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Bibliographic review

Use of biotechnological techniques in garlic crop (*Allium sativum* L.)

Empleo de técnicas biotecnológicas en el cultivo del ajo (*Allium sativum* L.)

Marian Rodríguez Hernández*

Instituto Nacional de Ciencias Agrícolas (INCA), carretera San José-Tapaste, km 3½, Gaveta Postal 1, San José de las Lajas, Mayabeque, Cuba. CP 32 700

ABSTRACT: Plant biotechnology has become a viable strategy for agriculture, its applications include strategies aimed at obtaining and maintaining pathogen-free cultivars, genetic improvement (anther cultivation, embryo rescue, etc.) and micropropagation, used to obtain a greater number of individuals in less time and reduced spaces. Each of its phases depends largely on an adequate culture medium, since the growth, development and morphogenesis of the explant is mainly due to its chemical composition. For the production of garlic "seed" (*Allium sativum* L.), all phases are carried out in a growth chamber under automatically controlled aseptic and artificial conditions and at a temperature ranging between 22-25 °C. Somatic embryogenesis allows complete plants to be regenerated from a single cell. Garlic is an annual plant that only reproduces asexually, which is why bacteria, fungi and mainly viruses, which contribute to a 50-80 % decrease in its yield, affect it. An alternative to avoid these problems is the use of chemotherapy, thermotherapy or the combined use of one of these techniques with the cultivation of meristems, to obtain plants of high biological quality. Depending on the behavior of the culture during its *in vitro* stage, its response will be in the acclimatization phase and later in field conditions. This work was carried out with the objective of deepening the knowledge of some biotechnological techniques that are applied in the cultivation of garlic.

Key words: Garlic, viral load, *in vitro* culture, micropropagation, virus-free plants.

RESUMEN: La biotecnología vegetal se ha convertido en una estrategia viable para la agricultura, sus aplicaciones contemplan estrategias dirigidas a la obtención y mantenimiento de cultivares libres de patógenos, mejoramiento genético (cultivo de anteras, rescate de embriones, etc) y la micropropagación, empleada para obtener un mayor número de individuos en menor tiempo y espacios reducidos. Cada una de sus fases depende, en gran medida, de un medio de cultivo adecuado, pues el crecimiento, desarrollo y la morfogénesis del explante se debe, principalmente, a la composición química del mismo. Para la producción de “semilla” de ajo (*Allium sativum* L.) de alta calidad, todas las fases se llevan a cabo en una cámara de crecimiento en condiciones asépticas y artificiales controladas automáticamente y a una temperatura que oscila entre 22-25 °C. La embriogénesis somática, permite regenerar plantas completas a partir de una célula. El ajo es una planta anual que solo se reproduce de forma asexual, por ello, lo afectan bacterias, hongos y, fundamentalmente, virus, que contribuyen a la disminución de su rendimiento entre 50-80 %. Una alternativa para evitar estos problemas, es el empleo de la quimioterapia, termoterapia o el empleo combinado de una de esas técnicas con el cultivo de meristemos, para obtener plantas de elevada calidad biológica. Según el comportamiento del cultivo durante su etapa *in vitro* será su respuesta en la fase de aclimatización y, posteriormente, en condiciones de campo. Este trabajo se realizó con el objetivo de profundizar en el conocimiento de algunas técnicas biotecnológicas que se aplican en el cultivo del ajo

Palabras clave: Ajo, carga viral, cultivo *in vitro*, micropropagación, plantas libres de virus.

*Author for correspondence: marian@inca.edu.cu

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INTRODUCTION

In recent years, plant biotechnology has become a viable strategy for agriculture thanks to its great advances, which include the use of new technologies generated by the knowledge of Genetics and Molecular Biology, which are the techniques related to the *in vitro* culture of immature organs, the manipulation of recombinant Deoxyribonucleic Acid (DNA) and molecular genetic markers. One of its branches is *in vitro* culture or tissue culture, which is nothing more than the capacity of plant cells, protoplasts or organs to regenerate a complete plant, which is what is called totipotency or cell totipotentiality, a concept explained by a researcher (1). Garlic (*Allium sativum* L.) is an annual plant, which in our country only reproduces asexually due, among other factors, to climatic conditions (2). In addition, it does not produce viable "seeds", so "seeds" from bulbs harvested in the previous season are planted (3).

Under these conditions, phytopathogenic diseases are easily transmitted to the offspring, leading to a progressive and irreversible weakening of the varieties or clones. Several phytosanitary problems are involved, capable of causing yield losses of 50 % or more, either by the presence of nematodes, fungal, bacterial and viral diseases, which are the most influential in the deterioration of the crop (2,4). One of the most efficient combat measures is the use of virus-free "seed" by means of tissue or meristem culture (5).

The success of tissue culture depends largely on the culture medium or nutrient medium suitable for each of the stages of *in vitro* micropropagation, as well as for the growth, development and morphogenesis of the explant (6).

For the production of garlic "seed", all the micropropagation stages are carried out in the growth chamber, which is usually located in the laboratory under automatically controlled aseptic and artificial conditions, to allow the development of seedlings *in vitro*. The optimum temperature for their development ranges between 22-25 °C (7).

In turn, somatic embryogenesis allows the regeneration of complete plants, and it is widely used by biotechnology and embryology studies, its main advantage is the production of numerous seedlings from one cell and allows the evaluation and genetic improvement. In these techniques, regeneration will depend on the explant type, concentration, combination of growth regulators and nutrients (8).

There are currently numerous techniques used to detect possible genetic variability in seedlings grown *in vitro*. In morphological studies, characters must be discovered and be delimited usually without any explicit criteria for selection or character coding, so they have the potential to be arbitrary. Morphologists do not generally disclose their criteria for including or excluding characters, and when criteria are given, they vary considerably among studies. However, they have the advantage of allowing much more careful taxonomic sampling than is done with molecular analyses, which is important for systematic reviews, studies of character evolution, and phylogenetic assessment (9).

