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Biotechnological Tools for Garlic Propagation and Improvement

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1. Introduction

Garlic (*Allium sativum* L.) is a monocotyledonous herb belonging to the genus *Allium* and the family Alliaceae and it is the second most widely distributed species of this genus throughout the world, (Kamenetsky, 2007). Garlic is used as food flavoring or as a medicinal plant. It can be preserved in oil or vinegar or processed into products such as garlic salt, garlic juice, concentrated garlic or most commonly, dehydrated garlic (Brewster, 1994). Although there have been different hypothesis as to the origin of garlic and it was even thought that it was a Mediterranean plant, Vavilov (1926) and Kazakova (1971) suggested Central Asia as its primary center. Years later, this hypothesis was confirmed by the discovery of fertile clones of a primitive garlic type in the Tien-Shan mountains in Kyrgyzstan (Etoh, 1986; Kotlinska et al., 1991) and by studies using biochemical and molecular markers (Pooler & Simon, 1993).

Fritsch & Friesen (2002) put forward the idea that *Allium sativum* is a complex made up by three main groups: (a) Sativum, (b) Longicuspis and (c) Ophioscorodon, and two subgroups: Subtropical and Pekinense. The commercial types of garlic can be divided into: (1) violet or Asian, which is cultivated in subtropical regions, (2) pink, which needs long photoperiods and has low requirements for cold, (3) white, which needs long photoperiods, has medium to high requirements for cold, and (4) purple, which needs long photoperiods and periods of cold (Heredia-García, 2000). They can also be classified into hard-neck and soft-neck garlic. Hard-neck garlic forms a floral scape whose flowers normally abort and whose end produces topsets, while soft-necked garlic does not form a scape. The majority of garlic cultivated for commercial purposes is soft-neck type because it is easier to cultivate and it has a longer shelf life (Kamenestsky, 2007).

Garlic is grown all over the world from temperate to subtropical climates (Fritsch & Friesen, 2002). Production and world cultivated area have increased over years. In 2004 production was of 14'071,335 t obtained from an area of 1'129,714 ha; while in 2007 15'799,909 t were produced on 1'220,314 ha. The main producer of garlic is China, with 17'967,857 t, accounting for 80.6% of the world production, followed by India (1'070,000 t) and the Republic of Korea (380,000 t) (FAOSTAT, 2009) (Table 1). Garlic bulbs are composed of aggregate of cloves which

have their origin in the axillary buds. The cloves are made up of a protective sheath (which is dry and thin at maturity), a thickened storage sheath leaf (which represents the major part of the clove and it is also the usable part), and sprouting and foliage leaves which protect the apical meristem (Mann, 1952; Purseglove, 1988). The number of cloves per bulb varies with the cultivar, but bulbs with a maximum of 16 cloves are preferred.

Rank	Country	Production (t)	Production (%)
1	China	17'967,857	80.64
2	India	1'070,000	4.80
3	Republic of Korea	380,000	1.71
4	Russian Federation	227,270	1.02
5	Myanmar (before Burma)	200,000	0.90
6	United States of America	178,760	0.80
7	Egypt	174,659	0.78
8	Bangladesh	154,831	0.69
9	Spain	154,000	0.69
10	Ukraine	150,100	0.67
	World	22'282,060	

Table 1. Main garlic-producing countries in the world (FAOSTAT, 2009).

Currently, garlic propagates vegetatively through cloves or through topsets that develop in the plant´s inflorescences (which can prevent the plant from producing flowers and seeds). Kamenetsky & Rabinowitch (2001) explain that lack of fertility could be due to the fact that in past the floral scapes were removed and plants with low flowering ability were selected in order to obtain bigger bulbs (Kamenetsky & Rabinowitch, 2001; Etoh & Simon, 2002). Nowadays, in some places the bulbs are harvested before the flowering time to avoid their rotting or to use the scapes as vegetable (Etoh & Simon, 2002). In addition, the sterility of the garlic has been mainly attributed to chromosomal deletions, and also to differences in the length of homologous chromosome, to loss of genes involved in gametogenesis, to hypertrophy of the tapetal layer of the anthers at the post-meiotic stage, to microspore degeneration before or after the tetrade stage, to nutritional competition between the topsets and flowers, and to infestation with microorganisms (rickettsias) (Novak, 1972; Konvicka et al., 1978; Etoh, 1985; Pooler & Simon, 1994).

2. Chemical composition and medicinal traits

The main components of the garlic bulb are water (65%) and carbohydrates (26-30%), especially fructose polymers (Table 2). Other components are lipids, proteins, fiber, minerals and saponins (Lawson, 1996). Elements such as selenium (700µg per 100g of fresh weight), sulphur, zinc, magnesium, iron, sodium, calcium, as well as vitamins A, C, E and B-complex vitamins (thiamin, riboflavin, niacin) and phenols are also present in the garlic bulb (Koch & Lawson, 1996; Vinson et al., 1998). Garlic produces organosulphur compounds such as the γ-glutamylcysteines and alliin ((+)-S-(2-propenyl)-L-cysteine sulfoxide) which confers its flavor, odor and biological activity (Block, 1985). The alliin can account for 1.4% of the fresh weight of bulb (Keusgen, 2002). It has been found that the activity of alliinase, the enzyme

that hydrolyzes the sulphur compounds in garlic, is 10 times higher in bulbs than in leaves (Rabinkov et al., 1994) (Table 2).

Apart from its use for food flavoring, garlic also has medicinal uses for the relief of various ailments such as those caused by aging, arthritis, cancer, artheroesclerosis, immune deficiencies, blood glucose level, respiratory diseases, etc. (Keusgen, 2002; Raham, 2001). Likewise, it has been observed that garlic has antioxidant properties, it reduces blood cholesterol and triglycerides levels, lowers blood pressure and the possibility of blood clot formation and improves arterial oxygenation (Augusti, 1990; Abrams & Fallon, 1998; Bordia et al., 1998). Garlic's effect on reduction of lipids has been most extensively studied. The properties mentioned above are directly related to the sulphur compounds found in the garlic bulb. Alliin is also attributed the antibiotic effect on microorganisms such as *Helicobacter pylori* (bacterium which is associated with stomach cancer), *Salmonella typhi*, yeasts, *Trypanosoma* and *Staphylococcus epidermis.* Its inhibitory effect has also been observed on pathogenic fungi (*Aspergillus*, *Cryptoccocus neoformis*, dermatophytes) (Keusgen, 2002).

