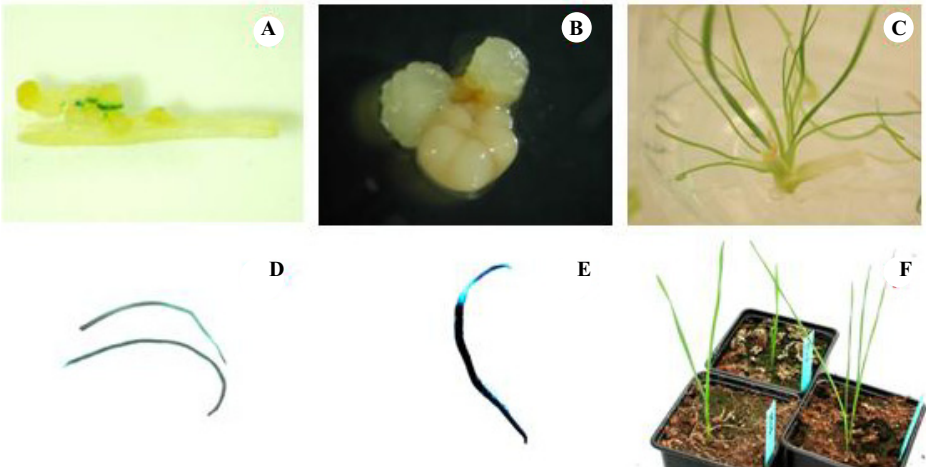

Fig. 2 Garlic transformation. (**A**) Transient expression of GUS in two-month old callus derived from root segment of cv. 'Printanor'; infection with AGL0 (pPB36) after four days of co-cultivation, (**B**) Hygromycin-resistant callus of cv. 'Printanor' after two months growth on selective medium, (**C**) Plant regeneration of cv. 'Printanor' transformed with AGL0 (pPB34). The photograph was taken four weeks after the hygromycin-resistant shoot had been transferred to rooting medium with hygromycin, (**D**) Expression of GUS in the leaves of a transformant, (**E**) Expression of GUS in the root of a transformant, (**F**) Transgenic garlic plants in the greenhouse. From: Zheng *et al*. (2004a), with kind permission of Springer Science and Business Media.

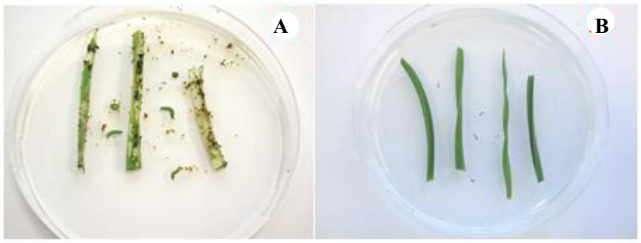

Fig. 3 Transgenic *Bt* garlic. Transgenic cv. 'Printanor' transformed with pPB36 resistant to beet armyworm (right image) and non-transgenic cv. 'Printanor' (left image). From Zheng *et al*. (2004a), with kind permission of Springer Science and Business Media.

Agrobacterium-mediated gene transfer. Zheng *et al*. (2001a, 2001b, 2004b, 2005) developed a reproducible and stable transformation protocol using calli derived from mature embryos of onion and shallot (Zheng *et al*. 2004b), and stably transformed garlic mediated via *A. tumefaciens* using young callus from different sources (Zheng *et al*. 2004a). Calli derived from apical and non-apical root segments of *in vitro* plantlets of garlic were used and via *Agrobacterium tumefaciens* mediated transformation GUS (β-glucuronidase) and GFP (green fluorescent protein) reporter systems were introduced into garlic (**Fig. 2**). Several independent transformed garlic callus lines were obtained within 6 months. The highest transformation frequency in a single experiment (1.47%) was obtained using garlic cv. 'Printanor' and differences between cultivars in transformation frequency were not significant. After transfer to the greenhouse of *in vitro* regenerants, transgenic garlic harbouring the *uidA* (GUS) gene survived easily and grew well, whereas some *gfp* transgenic garlic gradually died under these conditions.