Cytogenetic techniques such as FISH (fluorescence *in situ* hybridization) and GISH (genome *in situ* hybridization) use dyes to mark distinct regions of DNA. Both techniques are used to identify foreign chromosomes or to detect translocations (changes in the position of chromosome parts) in hybrids, which is not possible with classical cytogenetic techniques such as staining or banding (10).

With technological progress, the use of starch gels and histochemical staining of proteins, the existence of isoenzymes, multiple molecular forms within an organism that catalyze the same reaction, was demonstrated within an organism. The effect of an allelic modification can be detected with certainty, due to a change in electrophoretic mobility. This electrophoretic sensitivity has revolutionized genetic diversity studies in several species such as lettuce (*Lactuca sativa* L.), lentil (*Lens culinaris* M.) and bean (*Phaseolus vulgaris* L.) (11).

As for molecular techniques, protein-based polymorphism has been very useful in plant research, but with the development of DNA-based technologies, research in this area has been favored with the availability of a greater number of markers, those based on Polymorphic Restriction Fragments (RFLP) and Polymerase Chain Reaction (PCR). Both techniques have derived in multiple techniques such as Random DNA Amplification (RAPD), Amplified DNA Polymorphic Fragments (AFLP), minisatellites (VNTR) and microsatellites (SSR), among others (12).

The present work aims to deepen the knowledge of some biotechnological techniques applied in garlic cultivation.

DEVELOPMENT

1. Culture media used in garlic

A culture medium can be defined as a formulation of inorganic salts and organic compounds required for crop nutrition. Numerous formulations exist, each comprising between 6 and 40 compounds (13). Culture media supply:

- *A carbon source:* sucrose, in concentrations of 2 to 5 %, is the most commonly used sugar. In some media, it is replaced by glucose. In particular cases, the use of maltose or galactose is mentioned. In addition, myo-inositol (100 mg L^{-1}) is usually incorporated to the media and a better growth of the cultures is achieved.
- *Mineral nutrients:* Culture media should supply macro and micronutrients, which are essential for seedling growth. In general, relatively high concentrations of nitrogen (supplied in the form of ammonium and nitrate) and potassium stand out. Urea, glutamine and hydrolyzed casein can also be used. It is essential that iron be incorporated in conjunction with a chelating agent (Na2EDTA), which makes it available over a wide pH range.

- *Vitamins:* From all those commonly incorporated into media, it would seem that thiamine is the only one essential for good crop growth.
- *Growth regulators:* In most cases the media used for crop establishment contain auxins [ANA (naphthalene-acetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), IAA (indolacetic acid), AIB (3-indolbutyric acid), NOA (Naphthoxyacetic acid), Dicamba, Picloram] and/or cytokinins [BA or 6-BAP (6-benzyl amino-purine), KIN (kinetin), ZEA (zeatin), 2-iP (2-isopentenyladenine,) Thidiazuron]. Gibberellins [especially GA3 (gibberellic acid)] are sometimes required for meristem growth or shoot elongation. ABA (abscisic acid) is used in some cases.
- *Gelling agent* (in the case of semisolid culture media): Agar (between 0.6-1 %) is the most commonly used compound. Agargel (0.40-0.60 %), Transfergel (2.0-2.60 %), Phytagel (0.25- 0.40 %), agarose (0.80-0.90 %) and Gelrite (0.10-0.20 %) can also be used.
- *Other compounds:* Many substances, of varied chemical composition, are added often to culture media. In addition to glycine, other amino acids can be added, such as L-tyrosine, asparagine and cysteine. In some culture media, organic acids such as malic, citric, pyruvic and succinic acids are incorporated and L-glutamine and hydrolyzed casein are frequently used.

In studies carried out on *in vitro* bulbification of garlic, the mineral salts of Murashige and Skoog (MS) were used, to which were added 2-iP (2.0 mg L^{-1}), ANA (0.1 mg L^{-1}), thiamine (0.1 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), pyridoxine (0.5 mg L^{-1}), inositol (100 mg L^{-1}) and 30 g L^{-1} of sucrose. In the effect of 2-iP concentration, significant differences were only detected for the equatorial diameter variable, and the highest value was obtained with the 2.0 mg L^{-1} treatment with 10.93 mm. With all growth regulators, bulbification was 100 %, and only in the treatment with 2-iP was there formation of multiple bulbs and in 2 % of the cultures, between 2-3 microbulbs of 2-5 mm in diameter were obtained (14).

For the establishment of an *in vitro* protocol for garlic cultivation in Costa Rica, the culture medium used was that of Murashige and Skoog (MS), which was supplemented with 2.5 mg L^{-1} of IAA. Additionally, one of the following growth regulators was added: 6-BAP, Kinetin or 2-iP (15).

Studies carried out using two culture media: MS and LS (Linsmaier and Skoog) supplemented with 6-BAP for the seedling length variable. After 14 days of subculture it was observed that, the *in vitro* seedlings of the control treatment, which correspond to the MS culture medium enriched only with vitamins, had the greatest longitudinal growth where the mean was 2.56 cm followed by the seedlings of treatment 2 (T2). Basal salts of the LS medium supplemented with 6-BAP were used and the explants reached a height of 2.28 cm. Statistical analysis of that variable showed significant differences among treatments ($p \leq 0.05$) (16).

2. Main chemical components used for *in vitro* garlic establishment

When studying the clones 'Criollo-3', 'Criollo-6', 'Criollo-9', 'Martinez' and 'Vietnamita' in each phase for the micropropagation of the crop, it was found that both calcium hypochlorite (5 %-20 minutes) and sodium hypochlorite (10 %-15 minutes, with 5 % active Cl2) were effective in the disinfection of explants, survival and vigor of the seedlings. Explants established better and in less time on basal MS medium supplemented with ANA (0.1 mg L^{-1}) and KIN (0.1 mg L^{-1}). Between 4.9-7.3 shoots per explant were obtained, depending on the subculture and clone on the above medium enriched with ANA (0.1 mg L^{-1}) and 2-iP (4 mg L^{-1}). The highest percentage of *in vitro* bulbification was induced on the above basal medium when 75 g L^{-1} sucrose was added. Microbulbils were superior to *in vitro* seedlings, as they reached 95.35-99.40 % survival when planted in expanded polystyrene trays, in a substrate composed of Zeolite [Litonite] (25 %) and O.M [decomposed Cachaça] (75 %) (17).