Component	Amount (fresh weight; %)
Water	62-68
Carbohydrates	26-30
Protein	1.5-2.1
Amino acids: common	1-1.5
Amino acids: cysteine sulphoxides	0.6-1.9
γ-Glutamylcysteines	0.5-1.6
Lipids	0.1-0.2
Fibre	1.5
Total sulphur compounds*	1.1-3.5
Sulphur	0.23-0.37
Nitrogen	0.6-1.3
Minerals	0.7
Vitamins	0.015
Saponins	0.04-0.11
Total oil-soluble compounds	0.15 (whole) – 0.7 (cut)
Total water-soluble compounds	97.00

*Excluding protein and inorganic sulphate (0.5%)

Table 2. Chemical composition of garlic bulb (Lawson, 1996).

3. Pests and diseases during garlic cultivation and storage

Garlic plant can be affected by various diseases caused by viruses, fungi and bacteria. The viruses that tend to cause it severe damages are potyviruses, such as Leek Yellow Stripe Virus (LYSV), Garlic Yellow Streak Virus (GYSV) and Onion Yellow Dwarf Virus (OYDV) (Bos, 1982; Walkey, 1987). Some carlaviruses, like Common Latent Virus (GCLV) and Shallot Latent Virus (SLV) can also infect the garlic plant (Messiaen et al., 1994). One of the most widely spread diseases in garlic producing countries is white rot, caused by the fungus *Sclerotium cepivorum*, which provokes wilting of the plant and rotting of the bulb.

Its sclerotia can survive in soil for up to 20 years, therefore limiting garlic production (Delgadillo-Sánchez, 2000). As far as the fungus *Penicillium corymbiferum* is concerned, this attacks plants weakened by other pathogens, and although infested plants survive the infection, bulbs present symptoms during the storage period. Various bacteria (*Bacillus* spp., *Erwinia* spp., *Pseudomonas* spp.) can also cause damages on bulbs upon storage. Garlic can also be affected by pests like thrips (*Thrips tabaci*), which are insects that infest plants from early developmental stages and cause severe foliage damages. For this reason, thrips are considered the most noxious pest affecting this crop plant. Mites (*Rhizoglyphus* spp.) are another garlic pest that invade the bulbs and limit their sprouting ability (Bujanos-Muñiz & Marín-Jarillo, 2000). On the other hand, bulb nematode (*Ditylenchus dipsaci*) causes the root knot disease, characterized by yellowing and rolling of leaves, as well as rotting of the bulb's base.

4. Breeding

Commercial garlic cultivars only propagate themselves vegetatively, the increase of genetic variation through conventional crossing is very low, or even absent. For this reason, clonal selection, induced mutations, somaclonal variation or genetic engineering are the only options for breeding improved cultivars (Robinson, 2007). Clonal selection has been the most widely used method for generating new garlic material. It is based on the variability existing in populations as a result of cross pollination between various garlic types and its ancestors when this plant still had the ability of sexual reproduction (Etoh & Simon, 2002; Koul et al., 1979). On the other hand, although mutations may be a source of variability, they are rather limited; therefore, breeding using this strategy has not resulted in significant progress (Etoh & Simon, 2002). The lack of sexuality in garlic limits the increase of variability that is useful for breeding for economically important traits, such as tolerance to biotic and abiotic stress, earliness, yield and quality (Kamenetsky, 2007). Moreover, vegetative propagation has various disadvantages for the crop: (a) a low multiplication rate (5 to 10 per year), (b) expensive and short-term storage that requires wide spaces, (c) transmission of phytopathogens (fungi, viruses, nematodes) through generations and from one production area to another, which can cause a yield decrease of up to 70%, and (d) loss of product quality (Kamenetsky, 2007; Walkey, 1990; Nagakubo et al., 1993).

5. Biotechnology for garlic propagation, preservation and breeding

Biotechnological tools such as micropropagation, meristems culture (in order to obtain virus-free plants), somaclonal variation, and genetic transformation, have contributed to propagation, preservation and breeding of garlic.

5.1 Micropropagation

Studies related to the application of tissue culture techniques such as micropropagation for garlic production started in 1970. This technique proved to be advantageous over clove reproduction, as it only requires cells or small tissue fragments to generate high number of plants. Micropropagation can be carried out via two morphogenetic ways: (1) organogenesis, which results in the formation of organs (shoots or roots), and (2) somatic embryogenesis, which leads to the formation of structures having a similar or equal

morphology to that of a zygotic embryo. Both processes can involve (indirect) or not (direct) a previous callus phase. Morphogenetic ability in garlic decreases as the callus grows older and the emergence of abnormal plants increases (Novak, 1990). For this reason, regeneration that does not involve a previous callus phase is preferred. Embryogenesis possesses a series of advantages over organogenesis, such as higher potential for high plant output, lower labour requirement and lower cost (Sata et al., 2001). Several micropropagation protocols have been established using both ways of morphogenesis and different explant types; however, most protocols have been developed following the organogenetic way.

5.1.1 Organogenesis

Meristem culture is a technique used for obtaining virus-free plants, and also for micropropagation. Messiaen et al. (1970) were the first in regenerating garlic plants from meristems. Shoot or bud formation from callus was achieved using a combination of 6-furfurylaminopurine (kinetin), indol-3-acetic acid (IAA) and 2,4-diclorophenoxiacetic acid (2,4-D) (9.28µM, 11.4µM and 4.5µM, respectively). Likewise, Havránek & Novak (1973) obtained numerous growth areas on calli produced from meristems on a culture medium with 2,4-D. The subculture of calli to a medium containing IAA (11.4µM) and kinetin (46.5µM) induced formation of adventitious shoots.

In a different work, calli obtained from meristems of three garlic varieties (*Rose de Lautrec*, *California Early* and *California Late*) cultivated in a medium with 2,4-D (4.5µM) and IAA (5.7 µM) produced adventitious shoots when transferred to a medium with IAA (5.7µM) and kinetin (4.6µM) (Kehr & Shaeffer, 1976). For his part, Abo-El-Nil (1977) started cultures from meristems, stems and leaf discs of the variety *Extra Early White*, on a medium with p-chlorophenoxyacetic acid (p-CPA) (10µM), 2,4-D (2µM) and kinetin (0.5µM), from which callus formation was achieved, which in turn resulted in the formation of adventitious shoots in the presence of kinetin (10µM) and IAA (10µM). In other works, meristems were cultivated on B5 medium (Gamborg et al., 1968) with 2.5µM 2-isopentenyladenine (2ip) and 0.55µM α-naphtalenacetic acid (NAA) (Bhojwani, 1980), or in Linsmaier and Skoog (LS) (Linsmaier & Skoog, 1965) medium with 9µM N^6-benzyladenine (BA) alone or with 11.1µM NAA, and multiple shoots were obtained (Osawa et al., 1981).