Using the aforementioned protocol, for the first time, transgenic garlic resistant to beet armyworm (*Spodoptera exigua*) using *cry1Ca* and *H04* resistance genes from *Bacillus thuringiensis* were developed (Zheng *et al*. 2004a). Southern hybridization showed that the *cry1Ca* sequence was stably integrated into the garlic genome. The *cry1Ca* transgenic garlic plants developed normally in the greenhouse to maturity producing normal bulbs, but none of the transgenic *in vitro H04* garlic plants survived the transfer to the greenhouse. Transgenic *cry1Ca* garlic plants proved completely resistant to beet armyworm in a number of *in vitro* bio-assays (**Fig. 3**), thus providing good perspectives for the development of new garlic cultivars resistant to beet armyworm.

ELITE LINE MASS PROPAGATION THROUGH EMBRYOGENIC CELL SUSPENSION CULTURES

Rapid dissemination of new improved garlic varieties is possible depending upon the availability of efficient vegetative mass propagation methods, which will also be useful for virus-free garlic production systems. Indeed, the main constraint in garlic cultivation is the damage caused by virus diseases. Two potyviruses (OYDV and LYSV) and two latent viruses (Allexivirus and Carlavirus) are the major biotic factors affecting garlic clones, reducing plant development and bulb production significantly (Lot *et al*. 1994). The traditional scheme of a vegetative propagation system usually involves a four year process including the production of virus-free stock material, a mass propagation method, a vegetative propagation system and the distribution of high quality virus-free bulbs. Methods for eliminating viruses and producing virus-free material have been developed in the past 30 years, using meristem-tip culture (Walkey *et al*. 1987; Chovelon *et al*. 1990). Mass propagation methods were developed and several protocols of micropropagation were reported (**Table 1**). However, the multiplication rates of the regenerated garlic material were rather low and costly. Therefore suspension cultures, which are often used in other species with high efficiency and low cost, may provide a solution (Nagasawa and Finer 1988; Aitken-Christie 1991; Barrueto Cid *et al*. 1994).

Establishment of embryogenic suspension cultures

Young leaf and root explants produce calli on induction medium. Following size increase two distinct types are recognizable, one hyperhydric, translucent and nodular, the other nodular with a yellowish colour. The two types of calli were generally located at leaf edges near a vein or a root section. Clumps of the second type gave rise to somatic embryos, indicating their embryogenic potential (Fereol *et al*. 2002). Histological sections of these nodular yellowish calli showed globular masses with peripheral meristematic cells and isolated embryogenic cells containing large amounts of storage proteins.

It is well established that high percentage of root segments incubated in 2,4-D developed calli. Furthermore it has been shown that young leaf explants, excised from the three inter-most leaves of the garlic shoot, are more suitable for the development of embryogenic calli. A low level of 2,4-D (0.3 mg/l) resulted in a success rate of 90% callus production (Barandiaran 1999a, 1999b), with over 30% embryogenic calli. Monthly sub-cultured nodular, compact embryogenic calli became friable after 6 months. They mostly consisted of embryogenic and pro-embryos cells at an early developmental stage. Up to 60% of the explants produced this type of embryogenic friable calli.

Cell suspension cultures can be initiated from friable

Table 1 Overview *in vitro* propagation techniques used in garlic.