Results obtained from a disinfection methodology for the *in vitro* implantation of garlic explants (18) showed that the same behaved unevenly, since statistical differences were observed between the disinfection variants that were analyzed. In terms of explant survival, the best results were achieved in the treatments: 7 [Ca_2OCl (5 %-10 minutes)], 8 [Ca_2OCl (5 %- 15 minutes)], 9 [Ca_2OCl (5 %-20 minutes)], 11 [NaOCl (10 %-10 minutes)], 12 [NaOCl (10 %-15 minutes)] and 13 [NaOCl (10 %-20 minutes)], all with 90 % and outperformed control 1 [alcohol (70 %-30 minutes)] which achieved 59.60 % survival.

in vitro seedling establishment of Peruvian garlic clones from two chemical compounds showed that treatment of explants with calcium hypochlorite (1 %) and sodium hypochlorite (0.1 %) for 10 and 15 minutes, respectively, allowed total elimination of contaminating microorganisms from Peruvian clones 'P-007A' and 'P-007B' (19).

As can be seen, the use of chemical compounds at adequate concentrations is of utmost importance in the disinfection of garlic explants for *in vitro* propagation, as it reduces their contamination, as well as that of the culture medium used.

3. Use of biotechnological techniques in garlic cultivation

Numerous biotechnological techniques have been developed including tissue and organ culture, embryo rescue, protoplast fusion, molecular markers, molecular embryo rescue, protoplast fusion, molecular markers, protein and DNA sequence the establishment of protein and DNA sequences, and genetic engineering (20).

3.1. Main viruses affecting garlic cultivation

Worldwide there are 15 viruses associated with garlic cultivation belonging to five different genera (21). These include:

- 8 Allexiviruses: Garlic Virus-A (GarV-A), Garlic Virus-B (GarV-B), Garlic Virus-C (GarV-C), Garlic Virus-D (GarV-D), Garlic Virus-E (GarV-E), Garlic Vvirus-X (GarV-X), Shallot Virus X (ShVX) and Garlic Mite-borne Filamentous Virus (GarMbFV).
- 3 Potyviruses: Onion Yellow Dwarf Virus (OYDV), Leek Yellow Stripe Virus (LYSV) and Tobacco Etch Virus (TEV).
- 2 Carlaviruses: Garlic Common Latent Latent Virus (GarCLV) and Shallot Latent Virus (SLV).
- 1 Tospovirus: Irish Yellow Spot Virus (IYSV).
- 1 Fijivirus: Garlic Dwarf Virus (GDV).

Losses caused by viral infections vary and may depend, partially, on the viral agent-genotype interaction. In Argentina, Garlic Virus A caused reductions between 14 and 32 % in bulb mass and between 6 and 11 % in diameter in 'Morado-INTA' and 'Blanco-IFFIVE' varieties (22).

In Mexico it has been reported that diseases caused by viruses can cause a yield reduction between 33-50 % and up to 30 % of bulb mass, especially when the frequency of viruses such as Onion Yellow Dwarfism (OYDV), Tobacco marbling (LYSV) and Latent Shallot (SLV), among others, is high (23).

To reduce these sources of inoculum, the plant material must be prepared before being taken to the laboratory, in a greenhouse phase or phase 0. This involves taking the plants to a greenhouse, where for a period agrochemicals are applied to them to reduce potential sources of contamination, which can be fungi or bacteria. Among the substances used to reduce contamination are sodium hypochlorite, calcium hypochlorite, mercury (II) chloride, 70 % alcohol and hydrogen peroxide. Their use will depend on the type of plant tissue (herbaceous or woody) or the amount of contaminating inoculum present in the explant (18).

The use of mercuric chloride II ($HgCl_2$) as a disinfecting agent is a simple, fast and effective method for obtaining viable explants free of contamination. However, its use continues to be controversial due to its high toxicity and high level of environmental contamination, in addition, it can cause problems of tissue browning.

3.2. Obtaining pathogen-free plants

Several factors are involved in the contamination of *in vitro* explants, from the age of the plant material to its origin, disinfectant solutions, the way the operator works in the laminar flow chamber and the asepsis of the growth room (14).

In the tropics, there are conditions of high temperatures and relative humidity, heavy rainfall and abrupt climatic changes, which cause microclimates, which affect the multiplication and dissemination of fungi, bacteria and yeasts, contaminating agents of the culture media and explants. These contaminants compete for space and nutrients found in the culture medium, besides the release of toxic substances by some of them, which can cause the explant death (24).

Studies carried out in Cuba (25), have shown that in the disinfection of vegetal material for *in vitro* establishment, the best results were reached with 93.37 % of efficiency when ethanol at 70 % and sodium hypochlorite at 3.0 % were used, during 25 minutes. These results corroborated with those reported by other authors (26), where it is stated that sodium hypochlorite has been traditionally used alone or in combination with other disinfectants in the decontamination of plant materials to be established *in vitro*, due to its high redox potential of 1.36 eV.

In studies, high-yielding garlic genotypes were selected (27), of which the genotype 'Criollo-3' was sanitized from viruses, affecting the crop and propagated *in vitro*. The clone showed good agronomic behavior to pests, high yield and good "seed" quality.

Other results show that by increasing the concentration of the disinfectant agent (20 % v/v NaOCl) with a time of 15 minutes, there was no contamination during the sprouting phase and the tissues were kept in optimal conditions for handling (28).

The sanitation of garlic plants is carried out by means of meristem culture, which consists of cultivating, under *in vitro* conditions, the meristematic dome extracted from the basal part of the garlic bulblet. Because the tissue can dehydrate quickly, this technique requires the skill of a specialist. The extraction of the meristem dome is done after the dormancy period is over, when the bulblet is close to sprouting. It is important to note that one of the reasons postulated to explain the virus-free condition of the meristematic tissue is because the speed of replication and movement of viral particles is lower than the rate of cell multiplication of this tissue (29).