There are only a few reports on suspension cell culture in garlic. For instance, Nagasawa & Finer (1988) were the first to establish suspension cell cultures obtained from calli derived from meristems of the cultivar *Howaito-Roppen* grown in presence of NAA (5.5µM) and BA (9µM). Adventitious shoots with leaf primordia started differentiating only after transferring the calli to agar-solidified medium. Likewise, Kim et al. (2003) obtained cells in suspension after cultivating shoots of the cultivar *Danyang* differentiated *in vitro* in Murashige and Skoog (MS) (1962) medium with 2.5µM 2iP. These cultures regenerated an average of 21.5 shoots per explant when they were exposed to a light intensity of 50µmol m^{-2} s^{-1}. Thirty bulbs developed per explant in a medium containing 11% sucrose and 135 bulbs in the presence of 10µM jasmonic acid.

Subsequently, Nagakubo et al. (1993) developed a micropropagation protocol for six varieties (*Isshuwase*, *Isshu-gokuwase*, *Shanhai*, *Santo*, *Furano* and *Howaito-roppen*) starting from shoot-tips which were cultivated in a medium supplemented with NAA (1µM) and BA (1µM). Regenerated adventitious shoots were subcultured for four generations in presence

of NAA (5μM) and BA (10μM) for their multiplication. The application of this protocol enables the production of 256 plants from one shoot-tip in 10 months. A novel regeneration protocol was developed by dissecting and sectioning longitudinally the shoots developed from cloves of the cultivar *Extra Select Sets*. These shoots were cultivated on a medium with BA (8μM) and NAA (0.1μM), and after five weeks they produced eight more shoots compared to the ones that had been kept intact (Mohamed-Yassen et al., 1994).

The roots produced by cloves have proved to be a good explant for plant regeneration. When the root tips are cultivated in a medium with NAA (1μM) and BA (10μM) the shoot formation is achieved for 75% of the explants, without an intermediate callus phase. It is estimated that by using this method up to 380 shoots could be produced starting from a single clove (Haque et al., 1997). Other protocols have been developed, which involve callus formation from this type of explants upon their cultivation in MS or N6 culture media (Chu et al., 1975) supplemented with 2,4-D (4.5μM) alone or combined with kinetin (4.7μM). Transferring the calli to a medium with 4.4μM BA allows the regeneration of 169 plants per gram of callus, which have the ability of forming microbulbs (Robledo-Paz et al., 2000). Khan et al. (2004) also regenerated adventitious shoots from calli developed from root tips of two garlic varieties. The highest callus formation frequency was observed when a combination of 2,4-D (6.8μM) and kinetin (23.8μM) was used. Shoot differentiation rose exponentially with increasing BA concentration, reaching the highest value at 45μM BA, while shoot rooting occurred in the absence of growth regulators. A variation in the number of shoots and their regeneration time was observed depending on the genotype used. Approximately 75% of the regenerated plants established successfully when transferred to greenhouse.

The roots developed from adventitious shoots obtained *in vitro* also allowed garlic micropropagation when cultivated on a medium that induced callus formation and then transferred to a medium with BA (13.3μM) and 4-amino-3,5,6-trichloro-picolinic acid (picloram) (1.4μM). This method enables the regeneration of 5.4 shoots per explant (Myers & Simon, 1998a). A protocol named one-step was developed when the same type of explants was cultivated on a modified B5 medium supplemented with 0.1μM 2,4-D, 11.1μM NAA and 13.6μM BA, under two light conditions (16 hours photoperiod and complete darkness). In general, the root tips cultivated under low light conditions displayed the highest percentage of organogenic calli. The application of this protocol allowed the formation of callus and the regeneration of 250 shoots per gram of callus in the same culture medium and under the same light conditions (Martín-Urdíroz et al., 2004). Zheng et al. (2003) also obtained adventitious shoots by using apical and non-apical root fragments, originating from plants generated *in vitro* of four cultivars grown in Europe (*Messidrome*, *Morado de Cuenca*, *Morasol* and *Printanor*). The explants were cultivated on MS medium with 4.5μM 2,4-D and 0.5μM 2iP in order to induce calli formation, which were then transferred to a medium containing 4.7μM kinetin to promote shoot differentiation. The highest regeneration rate was obtained when non-apical fragments were used, although the difference was not significant.

In a different work, Ayabe & Sumi (1998) used the stem disc (consisting in the apical meristem and the lateral buds of the clove) to regenerate plants of the cultivar *Fukuchi-howaito*. When this was cut into various fragments and then cultivated on a medium with BA (0.4μM), 20-25 adventitious shoots were obtained. The same result was observed when

protoplasts isolated from shoot primordia were cultured in the presence of NAA (0.5µM) and 2iP (2.4µM), adenine and coconut milk (Ayabe et al., 1995). Barandiaran et al. (1999) used immature bulbs of 23 accessions as a source of axillary buds, which were cultivated during six weeks on B5 medium with 2.5µM 2iP and 0.55µM NAA (establishment phase). Multiplication of regenerated shoots was done on the same culture medium and 20 weeks later shoot clusters were separated in order to cultivate them individually and to induce bulb formation at a low temperature (4°C). Although plants and bulbs were obtained for all accessions under tested conditions, response depended on genotype (accession). Three months later, 60% of bulbs that were transferred to soil survived and produced shoots. This protocol allowed the use of the same culture medium for all phases of micropropagation (establishment, multiplication and bulb formation) and for all accessions, which enabled the handling of all materials tested at the same time, as only three subcultures were required over a period of seven months. Primordial leaf obtained from cloves are also able to produce adventitious shoots when cultivated on a medium with 2,4-D (4.5µM), and develop into plants when transferred onto a medium containing picloram (1.4µM) and BA (13.3µM) (Myers & Simon, 1999). Haque et al. (2003) developed a protocol for plant regeneration and bulb formation from shoot and root meristems of the cultivar *Bangladesh Local*. Meristems were cultivated on MS medium without growth regulators or containing various concentrations of BA (1-10µM) and NAA (1-5µM). None of the combinations of regulators tested produced a higher response than the one observed in their absence (95.5%). In fact, the presence of these compounds suppressed shoot formation in a directly proportional manner to concentration; 45% of root explants formed adventitious shoots, 60% of which produced bulbs. Although a higher number of buds resulted in shoot formation, the root meristems produced more shoots per explant (20). Bulbs derived from root meristems were smaller than the ones derived from bud meristems.

