Current Progress in Biotechnology

Suzy Hill

Current Progress in Biotechnology

Edited by **Suzy Hill**

Callisto Reference

New York

Published by Callisto Reference,
106 Park Avenue, Suite 200,
New York, NY 10016, USA
www.callistoreference.com

Current Progress in Biotechnology
Edited by Suzy Hill

International Standard Book Number: 978-1-63239-140-7 (Hardback)

Printed in China.

Preface

The world is advancing at a fast pace like never before. Therefore, the need is to keep up with the latest developments. This book was an idea that came to fruition when the specialists in the area realized the need to coordinate together and document essential themes in the subject. That's when I was requested to be the editor. Editing this book has been an honour as it brings together diverse authors researching on different streams of the field. The book collates essential materials contributed by veterans in the area which can be utilized by students and researchers alike.

This book is a concise and sophisticated introduction to innovations in biotechnology. It presents cutting edge research topics in microbial and animal biotechnology. It also enables the reader to understand the role of biotechnology in society, answering obvious questions pertaining to biotech procedure and principles in the context of research advances. In an age of multidisciplinary cooperation, the book serves as an outstanding in-depth text for a wide variety of readers ranging from experts to students.

Each chapter is a sole-standing publication that reflects each author's interpretation. Thus, the book displays a multi-facetted picture of our current understanding of application, resources and aspects of the field. I would like to thank the contributors of this book and my family for their endless support.

Editor

Contents

Part 1

Plant Biotechnology

1

Biotechnological Tools for Garlic Propagation and Improvement

Alejandrina Robledo-Paz[1] and Héctor Manuel Tovar-Soto[2]
[1]*Postgrado en Recursos Genéticos y Productividad-Semillas*
Colegio de Postgraduados Km. 36.5 Carretera México-Texcoco, Montecillo, Edo. Méx.
[2]*Instituto Tecnológico de Cd. Altamirano*
Pungarabato Pte. S/N. Ciudad Altamirano, Guerrero
México

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