In studies for the molecular detection of potyvirus in garlic leaves and minibulblets associated with a healthy seed production program, 730 meristems were introduced. Disinfection of the "seed" produced 100 % of explants free of bacterial or fungal contamination. Losses occurred in 31 % of the explants introduced, 18 % of the meristems were lost due to tissue death or viability. The establishment percentages allowed validating the *in vitro* production of garlic in the clones 'Cerrito', 'Santa Rosa' and 'Bogotá'. Seventy-one percent of the seedlings established in a culture medium supplemented with the regulators 2-iP and ANA, showed after four weeks an adequate formation and vigor, according to the requirements of the stage; they presented desirable characteristics, such as an average height of 1.5 cm, and the development of seedlings with 2.5 leaves per seedling and without roots (30).

3.2.1. Thermotherapy

The use of high temperatures (40-75 °C) to eliminate or reduce the "viral load" has been defined as the production of a progressively less suitable cellular environment for viral agents (31) and it is a step prior to the culture of meristems to provide virus-free plants. The latter is a process that requires at least five years (32).

In other studies, it was shown that the results of *in vitro* establishment in garlic culture were positive for OYDV (14), so thermotherapy was subsequently used with different thermal treatments and combined with plant tissue culture in order to eradicate the potyvirus from the seedlings. The results presented in the eight treatments of the cultivar 'Don Fermín' showed that thermotherapy in combination with tissue culture has a positive response when using as a source of explant meristematic domes of a size of approximately 1 to 2 mm because it was possible to obtain plant material free of OYDV.

The use of thermotherapy and tissue culture for the elimination of the Onion Yellow Dwarfing Virus (OYDV) showed that the "teeth" (cloves) exposed to 40 °C for 40 days did not produce any amplification product, that is, this virus did not infect the *in vitro* seedlings subjected to this process. It is important to mention that the tissue culture technique in combination with thermotherapy on caulinar apices (5-8 mm) of a relatively large size proved to be efficient in the elimination of OYDV (6).

An alternative management method available to garlic growers is the application of high temperature directly to the bulbs to eliminate or reduce the "viral load" without damaging their germination capacity or subsequent development (33). It has been proven that thermotherapy is an effective and easily applied method to reduce the amount of inoculum of the *Sclerotium fungus* and nematodes of the genus *Ditylenchus* in garlic "cloves" (34).

Results obtained proved that thermotherapy has no depressive effect on future plants when they come from seeds treated between 47-49 °C; and neither is production negatively affected. Thermotherapy at 49 °C, being lethal to parasites already installed in the planting material, avoids infection of the garlic growing soil when this seed is used, which achieves a positive overall health effect (35).

The use of high temperatures positively influences the eradication of different viruses present in the garlic crop, both in field conditions and at laboratory level by means of tissue culture. In addition, the use of this technique avoids contamination of the soil or culture medium when using contaminated material.

3.2.2. Chemotherapy

Chemotherapy is defined as the therapy using chemical products. The first works were carried out with Malaquine green against the potato weak or latent mosaic virus (PVX) (36), with very low effectiveness. Subsequently, numerous products have been used against different viruses, but in most of the substances used, non-phytotoxic doses can only reduce viral multiplication rates to a very limited extent (37).

Some authors showed that, garlic plants cultivar 'Taiwan' from *in vitro* meristem culture, after the application of the chemical product Rivabirin, only 32.5 % of them presented negative reading to the presence of virus (38).

In results obtained with chemotherapy, the incidence of the OYDV and LYSV viruses in the garlic crop was eliminated. In the case of virus *per se*, it was possible to reduce its incidence up to 85-95 % (39). Other studies with the application of virazole showed 100 % of virus-free plants when 0.3 mm meristems were cultivated in a MS culture medium with 50 mg L^{-1} of the product (40). Other authors reported Potyvirus-free garlic plants by clove chemotherapy with Ribavirin 205 µM with a survival rate ranging from 27.0-34.8 % (41).

It was demonstrated that the use of chemical compounds could positively influence the garlic crop for the elimination of different viruses, although sometimes the use of some products only manages to reduce the viral load in a low percentage. In addition, treatments with chemical products usually have to be repeated more than once.

3.2.3. Electrotherapy

The concept can be defined as the use of electric current for therapeutic purposes. It is based mainly on the phenomena caused in the tissues by the passage of electricity, i.e. heating by the joule effect and electrolysis. In practice, galvanic, faradic and alternating currents are used (38).

The first experiences in the application of electric current through plant tissues, reported that they found an increase in the levels of cell and tissue regeneration. The electrotherapy to garlic "cloves" has been used in the sanitation of the viral complex of this species; it has been achieved between 53-100 % of healthy plants, when applying direct current (10-13 v) between 5-30 minutes (35).

Studies carried out with the use of this technique showed that doses higher than 30 V during 10 min were lethal for the garlic crop (42). At the same time, other authors showed a stimulation of the growth of garlic plants by means of this technique (43).

According to the results, the use of electric current is a favorable technique for eliminating a large part of the viral load present in the garlic crop, although it is not widely used.

3.2.4. Use of combined techniques for garlic virus sanitation

Within the field of crop protection, biotechnology is a dynamic process without which competitive agriculture cannot be carried out. Biotechnological applications include strategies aimed at pathogen detection, obtaining and maintaining pathogen-free cultivars, as well as new strategies for the control of diseases for which there are no conventional ways. In the last century, new technologies were successively incorporated that made it possible to deepen the improvement of cultivars, including interspecific crosses and *in vitro* cultivation (37).

Some authors used thermotherapy, chemotherapy and meristem culture in combination to obtain garlic plants free of viruses of the Potyvirus group. Embryos were obtained from cloves (explants) that had undergone chemotherapy and plants regenerated from embryos received thermotherapy treatment. These meristems were cultured and ELISA indexed the plants obtained and it was found that thermotherapy has a negative effect on plant establishment. Nevertheless, thermotherapy in combination with meristem culture proved to be a more effective method for virus elimination (60.0-70.9 %) than meristem culture alone (64 %). However, they reported that chemotherapy was not effective for the elimination of the Potyvirus group (41).