On the other hand, Luciani et al. (2006) tested different explants for micropropagation of variety *069*, which were cultivated on BDS medium (Dustan & Short, 1977), supplemented with picloram, 2,4-D and BA. The basal plates and meristems resulted in the highest values of shoot regeneration, and 2,4-D proved to be better than picloram for inducing callus and shoot formation. By using a combination of 0.25µM 2,4-D and 4.43µM BA, 100% of explants were able to produce calli, which differentiated into both embryos and shoots. It is worth mentioning that *in vitro* propagation is frequently associated with a process known as hyperhydricity or vitrification, which is a physiological disorder caused by the *in vitro* culture conditions that affects the behavior of regenerated plants. This disorder promotes abnormalities at physiological, anatomical and morphological level, which limit the successful establishment of differentiated plants upon their transfer to greenhouse. Hyperhydric plants have a slow growth rate, thick and deformed stems. Their leaves are translucent, thick and wet (Olmos & Hellin, 1998; Kevers et al., 2004).

A study of biochemical and ultrastructural traits of hyperhydric garlic shoots regenerated *in vitro* was carried out by Wu et al. (2009), who observed that organelles such as mitochondria and chloroplasts were compressed against cell wall, in these shoots. In addition, protein content decreased significantly and O_2 and H_2O_2 generation rate increased 45.3% and 63.9%, respectively. Activity of oxidative stress-related enzymes (lipoxygenase, superoxid dismutase, peroxidase, catalase, ascorbate peroxidase) also increased. Moreover, a rise in the level of electrolytes lixiviation was observed, indicating a damage of membrane lipids. Authors concluded that hyperhydric condition of tissues is closely linked to oxidative stress.

5.1.2 Somatic embryogenesis

Formation of structures called embryoids was reported for the first time in 1977. They differentiated from calli obtained from stem tips, bulb leaf discs cultivated in the presence of kinetin (20µM) and IAA (10µM) (Abo-El-Nil, 1977). This response was observed again after a long time when basal plates and floral receptacles were cultivated on a medium containing NAA (1µM) and BA (10µM) (Xue et al., 1991; Al-Zahim et al., 1999). Likewise, Ali & Metwally (1992) induced embryo formation from calli generated from root segments; however, regeneration rate was low. In a different work, Barrueto-Cid et al. (1994) established cultures in suspension of the variety *Chonan* starting from calli initiated on MS medium with 5µM 2,4-D, 5µM picloram and 10µM kinetin. Calli were subcultured onto a medium with 4.5µM 2,4-D and hydrolyzed casein before using them for cell suspension cultures. Plant regeneration occurred after transferring cells to a medium containing 77-153µM adenine.

Later, Sata et al. (2001) obtained somatic embryos directly from basal sections of cloves of the cultivar *Malepur* grown on White medium (White, 1963) supplemented with 4.5µM 2,4-D and 2.3µM kinetin. Under these conditions, each explant formed 20 to 25 embryos, which in the presence of higher concentrations of 2,4-D and kinetin turned into masses of hyperhydric tissue. In the same way, Fereol et al. (2002) produced somatic embryos and plants of the variety *Rouge de la Réunion* after cultivating calli obtained from root tips on a modified B5 medium supplemented with 2,4-D (0.4µM) and kinetin (2.3µM). Thirty percent of the somatic embryos developed into plants which acclimated successfully to greenhouse conditions. Later, Fereol et al. (2005b) established a protocol for embryo regeneration through suspension cultures by using young leaf sections from cloves of the variety *Morasol*. Embryogenic calli were obtained when explants were grown on B5 medium with 4.5µM 2,4-D and 0.47µM kinetin, then transferred to a modified B5 medium with 2.2, 1.1, 1.1, 0.4µM 2,4-D, IAA, NAA and kinetin, respectively, plus 175mM sucrose and 2mM proline. Calli were maintained on this medium for five months and were later used to initiate suspension cultures in a modified N6 medium supplemented with 1.3µM 2,4-D, 0.4µM BA and 131mM sucrose. Embryo production was induced on a medium with 2.3µM kinetin and 0.4µM 2,4-D. Embryos developed into plants, which could produce microbulbs *in vitro*. By using the same explants type and the same culture conditions described above, induction of embryogenic suspension cultures of four garlic cultivars (*Rouge de la Réunion*, *Morasol*, *Messidrome* and *Printanor*) was achieved. Ninety percent of calli differentiated into embryos at globular stage after two months of culture. Out of the regenerated embryos, 50% developed into plants that were successfully established in greenhouse. The histological analysis of the culture revealed that regenerated somatic embryos had a unicellular origin (Fereol et al., 2005a).

5.2 Meristem culture

Meristem culture technique has been widely used for the production of virus-free clones. Virus elimination through meristem culture is based on the fact that these meristematic cells are free or almost free of virus and therefore plants regenerated from them will also be virus-free (Salomon, 2002). For this purpose, it is recommended to isolate explants of maximum 5mm, although sometimes their size may limit their establishment *in vitro*. Meristem culture enabled virus-free plants to be produced in various regions in the world

(Walkey, 1987; Bhojwani et al., 1982; Peña-Iglesias & Ayuso, 1982; Bertacinni et al., 1986; Conci & Nome, 1991). In Slovenia, Eastern Europe, a trial was conducted to eliminate the OYDV in plants of the cultivar *Ptujksi-spomladanski* through thermotherapy and meristem culture. Meristems of 0.3-0.6mm were first cultivated on B5 medium with 1μM IAA and 1μM BA, then transferred to a multiplication medium containing 5μM jasmonic acid and 5μM 2iP. Meristems obtained from plants that had undergone thermotherapy regenerated a lower number of shoots (1.0-2.2) than the non-treated plants (9.3); 90 to 100% plants were found to be free of the OYDV (Ucman et al., 1998).

Sidaros et al. (2004) attempted to produce plants of three garlic cultivars (*Chinese*, *Italian* and *Balady*) through meristem culture and chemotherapy. Chemotherapy was carried out by using virazole [or ribavirin (1-β-D-ribofuranosil-1,2,4-triazole-3-carboxamide)] in culture medium. The highest percentage (100%) of virus-free plants was obtained when meristems of 3mm were cultivated on MS medium containing 50mg L^{-1} virazole. In a different study, thermotherapy, chemotherapy and meristem culture were combined in order to obtain plants of the varieties *Taiwan* and *Chileno* free of potyvirus. Thermotherapy consisted in maintaining regenerated plants from embryos dissected from cloves that showed negative results on an ELISA (Enzyme Linked Immuno Absorbent Assay) for potyvirus during one week at 32°C, followed by two weeks at 36°C, and three weeks at 38°C. Embryos were removed from cloves of these plants and cultivated in presence of 205μM ribavirin. Meristems (0.1-0.5mm) of regenerated plants that showed negative results by ELISA were used to generate new plants. Thermotherapy had a more negative effect on plant survival than meristem culture and chemotherapy. However, thermotherapy proved to be more efficient for virus elimination (60.0 to 70.9%) than meristem culture (64.0%), while chemotherapy was not efficient for potyvirus elimination. On the other hand, 10.7% of plants of the cultivar Taiwan grown in field became reinfected, while the Chileno cultivar showed an 8.9% of reinfection after three consecutive cycles of the crop (Ramírez-Malagón et al., 2006).