Cultivar	Explant	Basal medium	PGR	Sucrose Conc.	Morphogenic pathway	Efficiency	References
Extra Early White	Stem tip, Stem segment, Bulb leaf	AZ	C: p-CPA/2,4-D/Kin: 10/2/0.5 µM R: IAA/Kin: 10/20 µM	2%	Organogenic C	No data presented	Abo El Nil 1977
Isshuwase, Isshu-gokuwase, Shanhai, Santo, Furano, White-roppen	Meristem	LS	S induct: IAA/BA: 1/1 µM S prolif: NAA/BA: 5/10 µM	3%	S proliferation and *in vitro* bulblet formation	86% of meristems gave shoots 138 shoots from one shoot in 7 months	Nagakubo *et al*. 1993
White-roppen	Basal end of bulblets	MS modified	S induct: NAA/BA: 0.5/0.5 mg/l S prolif: NAA 0.05 mg/l	2%	Adventitious S	10 shoots/bulbet in 8 weeks	Masuda *et al.* 1994
Chonan	Inner leaves of small bulbs	MS	C: 2,4-D/Picloram/Kin: 1.1/1.2/2.1 mg/l, R: NAA/BA/Ad: 1/0.5/10 mg/l	3%	Cell suspension culture		Barrueto Cid *et al.* 1994
White-roppen	Root tip of micropropagated plants	B5	S induct: NAA/BA: 1/10 µM S prolif.: BA: 0.5 µM	3%	Direct S regeneration	70% explt. gave shoots, 10 shoots/explt. in 1 month	Haque *et al.* 1997
DDR7099, PI383819, Piacenza	Roots segments	B5	C: 2,4-D 4.5 µM then Picloram/ 2iP: 4.7/0.49 µM R: Picloram/BA: 1.4/13.3 µM	3%	Somatic emb.	85% cal. regenerated 5 shoots/cal.	Myers and Simon 1998
Fukuchi-White	Stem disc 1 mm thick	LS	Hormone free		S proliferation	20-30 S/month from 1 clove	Ayabe and Sumi 1998
20 cvs., different physiological groups	Root tip of micropropagated plants	B5	C: 2,4-D/ 2iP: 0.3 /0.5 ppm R: BAP: 3 ppm	3%	Organogenic callus	44% organogenic C 15 S/g C	Barandiaran *et al*. 1999
Aben, GT96-1	Root tip of micropropagated plants	N6 or MS	C: 2,4-D/Kin: 4.5/4.6 µM R: BA: 4.4 µM	87.6 mM	Organogenic callus	170 S/g C in 4 months	Robledo Pas *et al*. 2000
Uru-b, Fc	Immature inflorescence bulbils	MS	S induct: NAA: 5.4 µM	3%	Development of bulbils	33 to 46 bulbils induced per inflorescence	Ebi *et al.* 2000
Malepur	Basal tissue of clove	White's	Direct emb.: 2,4-D/Kin: 1/0.5 mg/l	2%	Direct somatic emb.	60% explt. gave 20-25 embryos/explt.	Sata *et al.* 2002
Rouge Reunion	Young leaves Root sections	B5	C: 2,4-D/IAA/NAA/Kin: 0.5/0.2/ 0.2/0.1 mg/l R: 2,4-D/Kin: 0.1/0.5 mg/l	6%	Somatic emb.	37 embryos per 150 mg of C	Fereol *et al*. 2002
	Leaf bases 2-3 mm	MS	C: 2,4-D/IAA: 1.5/1 mg/l R: Kin: 6 mg/l		Somatic emb.	70% embryogenic C, 6.8 P/C	Chowdhury *et al.* 2003
Danyang	shoot of micropropagated plants	MS	S prolif: 2-iP: 0.5 mg/l B: NAA: 0.1 mg/l	2%	Shoot proliferation in liquid medium	15 shoots/explt. in 3 weeks	Kim *et al.* 2003
Messidrome, Morado, Morasol, Printanor	Root segments 1cm	MS	C: Picloram/2-iP: 20.7/0.5 µM R: Kin: 1 mg/l	3%	Organogenic callus	C: 34% explt. R: 47% C	Zheng *et al.* 2003
Danyang	shoot	MS	S prolif: hormone free B: NAA: 0.1 mg/l	11%	Shoot and bulblet proliferation in bioreactor	27 S/explt. in 3 weeks	Kim *et al.* 2004
Rouge Reunion, Messidrome, Morasol, Printanor	Young leaves	N6	Liq. Med.: 2,4-D/BAP: 0.3/01 mg/l R: 2,4-D/Kin: 0.1/0.5 mg/l	4.5%	Cell suspension culture	10^{11} embryos annually from 1 clove	Fereol *et al.* 2005b

Usually, for all these techniques the other *in vitro* culture conditions are as follows: temperature 25-28°C, photoperiod 16 h of light, light intensity 50 µmol m^{-2} s^{-1}; bulbs were used 3-4 months after harvest and maintained 3 weeks at 5°C in order to break the dormancy before the actual experiment took place.