Other authors tested electrotherapy, thermotherapy, chemotherapy, or meristem dissection (*in vitro* culture) methods to eliminate OYDV from *Allium sativum* L (44). They combined electro- and chemotherapy methods (15 mA/10 min + 20 mg L^{-1} Virazol) and obtained the best results, 85 % of the surviving seedlings were free of OYDV.

In studies (45) garlic "cloves" were treated for sanitation with hot water and air in growth chamber and greenhouse. The hot water treatments were 50 and 55 °C for 5, 15 and 20 minutes, with an efficiency of only 13 % sanitation. With hot air, a temperature of 36 °C was applied for 30, 40 and 60 days; the percentage of regeneration decreased as the application time increased and the efficiency of sanitation increased, reaching up to 100 % with 60 days of application.

3.2.5 Techniques used for virus diagnosis in garlic

Reliable, rapid and cost-effective methods for the detection of garlic viruses play an important role in the control and management of viral diseases. Enzyme-linked immunoadsorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) have been used for the diagnosis of garlic viruses (46). However, the antisera used in the ELISA method are not always available for all viruses and their diagnosis becomes complex due to multiple viral infections; in contrast, RT-PCR has been shown to be more sensitive by detecting viruses at very low concentrations (47).

For the detection of viral complexes in the garlic crop (48), the enzyme immunoadsorption technique (ELISA) was used. By means of this test, the presence of five viruses was observed: Potyviruses: Leek Yellow Streak Virus (LYSV) and Onion Yellow Dwarfing Virus (OYDV); Carlaviruses: Shallote Latent Virus (SLV) and Garlic Common Latent Virus (GCLV); and Tospovirus: Iris Yellow Streak Virus (IYSV) in the foliage of the ecotypes evaluated. In three different sampling dates, it was detected that LYSV had a frequency of 100, 100 and 100 %; in OYDV the sampling frequencies were 50, 75 and 77.7 %; in SLV 19.4, 30.5 and 0 %; in GCLV 100, 97.2 and 91.6 %; and in IYSV 0.0, 2.7 and 22.2 %.

Studies carried out on this crop (49) revealed for the 54 samples analyzed by RT-PCR the presence of two to five viruses in each plant, with a co-infection rate of 100 %. Two samples (3.7 %) contained 2 viruses, six samples (11.11 %) contained 3 viruses, ten samples (18.52 %) contained 4 viruses and 36 samples (66.67 %) contained 5 viruses. The garlic varieties 'Tigre' and 'Fermín' showed a higher frequency of viruses than the variety 'California'.

3.3. Garlic micropropagation

The vegetative propagation of plants using *in vitro* culture techniques is known commonly as micropropagation, a term that refers to the fact that the amount of plant material needed to initiate cultivation is small, much smaller than in traditional vegetative propagation techniques. There are different options for vegetative propagation by *in vitro* culture (50):

Multiplication from pre-existing buds (apical or axillary).

The formation of adventitious shoots or adventitious somatic embryos, from: explants consisting of portions of tissues or organs extracted from the mother plant, disorganized cells (cell suspensions) or tissues (callus cultures) established by cell proliferation in the explant itself.

Micropropagation can be used with a conservation approach, making it possible to obtain numerous seedlings, in short times and reduced spaces (51). The main advantages it offers (52), compared to traditional methods, and they are as follows:

- Seedling regeneration is faster because it occurs under controlled conditions of temperature, photoperiod, as well as chemically defined media in which the amount of nutrients and growth regulators are manipulated.
- Small proportions of plant tissue are required to initiate aseptic establishment.
- It is possible to propagate genotypes that with traditional methods present a certain degree of difficulty and allows obtaining a greater quantity of vegetative material of high biological quality.
- It is possible to produce seedlings *in vitro* at any time of the year.

The laboratory development phases (53) involved in the technology are three:

- *in vitro* establishment phase: This begins with the implantation of meristems (apical tissue) in a culture medium containing the nutrients necessary for *in vitro* seedling development.
- *in vitro* multiplication phase: Regenerated *in vitro* seedlings are transferred to a culture medium containing auxins and cytokinins for the induction of lateral shoot formation, which is minor in the first subculture and increases with the following subcultures. This multiplication cycle lasts approximately 4-8 weeks, depending on the genotype, and is repeated until the number of available seedlings is increased.

- *in vitro* microbulbing phase: Seedlings are separated and are placed in magentas or tubes and the formation of microbulblets is induced by increasing the sucrose concentration to 3, 6, 9 and 12 %. When they reach the appropriate size and diameter and take on a purple coloration, they are extracted and left to dry and then transferred to the greenhouse phase.

Studies from rapid multiplication of garlic *in vitro* (54) achieved micropropagation by using MS culture medium with 0.57 µM of IAA and 0.44 µM of BA for initial shoot culture, and then MS culture medium supplemented with 170 mg L^{-1} of NH_2PO_4.

3.3.1. Use of traditional growth regulators

Studies showed that shoot initiation occurred in 98 % of the apices grown in the MS basal culture medium salts supplemented with 0.5 mg L^{-1} of 2-iP and 0.1 mg L^{-1} of ANA, which allowed the development of only one shoot per test tube with an average height between 4-4.6 cm. The maximum height was observed in treatment T2 (0.01 mg L^{-1} of 2.4-D and 5 mg L^{-1} of Kin). It is with 9.9 cm height, followed by T3 (0.01 mg L^{-1} of 2.4-D and 5 mg L^{-1} of 6-BAP) with 8.2 cm, while in T3 the highest number of shoots per callus was obtained (3.9) followed by T2 with 2.2 shoots per callus. In the absence of growth regulators [T1 (MS)] there was no caulogenesis. These results indicated that the composition of the nutrient medium had an impact on the ability to regenerate shoots. In this regard, a higher proportion of cytokinins (Kin, 6-BAP) than auxins (2,4-D) was required in the growth medium. In this case, cytokinins seem to play a specific role for shoot regeneration from callus, probably by determining the differentiation pathway toward caulogenesis (55).