The use of stems and scape tips of the variety *Red Six Cloves* allows formation of adventitious shoots when cultivated on a medium with NAA (2.6μM) and kinetin (2.3μM). These shoots developed into plants free of the garlic mosaic virus (GMV) 65 days after starting the culture (Ma et al., 1994). Alternative protocols have been developed for generating virus-free plants starting from inflorescence meristems, bulbils or roots, as apart from being virus free they are available in higher numbers than the apical meristems (Appiano & D'Agostino, 1983). In this way, Verbeek et al. (1995) cultivated meristems obtained from cloves and bulbils (0.15-1.00mm), 71-71% of which regenerated plants; 38% of explants obtained from cloves and 25% of the explants obtained from bulbils were found to be virus-free. In addition, it was observed that meristems smaller than 0.4mm failed to produce shoots. Similarly, Ebi et al. (2000) established a system for elimination of mite-borne mosaic virus using meristems (0.2-0.4mm) obtained from bulbils. These meristems produced plants after being cultivated on MS medium with 5.4μM NAA. The immunoblot assay indicated that several of regenerated plants were virus-free. Senula et al. (2000) obtained plants of 87 accessions free of the viruses OYDV, LYSV, GCLV, SLV and MbFV by cultivating meristems of 0.3-0.8mm originated from bulbils. OYDV and LYSV were eliminated in 85-95% of the regenerated plants. Addition of ribavirin to culture medium reduced regeneration potential, but increased virus elimination. Later, Xu et al. (2001) regenerated virus-free plants from meristems obtained from inflorescences of nine lines. Explants were cultivated on B5 medium containing 0.22μM BA and 0.3mM adenine. By

using this protocol it was possible to obtain 50-90% plants free of the OYDV, 70-100% plants free of the LYSV and 60-80% free of the SLV. Production of bulbs from virus-free plants was higher than from infected plants. Four to five years would be necessary to obtain virus-free elite seeds that can be established in the field. The economical analysis indicated a net profit of 50.3 to 244.5% (depending on the genotype) for garlic seed producers.

5.3 Somaclonal variants

Tissue culture tools such as *in vitro* selection, embryo rescue, somatic hybridization, genetic transformation and somaclonal variation can be used to generate crop variation. Larkin and Scrowcroft (1981) defined somaclonal variation as the phenotypic variation seen in plants regenerated *in vitro* with respect to the original plant. At genetic level, somaclonal variation can be brought about by various DNA changes that include: (a) chromosomal rearrangements, (b) aneuploidy, (c) poliploidy, (d) modification of gene expression by methylation, amplification, inactivation or reactivation, (e) genetic conversion, (f) somatic recombination, (g) transposons movement, (h) genes mutations, etc. (Scowcroft, 1984; Peschke & Phillips, 1991).

Various experiments have been undertaken in garlic in order to generate somaclonal variants that could be used in its improvement. For instance, Novak (1983) treated meristems 0.5-0.7mm with a solution of colchicine (3g L^{-1}) to induce polyploidy. Meristems were treated in two different ways: (1) cultured for 7 days on a solid medium with colchicine, and (2) cultured for 2 days in a liquid medium with colchicine. The latter treatment proved to be more effective. By using this experimental strategy, 35% of regenerated plants were found to be tetraploid, and 14% chimaeras with diploid and tetraploid cells. Dolezel et al. (1986) pointed out that in garlic probability of generating somaclonal variation is higher when disorganized growth occurs, specially for longer periods of time. Similarly, plants generated from old explants or with a high level of differentiation have an increased possibility of suffering this type of variation. The cultivar *Frankon* is resistant to populations of the nematode *Ditylenchus dispsaci* found in Israel, but produces bulbs and cloves of small size and has low yield. A protocol for *in vitro* regeneration was developed to generate somaclonal variants that can produce bulbs with commercial traits. This protocol consisted in cultivating basal plates on BDS medium supplemented with 2.2µM 2,4-D and 2.3µM kinetin to induce callus formation, which were then transferred to a medium containing 9.5µM kinetin and 11.7µM IAA to develop adventitious shoots. Once shoots produced roots, they were transplanted to soil in greenhouse. Assessment of plant characteristics and bulb development revealed that there was variation in bulb size and color, and also in number and size of cloves per bulb compared to the original plant type, which indicates that this could represent a promising material for generation of improved somaclonal variants (Koch & Salomon, 1994).

In a different study, Madhavi et al. (1991) compared ability of calli (organized and disorganized) and bulbs to produce sulphur compounds like alliin. Apart from finding differences in sulphur level compounds, they also observed changes related to proteins, aminoacids, carbohydrates and enzymes. For instance, specific activity of the enzyme alliin lyase in callus was 50% lower than in bulb, while enzymes amino transferase, malate dehydrogenase, polyphenol oxidase, peroxidase and alkaline phosphatase displayed a higher activity than in bulb. Incorporation of precursors to volatile fraction was also higher

in organized calli. The somaclone *118.15*, derived from variety *Rosado*, which is grown commercially in Argentina, possesses agronomically desirable traits. A cytological and phenotypical study revealed that both somaclone and original type had the same chromosome number (2n = 16). In addition, some individuals of this clone contained polyploid, aneuploid and haploid cells, probably derived from processes such as endomitosis, nuclear fusion or homologue chromosome pairing in somatic cells. Binucleate cells and differences in length of chromosome pairs were also observed. Plants of clone *118.15* were taller, had a higher diameter of pseudostem and produced bulbs with less, but bigger cloves than the original type (Ordoñez et al., 2002).

El-Aref (2002) regenerated plants of cultivar *Balady* from leaves and roots cultivated first on BDS medium with 2,4-D (4.5µM) and kinetin (9.5µM) for callus initiation, then in presence of 9µM BA and 5.5µM NAA for plant regeneration. These plants formed bulbs which were planted in soil to generate new plants. Isoenzyme analysis showed that 9 out of 29 regenerated plants were different from original plant with regard to some of the enzymes analyzed (phosphatase acid, alcohol dehydrogenase, malate dehydrogenase, esterase). Parental bands were found in all plants, and 5 new bands were observed in 31% of them. Esterase and acid phosphatase displayed a higher polymorphism than alcohol dehydrogenase and malate dehydrogenase. Later, Mukhopadhyay et al. (2005) studied chromosome stability in plants of cultivar *Rossete* generated from callus. It was observed that plants generated from calli iniciated on solid MS medium with 2,4-D (9µM) and kinetin (0.93µM) and sub-cultured in liquid medium with NAA and kinetin exhibited chromosome stability, while the ones grown solely on the initiation solid medium contained hypo- or hyperdiploid cells along with the diploid ones. Frequency of aneuploid cells (2.2-48.9%) increased with callus age.