Ad: adenine; B: bulbing; C: callus; Conc: concentration, Emb: embryogenesis; Explt: explant; Ind.: induction; Liq Med: liquid medium P: plant; PGR: plant growth regulator; R: regeneration; S: shoot

BA: 6-benzyladenine; BAP: 6-benzylaminopurine; IAA: 3-indole acetic acid; Kin: Kinetin; NAA: α-naphtalene acetic acid; Picloram: 4-amino-3,5,6-trichloropicolinic acid; p-CPA: p-chloro-phenoxy acetic acid; 2,4D: 2.4-dichlorophenoxy acetic acid; 2iP: 6-(γ-γ-dimethylallylamino)-purine

AZ: Abo El Nil and Zettler (1976); LS: Linsmaier and Skoog (1965); MS: Murashige and Skoog (1962), B5: Gamborg *et al*. (1968); N6: Chu *et a*l. (1975); White's: White (1963)

embryogenic calli which are less than 18 months old. When the fresh calli were inoculated in liquid medium they released numerous individual cells and small cell aggregates. The cell density of the suspension continuously increased due to the cell divisions, and consequently a rapid cell suspension growth was observed with a 5-7 fold monthly increase (Fereol *et al*. 2005a). Histological examination of the suspension thus initiated revealed many cells with embryogenic characteristics: dense cytoplasm, large central nucleus with large nucleolus, large amount of storage proteins and some starch grains. The initial cell density in the suspension culture significantly affected the packed cell volume (PCV) rate and the potential of embryo production. Low cell densities proved to be best for mass cell production while high densities are best for embryo differentiation, probably due to the fact that medium composition is being altered by the cultured cells in a way that allows cell differentiation. Below a certain minimum density there are too few cells to affect this response (Ammirato 1983; Hari 1980) thus cells propagation continues. An initial cell density of 3-4% was particularly preferable both, for long term suspension culture and for maintaining a better potential for later regeneration of embryos. The periodicity of medium renewal significantly affected the PCV rate and the potential of production of embryos. Media refreshing every 14 days and subculturing the cell suspension every 28 days

were optimal for cell propagation and also for the further regeneration of embryos. 2,4-D concentrations in the suspension medium affected significantly the PCV rate and the potential for production of embryos. Similar to the results of Barandiaran *et al*. (1999a), Fereol *et al*. (2005a) found that at low concentration (0.3 mg/l) cell propagation was fastest and a high potential for further cell differentiation and regeneration capacity was maintained. Throughout culture development, and up to 14 months, growth rate of the cell suspension was on average rather similar, increasing at about 4.5 to 6 fold every 28 days period. However, in the first seven months the growth rate of the culture fluctuated widely (4.2-6.2), indicating some instability. Thereafter, growth rate fluctuation was much smaller, pointing at a more stable culture.

Plant regeneration

Aliquots of cell suspension, plated on semi-solid embryo production medium, produce numerous somatic embryos in eight weeks. The first pro-embryos differentiated 3 weeks after plating, being large globular structures (1-2 mm) covered with a smooth epidermis surface. They further developed into mature epidermis coated embryos expressing a bipolar structure with shoot and root apices connected to a haustorium-like structure by vascular bundles. These embryos were generated from the cell suspension cultures for longer than 16 months, thus 1 ml PCV, has the potential of producing 3,000 to 7,800 mature embryos.