Although 2,4-dichlorophenoxyacetic acid (2,4-D) is an auxin that at low concentrations can favor shoot induction, its use in micropropagation processes is not recommended because it can induce genetic variability in the regenerants obtained.

In garlic cultivation, the ecotype 'Colorado de Mendoza' achieved the highest number of roots (2.5 roots) with the basal culture medium MS salts (Murashige and Skoog with synthetic vitamins). The culture medium MSM + EQ (Modified Murashige and Skoog salts, substitution of KNO_3, $MgSO_4$, NH_4NO_3 + quinoa extract) was the one that obtained the lowest number of roots, one on average (56). The ecotype 'Rosado de Italia' achieved the highest average root number (3.3 roots) in the presence of MSM culture medium (Murashige and Skoog Salts Modified, KNO_3, $MgSO_4$, NH_4NO_3 substitution), while MSM +EQ culture medium obtained the lowest root number (1.6 roots) (57).

The MSM culture medium (Murashige, and Skoog Modified with commercial salts) supplemented with the growth regulators Kinetin (1 mg L^{-1}) and Indolaceic Acid (2 mg L^{-1}) showed a higher regeneration of adventitious shoots. An average of 2.1 shoots per explant was reached for the ecotype 'Colorado de Mendoza' and one (1) shoot per explant for the ecotype 'Rosado de Italia' (57).

3.3.2. Use of growth biostimulators

Biobras-6® and Pectimorf® can be used as growth regulators in all phases of micropropagation of garlic clone 'Criollo-9'. The combination of IAA (0.1 mg L^{-1}) and Biobras-6® (0.05 mg L^{-1}) or IAA (0.5 mg L^{-1}) and Pectimorf® (1 mg L^{-1}) increased the *in vitro* establishment of caulinar apices and decreased the time the explants remained in the culture medium by three to four days compared to the control treatment (IAA and 6-BAP). The combinations ANA (0.3 mg L^{-1}) and Biobras-6® (2 mg L^{-1}) and IAA (0.5 mg L^{-1}) and Pectimorf® (10 mg L^{-1}) were better for obtaining multiple shoots. Survival and rooting during acclimatization of microbulbils was superior with prior immersion in Biobras-6® (1 mg L^{-1}) or Pectimorf® (10 mg L^{-1}) for 15 min and subsequent planting in a substrate composed of charged zeolite [Litonite] (25 %) and organic matter [decomposed cachaça] (75 %). Agronomic evaluations showed that Biobras-6® produced a yield of 12.42 and Pectimorf® 11.67 t ha-1, higher by 4.47 and 2.72 t ha-1, respectively in relation to the control plants (2).

Other studies with the use of sucrose showed that the behavior of *in vitro* garlic seedlings and microbulblets of different genotypes ('Criollo-9', 'Martinez', 'Criollo-6', Criollo-3' and 'Vietnamita') with respect to the percentage of survival in the acclimatization phase, the most effective response was obtained from microbulblets induced *in vitro* with 75 g L^{-1} of sucrose. In the case of *in vitro* seedlings, survival ranged between 39.50-49.62 % and for microbulblets between 90.70-94.95 %, the best values were achieved by the clones 'Criollo-9' and 'Martinez' with 94.95 and 93.56 %, respectively (17).

3.4. Somatic embryogenesis in garlic

Somatic embryogenesis allows the regeneration of complete plants, and it is widely used by biotechnology and embryology studies, its main advantage is the production of numerous seedlings from one cell and allows the evaluation and genetic improvement (8).

Studies on garlic showed that the highest average number of somatic embryos (173.71 per 200 mg of callus) was achieved after 8 weeks of culture in a culture medium containing a combination of 1.0 mg L^{-1} of 6-BAP and 0.25 mg L of 2,4-D. However, the number decreased with increasing concentration of 2,4-D. The study also indicated that reducing the level of 2,4-D in the culture medium helped to promote a higher number of somatic embryos during subculture. However, embryogenic callus growth with globular embryos decreased with increasing concentration of BAP (2.0 mg L^{-1} or more) and 2,4-D (1.0 mg L^{-1} or more) (58).

Somatic embryogenesis is nothing more than the formation of an embryo from one cell, without the need for gamete fusion. This technique has been used worldwide for

the mass production of plants by *in vitro* culture in some plant species such as carrot (*Daucus carota* L.) and alfalfa (*Medicago sativa* L.), despite the fact that the biology of the process is not known exactly. There are several steps for plant regeneration by this route, which are related to each other: induction and development of somatic embryos, proliferation, maturation, germination and conversion into plants. Generally, media with high concentrations of salts, sucrose or mannitol are used and the presence of an auxin, usually 2,4-D, is required for the initiation of embryogenic callus. Since embryo maturation and germination do not occur in the presence of this auxin, it must be removed or be used at low concentrations to allow development. In addition, both the induction of somatic embryogenesis and the development of subsequent stages depend on the presence of reduced nitrogen.

3.5. *in vitro* conservation of garlic germplasm

in vitro germplasm banks are sites for the conservation of genetic resources under controlled laboratory conditions and involve various *in vitro* cultivation and storage techniques. They seek to maximize the diversity of specimens collected from populations in the field or in their center of origin (56).

Their germplasm is conserved through field collections or biotechnological methods. The INIFAT Germplasm Bank has been working for the last 10 years on the conservation of garlic germplasm by reduction of the growth rate and currently has a collection of clones of interest, which are conserved, depending on the genotype, for cycles ranging from 9 months to one year (59).

3.5.1. Medium-term

In short-term storage, explants remain *in vitro* for up to 12 months, with management of the culture conditions to delay growth and increase the intervals between subcultures (60).