Recently, Badria & Ali (1999) identified somaclonal variants regenerated from calli obtained from root meristems cultivated on MS medium with kinetin, 2,4-D and IAA. The somaclonal variants formed bulbs without division in the first generation, and displayed normal phenotype in the following generation. After four cycles in field, somaclonal variants that exhibited significant differences of bulb characteristics were found. Cytogenetical analysis revealed that these somaclonal variants had the same chromosome number as original plants. Quantification of alliicin production showed that some somaclones contained three times more of this compound (14.5mg g^{-1}) than control plants (3.8mg g^{-1}). Authors suggest that this technique could be useful for improving alliicin content in garlic.

5.4 Germplasm conservation

Conservation of valuable garlic accessions involves their yearly cultivation, as bulbs cannot be stored for long periods of time (6 months at -3°C). This practice is expensive as it requires land use and manpower. Moreover, germplasm grown in field is exposed to environmental changes, pests and diseases that reduces its quality (Panis & Lambardi, 2006). The majority of plant germplasm is stored in seed repositories at temperatures between -15 and -20°C. However, for species whose seeds are recalcitrant (they cannot be dried to humidity levels low enough for storage) or for species that do not produce seeds, like garlic, slow growth storage and cryopreservation are the only tools for conserving them. Slow growth storage involves a condition that maintains tissue growth at a minimum and it allows the medium term storage of material (Botau et al., 2005). It is

based on using organs cultured *in vitro* at 4°C and 10 to 15°C for plants growing in temperate and tropical areas, respectively (Keller et al., 2006).

Cryopreservation is one of the most commonly used tools for germplasm conservation because it requires minimum amount of space and maintenance. In addition, it reduces loss of accessions by contamination, human errors and somaclonal variation which may occur during slow growth storage (Panis & Lambardi, 2006; Sakai & Engelman, 2007). This technique involves the use of liquid nitrogen (which has a freezing temperature of -196°C) for long-term storage of plant material. At this temperature, the majority of biochemical and physical processes are effectively stopped. Cryopreservation is only useful if formation of intracellular ice crystals does not take place, as they may cause irreversible damage to the cell membrane (Panis & Lambardi, 2006). Ice formation without an extreme reduction of the cell water content can only be avoided by a process known as vitrification, in which an aqueous solution turns into an amorphus and glassy state (Sakai, 2000). This procedure substitutes cellular dehydration that occurs during freezing by a reduction of cell water content that is achieved by treating tissues prior to the cooling process with highly concentrated solutions (PVS2, PVS3) containing glycerol, ethylen glycol, dimethylsulfoxid and sucrose, or by air drying (Sakai & Engelman, 2007).

Some countries are already making use of the previously mentioned techniques to conserve their valuable germplasm. For example, China possesses *in vitro* virus-free germplasm banks and their respective databases. These banks have been established taking into account factors such as genotype, culture medium components, light conditions, temperature of incubation rooms, etc. Moreover, studies have been carried out in order to optimize conservation conditions. In this respect, Xu et al. (2005) studied the behavior of six genotypes during their *in vitro* storage. They found out differences in conservation period, depending on genotype. Two genotypes, namely *Cangshan Zaotai* and *Tianjin Baodi* could be stored for 25 months and had a survival percentage of 100%. They also observed that shoots grown at low temperatures on B5 medium with 1.3-2.2µM BA, 0.5-1.6µM NAA and 38-115µM of abscisic acid could be conserved for a longer time. Evaluation of stored material indicated that it was genetically stable and 0.1-0.2% of it became infected with virus. Similarly, the Institute of Plant Genetics and Crop Plant Research at Gatersleben, Germany, one of the biggest gene banks in Europe, possesses a collection of 3039 accessions of species of the genus *Allium*, including the European garlic core collection. Before storage of germplasm, virus elimination is undertaken through meristem culture, then either slow growth storage is carried out for 12 months at 2 and 19°C or cryopreservation-vitrification using a mix of glycerol and sucrose 1:1 as cryoprotectant inside the aluminum foil (Keller et al., 2006).

In the United States, investigations have been carried out for cryopreservation of garlic accessions at the Western Regional Plant Introduction Station, Pullman, Washington. In this respect, Ellis et al. (2005) tested two vitrification solutions to cryopreserve 12 accessions. Shoot tips excised from cloves were treated with the vitrification solutions 2 (PVS2; 15% DMSO, 15% ethylene glycol, 30% glycerol, 0.4M sucrose) and 3 (PVS3; 50% sucrose, 50% glycerol). Eleven out of the 12 accessions could be successfully cryopreserved by using vitrification solutions 2 and 3 as cryoprotectants. Cryopreservation resulted in better regrowth of 7 and 3 accessions when PVS2 and PVS3, respectively, was used. Only one

genotype displayed good recovery in both solutions, which indicated that response depended on genotype.

In Romania, investigations were carried out to establish conditions for slow growth of Romanian garlic landraces. Shoots regenerated from leaf discs were cultured under a slow growth condition that consisted in a medium lacking sucrose at 16°C and under normal conditions on a medium with 3% of sucrose at 24°C. After four weeks, shoots cultivated under slow growth conditions reduced their growth rate by 42%. Shoots grown on sucrose-free medium displayed shorter internodes, but higher foliar surface, and shorter roots. Some landraces proved to be more sensitive to lack of sucrose than to low temperature. Differences in response to growth conditions tested were also observed among the different genotypes under study. The developed protocol allowed medium-term preservation of landraces under study (Botau et al., 2005).

Other investigation groups have focused their efforts on defining the best conditions for slow growth storage or cryopreservation. For example, Makowska et al. (1999) studied the response of apexes from different sources (cloves and bulbils) to cryopreservation after being treated with vitrification solutions. They found out that after freezing, apexes treated with PVS2 solution (30% glycerol, 15% ethylene glycol, 15% DMSO, 0.4M sucrose) restored their growth in a higher proportion compared to the ones that had been submerged in solution PVS3 (40% glycerol and 40% sucrose). A higher number of apexes excised from big bulbils restored their growth, while the ones dissected from small bulbils failed to regrow. On the other hand, apexes obtained from cloves had higher survival rate than the ones obtained from bulbils.