Histological studies showed that individual cells underwent numerous internal cell divisions thus resulting in formation of pro-embryos, which differ of each other, in single aggregates. This compartmentalization indicates the unicellular origin of these pro-embryos. They further developed into mature embryos with two poles, a root and a shoot apex (**Fig. 4**). After culture on germination medium, they form a complete plantlet within 4 weeks. Those that failed to germinate within this period of time turned brown and died. The percentage of conversion into plantlets ranged between 36 and 51%. Germinated plantlets cultivated *in vitro* can produce bulblets within eight weeks (Kahane *et al*. 1992). Analyses of plants after acclimatisation and transfer to the field under insect-proof nets revealed some genotypes with an abnormal ploidy level. About 4% tetraploids were detected by flow cytometry. Such polyploidyzations might be a serious drawback for propagating plantlets by somatic embryogenesis, and has yet to be evaluated with regard to a mass propagation system.

Organo-sulphur analyses of bulblets from embryogenesis derived garlic plants (EP) and from standard vegetatively multiplied garlic (SP) showed that alliin plus γ glutamyl allyl cysteine (GLUAlCS) represented 70% of the organo-sulfur compounds in both types, but their distribution differed. In EP micro bulbs alliin was low while GLUAlCS was high as compared with SP micro bulbs (Fereol *et al*. 2005a). However, after two cycles of field culture, the differences disappeared.

Fig. 4 Embryogenesis in cell suspension cultures of garlic (*Allium sativum* L.). Mass production of embryos, Bar = 3 mm: (Photo: L. Fereol).

The annual multiplication rates, from one clove, ranged from 8 x 10^9 to 10^{11} for different cultivars belonging to different physiological garlic groups (Messiaen *et al*. 1993; Fereol *et al*. 2005b). Within such a range, the difference between cultivars was not a concern, and further efforts to gain productivity in "less productive cultivars" compared to "more productive ones" was useless. The aforementioned coefficients of mass propagation are up to 10^3-fold higher compared to the other garlic multiplication methods published (Nagakubo *et al*. 1993; Haque *et al*. 1997). Therefore the technique described by Fereol *et al*. (2005b) seems to be the preferred method for an efficient mass propagation of specific garlic genotypes. However, one or two cycles of traditional propagation may still be necessary to provide plants at a standard quality.

CONCLUDING REMARKS

Garlic improvement has been focussed until present on selection in existing landraces and yield has been a major selection criterium (**Table 2**). Garlic breeding has been hampered by the absence of adequate methods to generate variation in the existing germplasm. Futhermore the absence of efficient vegetative propagation methods added to the problem of efficient development of new garlic cultivars. In the current review the introduction of sexual hybridization into garlic was described and the ways it can be successfully implemented both via genetic and environmental methods. Another route to improve garlic is via genetic transformation. Both *Agrobacterium* and biolistic gene transfer systems were developed in the last years and the first transgenics have already been produced. Because of this the next step in the introduction of garlic GMOs into agriculture will be comprehensive safety assessments (Dale and Kinderlerer 1995) and providing answers to questions like: a) does the introduced gene changes the modified crop, b) is there evidence of an increased risk of toxicity and/or allergenicity, c) are effects visible on organisms in the environment, if transgenic garlic induces weediness or invasiveness in natural habitats or if gene flow between the transgenic and wild relatives is to be expected. When these questions have been answered satisfactorily then there is a possibility to introduce the novel transgenic garlic. However providing answers to these questions is costly for companies wanting to introduce transgenics therefore it remains to be seen if transgenic garlic will become a reality in the near future. Last but not least the recent successful developments in garlic embryogenic cell suspensions have been described. All in all, these developments in garlic breeding system innovation show that there are good opportunities for the production of improved garlic cultivars which are better suited for the market. Furthermore this review indicates that also on the fundamental level (e.g. garlic florogenesis, genome organization, genetic transformation and embryogenic cell suspension development) large steps forward have been made, but it is also clear that there are still large gaps present in our knowledge.

Table 2 Yield (tons/ha) of commercially grown garlic cultivars in France. Garlic cultivars California Late and California Early have the same yield as Printanor and Thermidrome, respectively (V. Chovelon, pers. comm., 2007).

Cultivar	Yield
Germidour	7.6
Messidrome	7.7
Printanor	9.1
Thermidrome	7.6
Morado	6.5

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