Results showed that at a temperature of 60 ºC, a reduction in the growth rate of the garlic crop was observed, since more time was required for the plants to reach a height of 5 cm, for bulbification to become evident and for profuse rooting to occur (3-5 roots). The effect of lowering the storage temperature on rooting was remarkable, as the time to reach rooting was approximately 5 times the time required at 24 ºC. The contamination observed was low, so it does not seem to be a limiting aspect. The culture medium content in the test tubes decreased by 25 % with respect to the initial one, so that the seedlings reached one year of preservation and approximately 75 % of the culture medium content was still present (61).

Other studies on *in vitro* preservation (62) reported that during the cultivation of *A. sativum* L. microbulbs in five *in vitro* preservation media stored at ¼ MS with 45 g L^{-1} sucrose (T4) showed survival percentages of 86.6, 76.6 and 73.3 % at 150, 180 and 210 days, respectively. Likewise, they registered the best vigor with average values of 1.16, 0.93 and 1.04 according to the established scale (1 = 75 % defined bulbs; 2 = 50 % defined bulbs; 3 = 25 % defined bulbs and 4 = absence of bulbs or undefined bulbs) at 150, 180 and 210 days of evaluation, respectively.

Other studies showed that, with respect to the bulbing index, all garlic plants stored at 24 ºC had bulbed at 4 months of storage. At 6 ºC, bulbing was not observed until 6 months and bulbing of all plants was observed at 10 months (63).

3.5.2. Long-term

Long-term storage is very safe and it is used extensively in agriculture, horticulture, and forestry for research and environmental monitoring. Long-term storage is considered a useful practice to avoid somaclonal variation in vegetatively propagated plants and allows keeping explants unaltered under these conditions (64).

Studies showed that shoot cultures of three garlic cultivars were maintained at various temperatures and media to maintain viability without subculturing (65). As a result, a high level of viability was recorded after 16 months of cultivation at 4 °C with 100 g L^{-1} sucrose in B-5 medium.

3.5.3. Cryopreservation

Cryopreservation allows the storage of living plant cells, tissues or organs at extremely low temperatures (-80 °C). This technique allows storage for prolonged periods (greater than one year) with the use of liquid nitrogen (NL), which is normally at -196 °C. On some occasions, NL is combined with other inert gases (such as helium and argon) (59).

In the case of garlic clones 'Castaño INTA', 'Sureño INTA' and 'Morado INTA', 96.7, 92.9 and 89.3 % of sprouted explants were obtained, respectively, when "clove" apices were cryopreserved using the frozen drop methodology (66).

Protocols for aerial bulblets and "tooth" apices were developed at IBONE (Institute of Botany of the Northeast), and the encapsulation-dehydration method with rapid and slow thermal descent was employed. Apices were encapsulated with 3 % calcium alginate. The capsules were pre-cultured in three solutions of increasing sucrose concentrations (0.5; 0.75; 1 M) under permanent agitation at 80 rpm. Dehydration was carried out with silica gel for 5 hours. Subsequently, the apices were placed in the programmed temperature lowering equipment and were placed in liquid nitrogen (24 hours). They were then thawed at 30 ºC for two minutes, rehydrated in liquid media with decreasing sucrose concentration (0.75 and 0.5 M) and recultured in modified Murashige and Skoog (1962) medium. This methodology allowed obtaining approximately 95 % regeneration for apices of "teeth" of 3 clones; however, aerial bulblets did not survive the previous treatments (67).

3.6. Techniques used to detect genetic variability in garlic seedlings obtained by *in vitro* culture

3.6.1. Morphological Techniques

In morphological studies, characters must be discovered and be delimited usually without any explicit criteria for selection or character coding, so they have the potential to be arbitrary. However, they have the advantage of allowing much more careful taxonomic sampling than molecular analyses, which is important for systematic reviews, studies of character evolution, and phylogenetic assessment (68).

Studies on garlic (63) showed that the clone 'UCLA-1' (group III) stood out from the rest of the materials for having the highest values for bulb and foliage characteristics. Clones 'AN-2' and 'AN-1' (group I), with identical electrophoretic profile, showed morphological differences, since 'AN-2' showed in comparison with 'AN-1', high averages for height and number of leaves per plant, as well as for bulb characteristics such as mass and diameter.

Regarding the characteristics related to plant foliage, cultivar 'PAL-1' presented the highest average for plant height, with a value of 59.2 cm followed by cultivars 'AN-1', 'BO-4' and 'AN-2'; while cultivar 'SAL-2' resulted with the lowest value for this foliage characteristic (63).

3.6.2. Cytogenetic techniques

Currently there are cytogenetic techniques such as FISH (fluorescence *in situ* hybridization) and GISH (genome *in situ* hybridization) in which dyes are used to mark different regions of the DNA. Both techniques are used to identify foreign chromosomes or to detect translocations (changes in the position of parts of the chromosome) in hybrids, which is not possible with classical cytogenetic techniques such as staining or banding (54).

Studies on the detection of somaclonal variation in garlic by cytological analysis examined the karyotypes of 75 regenerants derived from callus cultures of three parental garlic cloves and found that 9.3 % were tetraploid, 4 % aneuploid, and 2.6 % showed a change in the position of the secondary constriction. No association could be demonstrated between the rate of variation in molecular and cytological characters, either when comparing cultivars or examining individual regenerants (54).

Different concentrations and combinations of growth regulators (kinetin, indole-3-ylacetic acid, 2,4-dichlorophenoxyacetic acid) in plant tissue culture media at low concentrations have no effect on the induction of mitotic aberrations or inhibition of mitotic activity in *Allium sativum* L. meristem root tip cells. After studies, inhibition of mitotic activity, tendency to chromosome adhesion and agglutination, and a slight increase in the frequency of mitotic aberrations were observed at higher concentrations. Thus, it can be said that plant tissue culture media do not have a direct effect on the induction of mitotic aberrations in *in vitro* plant tissue cultures (69).