Later, Sudarmonowati (2001) tested different vitrification solutions in an attempt to define the most suitable method for cryopreservation of embryogenic calli of cultivar *Lumbu Hijau*. Calli with embryos at globular stage were precultured on MS medium with 9μM BA and 0.4M sucrose for 1 to 7 days. Next, they were submerged in MS medium with 2M glycerol and 0.4M sucrose (loading solution) in order to be later exposed to different vitrification solutions for various periods of time (5 to 60 min.). Calli were placed in cryotubes with one drop of vitrification solution, then plunged into liquid nitrogen for 30 minutes. After freezing, calli were first plated on MS medium with 5μM 2iP, 2.3μM kinetin and 0.4M sucrose, and later on the same medium containing a lower amount of sucrose. Of the three vitrification solutions tested, the one that contained a mix of glycerol (22%), ethylene glycol (17%), propylene glycol (17%) and DMSO (7%) proved to be better for calli conservation. The highest calli survival percentage was 30%, which indicated that it was necessary to improve the tested methodology.

Recently, Hassan et al. (2007) established slow growth cultures of two garlic varieties grown in Egypt (*Balady* and *Seds 10*). They cultivated bulblets on MS medium with 0.35M sucrose, 5g L^{-1} charcoal and 0.04μM BA, then on a medium with different concentrations of sorbitol and sucrose (0.1, 0.2 and 0.4M). Cultures were incubated in darkness at 4°C for their conservation. Bulblets did not develop neither shoots nor roots under these conditions during the first three months. Addition of 0.1M sorbitol to culture medium delayed growth of shoots and roots of cultivar *Balady* to 6, 12 and 18 months, while sucrose (0.1 or 0.2M) had the same effect on bulblets of cultivar *Seds* 40. The survival rate was of 100% after 18 months of maintaining cultures under these conditions.

5.5 Genetic transformation

Although several methods are available to introduce DNA into plant cells, most of them have been developed using the bacterium *Agrobacterium tumefaciens* (direct method) or biolistic (indirect method) as a vehicle. *A. tumefaciens* is a soil bacterium having natural ability to transfer part of its DNA to plant cells of various species causing formation of crown gall tumors (Hooykaas & Schilperoot, 1992). This ability is conferred by the Ti plasmid (tumor-inducing), which contains a region called T-DNA, that is transferred to the host cell with helping of virulence genes, also present in Ti plasmid. The T-DNA contains genes that are involved in production of cytokinins (2-isopentyl-AMP) and auxins (IAA), which are responsible for tumor formation (Leemans et al., 1982; Barry et al., 1984). Genetic manipulation of this plasmid has resulted in the replacement of genes contained in the T-DNA of the wild strain by genes that confer desirable traits to transformed plants (Christou, 1996).

A series of physical, electrical and chemical methods (e.g. electroporation and biolistic) have been generated to introduce DNA directly into plant cells (Songstad et al., 1995). Starting from the successful transformation of monocotyledonous plants, such as maize, and soybean by using the biolistic method (McCabe et al., 1988; Fromm et al., 1990), this has become one of the most used systems for gene transfer. This method consists in bombarding target cells with DNA-coated gold or tungsten microparticles accelerated to very high speeds by a gene gun, which allows them to cross the cell walls. Although there are various types of gene guns, the PDS1000 helium designed by Dupont has been the most widely used, specially for transformation of monocotyledonous plants (Vain et al., 1993; Christou, 1995). A great variety of genetic transformation protocols have been developed, but this technology has not been applied with the same efficiency in every species. The species of the genus *Allium* represent an example in this respect, particularly garlic, for which only a small number of publications are available.

5.5.1 Via biolistic

It was not until 1998 that a protocol for garlic transformation was reported for the first time. Barandiaran et al. (1998) bombarded leaf tissue, immature bulbs, cloves and callus of the cultivar *Morado de Cuenca* with four constructs (pDE4, pCW101, pActl-D and pAHC25). Out of these vectors, the one carrying the reporter *uidA* gene (*gusA*) (coding for β-glucuronidase) under control of the promoter 35S from cauliflower mosaic virus (CaMV35S) and the terminator of the nopaline synthase gene (NOS), allowed expression of the *uidA* gene in 43.3% of leaf explants, 76.7% of bulbs, 23.3% of clove tissue and 13% of calli. Transitory expression of the *uidA* gene could only be detected after treating tissues with a nuclease inhibitor (aurintricarboxylic acid). However, regeneration of transgenic plants could not be achieved by using this protocol. Similarly, Myers & Simon (1998) bombarded cell suspensions of *RAL27* clone with *gusA* and *nptII* (conferring resistance to kanamycin) genes which were under control of CaMV35S and NOS promoters, respectively. After 14-16 weeks on selection medium, shoots were regenerated on calli. Incorporation of *gus* and *nptII* genes into garlic transgenic plants was confirmed by PCR assays.

Later, Ferrer et al. (2000) used biolistics to introduce the reporting gene *uidA* and the selection gene *bar*, which codes for N-acetyl-transpherase, into leaf tissue, basal plate discs and embryogenic calli of cultivar *Moraluz*. The *uidA* and *bar* genes were under control of CaMV35S and maize ubiquitin (Ubi) promoters, respectively. Maximum expression of *uidA*

was observed in calli and leaves. In a different investigation, Sawehel (2002) developed a transformation system using calli derived from immature cloves of cultivar *Giza 3*. Calli were bombarded with the plasmid pBI22.23 containing the *hpt* gene (coding for the enzyme hygromycin phosphotranspherase that confers resistance to antibiotic hygromycin), and the reporting gene *gusA.* Calli had been previously treated with aurintricarboxilic acid to inhibit activity of endogenous nucleases. Southern blot assays and histochemical analysis proved that this system allowed the transfer, expression and stable integration of transgenes into the garlic genomic DNA.

At the same time, Park et al. (2002) obtained transgenic plants resistant to herbicide chlorsulfuron after bombarding calli of cultivar *Danyang* with the plasmid pC1301-ALS, which contains *gus, hpt* and *als* (coding for acetolactate synthase) genes, under control of the promoter CaMV35S. Out of 1900 calli, 12 grew and regenerated plants resistant to chlorsulphuron (3mg L^{-1}), which formed bulbs and reached maturity. PCR, Southern blot and Northern blot assays confirmed the expression and integration of transgenes into the genome.

In a different work, Robledo-Paz et al. (2004) established a transformation protocol using embryogenic calli derived from root tips of cultivar *GT96-1*. Calli were bombarded with the plasmid pWRG1515 containing *hpt* and *gusA* genes, both under the control of the promoter CaMV35S, and the 3' region of the *nos* gene. Putative transgenic calli were identified after four months of culturing them on a medium containing hygromycin (20mg L^{-1}), and later developed into plants. Molecular (Southern blot) and histochemical (GUS) analysis confirmed transgenic nature of regenerated plants. Transformation efficiency was of 2.2 clones per fresh weight gram of bombarded callus.