Studies conducted to validate the use of *Allium sativum* L. as a sensitive test model for genotoxicity evaluated the cytogenetic effects of a commercial formulation of the pyrethroid insecticide, cypermethrin, on root meristem cells of *A. sativum*. For the cytogenetic assay, root meristem cells were exposed to 1; 2; 4; 8 and 16 ppm (mg L^{-1}) of the test compound for 24 h, and either processed immediately for analysis or incubated in water for 24 h recovery and then processed. Cells analyzed immediately after exposure exhibited significant dose-dependent inhibition of mitotic index (MI) and induction of mitotic and chromosomal aberrations (MA and CA). The 24-h recovery period reduced the effect of the test compound on MI and the percentage of aberrations; however, cells exposed at 8 and 16 ppm showed a significant frequency of aberrations despite the recovery period. The data indicated that higher doses of cypermethrin produced toxicity, CA and MA in *A. sativum* L (70).

3.6.3. Isoenzymatic techniques

With the technological advances of the 1970s, the use of starch gels and histochemical staining of proteins, the existence of isoenzymes, multiple molecular forms within an organism that catalyze the same reaction, was demonstrated within an organism. The effect of an allelic modification can be detected with certainty, due to a change in electrophoretic mobility. This electrophoretic sensitivity has led to the technique revolutionizing genetic diversity studies in various species (54).

For these studies, several plant structures have been used, such as leaves, roots or flower buds, from which a crude protein extract is obtained. The technique consists of the separation of enzymes from the crude extract, on a permeable support (starch, PAGE) under the action of an electric field and followed by histochemical staining. Separation is performed by net electrical charge, molecular mass, isoelectric point and combination of these criteria (multidimensional separation). This separates enzymes encoded by different genes or products of different alleles of the same gene (54).

M tumefaciens according to the histochemical assay allowed detecting the activity of the GUS gene in the 30 clones obtained in the inoculation treatment I2, which consisted of drying the corns when exposed to the air of a laminar flow hood for 10 min. Then it immersed in the suspension for 5 min and placed on a sterilized paper towel, but with different magnitude. Forty-three percent of them (13 colonies) showed intense blue coloration, while in the rest the coloration was fainter (54).

Some authors evaluated the effects of an acute dose of γ-rays (10 Gy) to post-latent garlic cloves on shoot ingrowth and changes in peroxidases and soluble proteins up to 100 days of storage in the dark at 19±1 °C and 42±2 % relative humidity. Radiation-induced inhibition of shoot growth was evident after 25 days of treatment and was synchronous with a marked increase in peroxidase activity. Thin-layer isoelectric focusing revealed that radiation induced an increase in the number of anodic peroxidase isoenzymes at 100 days, suggesting modifications in the vascularization process. Neither soluble protein content

nor protein pattern was affected by irradiation. These results are discussed in terms of a possible mediating effect of peroxidase on radiation-induced shoot inhibition in garlic (71).

3.6.4. Molecular Techniques

Protein-based polymorphism has been very useful in plant research, but with the development of DNA-based technologies, research in this area has been favored with the availability of a larger number of markers, those based on Restriction Fragment Polymorphism (RFLP) and Polymerase Chain Reaction (PCR). Both methods have led to multiple techniques such as Random DNA Amplification (RAPD), Amplified DNA Polymorphic Fragments (AFLP), minisatellites (VNTR) and microsatellites (SSR), among others (72).

Studies on genetic diversity of garlic populations showed that DNA isolated from 71 samples analyzed by the AFLPTM technique detected a high level of polymorphism for the differentiation of the samples under study. It was expected that the genetic differences would not be so marked, because this is an obligate apomictic species and has required clonal reproduction for approximately 5000 years (73).

Studies performed with the AFLP technique showed 130 clearly separated bands. All quantified bands were monomorphic (100 %), so the phylogenetic distance was zero. The same number of bands was detected with each set of primers in a field-collected parental plant and in those subjected to three different culture treatments (MS medium, absolute control; MS medium + ANA (0.1 mg L^{-1}) + Kin (8 mg L^{-1}); MS medium supplemented with ANA (0.1 mg L^{-1}) + 2-iP (4 mg L^{-1}) in three subcultures, respectively. This analysis indicates that there were no differences at the molecular level between the *in vitro* seedlings regenerated in the different culture media, in the three subcultures that were performed and the field stock, for the primer combinations that were analyzed (72).

The techniques used to detect genetic variability in garlic seedlings obtained by *in vitro* culture are widely used worldwide with good results. With the use of these techniques, foreign chromosomes can be identified, translocations can be detected, and taxonomic sampling of plants is much more careful. In addition to detecting polymorphism levels from DNA-based technologies.

Other studies using the ASLSM2 primer combination produced 11 alleles (bands of different sizes) among the 25 garlic clones evaluated. On the other hand, the combination of ASLSM4 primers produced only one polymorphic allele, which was present only in garlic clones clustered in AFLP groups VII and X. Other primer combinations, ASLSM1 and ASLSM3, produced 7 and 5 polymorphic alleles, respectively (74).

The use of DNA-based molecular techniques has been widely used to detect the genetic integrity of germplasm banks or to detect the presence of genetic variation in materials subjected to mutagenic treatment, and therefore is considered a breakthrough in plant biotechnology.

CONCLUSIONS

Biotechnology allows, through tissue culture or *in vitro*, to obtain more resistant plants, free of pathogens and viruses, in short times and reduced spaces.

Micropropagation can be used with a conservation approach, since it allows obtaining numerous plants free of pathogens and viruses, in short times and reduced spaces. In addition, it allows the use of different growth biostimulators such as Biobras-6® and Pectimorf® to replace or complement traditional regulators.

Somatic embryogenesis, which allows the regeneration of complete plants, is widely used in biotechnology and embryology studies, its main advantage being the production of numerous plants from one cell.

The use of *in vitro* culture techniques allows the production of seedlings throughout the year and then subjecting them to the acclimatization phase where greater survival and development is achieved before moving to field conditions.

The use of high temperatures (40-75 °C), chemicals and electric current combined with meristem culture are effective techniques for the reduction or eradication of viruses present in the garlic crop, either in field conditions or at the laboratory level through tissue culture.

The techniques used to detect genetic variability in seedlings obtained by *in vitro* culture allow the detection of translocations and different levels of polymorphism, as well as the identification of foreign chromosomes.

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