5.5.2 Via *Agrobacterium tumefaciens*

Kondo et al. (2000) were the first to achieve the establishment of a transformation protocol in garlic using *A. tumefaciens* as a vehicle. They infected morphogenetic calli with the strain EHA101 carrying the plasmid pIG121, which in turn contained *nptII, hph* and *uidA* genes under control of the promoter CaMV35S. By using this protocol it was possible to regenerate 15 transgenic plants from 1000 inoculated calli grown on a selective culture medium for five months. Zheng et al. (2004) presented a transformation system that apart from producing plants resistant to antibiotics or herbicides, also enabled introduction of genes for resistance to insects. Inoculation of calli of three European cultivars was undertaken using the strain AGLO carrying four different plasmids containing *gusA* and *hpt* genes, and also *cry1Ca* and *HO4* genes from *Bacillus thuringiensis,* which confers resistance to the insect *Spodoptera exigua*. The highest transformation frequency (1.47%) was achieved with the cultivar *Printanor* and the plasmid pPB34. Of regenerated plants, only the ones that integrated the *cry1Ca* gene had a good growth under greenhouse conditions and had the ability to form bulbs. These plants were totally resistant to *Spodoptera exigua* in bioassays carried out *in vitro.* Later, Eady et al. (2005) inoculated immature embryos with the strain LBA4404 carrying the vector pBIN *m-gfp-ER* containing the gen *gfp* (encoding for the green flourescent protein) and the gene *nptII.* Out of the 3200 infected embryos, only two transgenic plants (0.06%) were regenerated. Khar et al. (2005) studied the transitory expression of the reporter gene *gusA* in two garlic cultivars after infecting them with a *A. tumefaciens* strain carrying two plasmids. Plasmid pCAMBIA 1301 induced a higher transformation frequency (7.4%) than plasmid pTOK233 (4.1%). Genes conferring resistance

to fungi are still not being commercially used for fighting diseases caused by these phytopathogens. In garlic, Robledo-Paz (2010, personal communication) incorporated chitinase and glucanase genes in an attempt to confer resistance to the fungus *Sclerotium cepivorum*. These experiments revealed that regenerated transgenic plants were not totally resistant to the fungus, but displayed a delay in the infection speed.

5.6 Molecular markers

In order to make a more efficient use of garlic germplasm cultivated in various regions of the world, it is necessary to evaluate and characterize the available genetic diversity (Ordás et al., 1994). As the descriptions based on anatomical and morphological characteristics are incomplete and they can be affected by environmental factors, other methods are required to perform this characterization (García-Lampasona et al., 2003). Polymorphism of molecules such as isozymes and DNA can be used to characterize plant germplasm, specially in cases where morphological and biochemical differences are not conspicuous. Although isozyme analysis represented the first application of molecular markers in the genus *Allium*, its main drawback is the low number of enzymatic systems available in garlic. In addition, these markers may suffer changes induced by the developmental stage of plant material analyzed and by environment (Pooler & Simon, 1993; Klaas & Friesen, 2002).

DNA-based markers are less affected by age, physiological condition of the sample and environmental factors. They are not tissue specific and can be detected in any developmental stage of an organism. DNA markers such as RAPDs (Random Amplified Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeats) and DNA fingerprinting have been of great use for various studies in garlic. Isozyme analysis, RAPDs and AFLPs have enabled the study of phylogenetic relationships between different garlic clones and determination of their place of origin (Pooler & Simon, 1993; Maaβ & Klaas, 1995; Bradley et al., 1996; Al-Zahim et al., 1997; Lallemand et al., 1997; Ipek & Simon, 1998; García-Lampasona et al., 2003; Buso et al., 2008; Ipek et al., 2008; Abdoli et al., 2009). The use of molecular markers is indispensable for the establishment of core collections that should contain unique, varied and completely identified accessions in order to reduce costs and labour required for maintenance of collections *in situ* (Ipek et al., 2008). On the other hand, germplasm exchange between garlic producing countries can give rise to that a clone be called in different ways in various countries. If this occurred, the germplasm banks could be constituted by duplicated accessions. In this respect, molecular markers such as DNA fingerprinting and AFLPs have been used to detect duplicated accessions in collections (Bradley et al., 1996; Ipek et al., 2003). The use of AFLPs revealed that 64% of the U.S. National Plant Germplasm System's garlic collection was duplicated (Volk et al., 2004). Moreover, molecular markers can be used for detection of somaclonal variants generated by *in vitro* culture (Al-Zahim et al., 1999; Saker & Sawahel, 1998; Sánchez-Chiang & Jiménez, 2009), for determination of fertile clones (Hong et al., 2000; Etoh & Hong, 2001), disease resistance (Nabulsi et al., 2001) and clones producing S-amino acids (Ovesná et al., 2007).

6. Conclusion

Biotechnological tools such as plant tissue culture can help overcome problems associated with vegetative propagation of garlic, specially the low multiplication rate and disease dispersion.

Although plant regeneration has been achieved from different explants types, use of root tips has advantages over other explants due to their virus-free condition and to their availability in a relatively high number (30 or more per clove). Moreover, roots developed from bulbs obtained *in vitro* can also be used for tissue culture (Robledo-Paz et al., 2000). On the other hand, production of virus-free plants via meristem culture combined with thermotherapy and chemotherapy can reduce losses caused by phytopathogens, even when propagation of virus-free material is relatively expensive (Salomon, 2002). Tissue culture has also been applied to the establishment of germplasm banks in various parts of the world where valuable garlic collections are maintained for medium (slow growth) and long term (cryopreservation).

Techniques such as somaclonal variation and genetic engineering could play an important role in the genetic improvement of garlic because they generate genetic variability. However, the somaclonal variants with commercial potential are scarce, and further experiments are necessary to identify the optimal explant type and the culture conditions that enable formation of somaclones (Novak, 1990). In addition, although there have been advances in the field of genetic transformation in garlic, more investigations are required to establish reproducible and efficient protocols. This task will require selection of suitable target cells for inoculation with *Agrobacterium* or biolistics (e.g. embryogenic calli) (Myers & Simon, 1998b; Robledo-Paz et al., 2004), strategies for transgene expression, a suitable selection method and efficient protocols for plant regeneration (McEloy & Brettell, 1994; Hansen & Wright, 1999). Furthermore, molecular markers will be key pieces in phylogenetic and taxonomic studies (Maaβ & Klaas, 1995) and germplasm conservation (Ipek et al., 2008). Moreover, they will be used for detection of somaclonal variants (Al-Zahim et al., 1999), fertile genotypes (Etoh & Hong, 2001), disease resistant genotypes (Nabulsi et al., 2001) and clones producing compounds of economical importance (Ovesná et al., 2007) which can be used for improving this important crop plant.

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