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Review on the Application of Biotechnology in Garlic *(Allium Sativum)* Improvement

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Abstract: *Garlic (Allium sativum L. 2n=16), belongs to the family Alliaceae and genus Allium, which is the most important edible bulbous crops of the world. The aim this paper was to review recent efforts achieved towards garlic crop improvement through biotechnology. Both asexual and sexual method of reproduction is possible. However; commercial garlic cultivars only propagated through vegetative method. The increase of genetic variation through conventional crossing is nearly absent. The lack of sexuality in garlic limits the source of variability for breeding program of economically important traits, such as tolerance to biotic and abiotic stress, earliness, yield and quality. Biotechnological tools such as micro propagation, meristems culture (in order to obtain virus-free plants), somaclonal variation, and genetic transformation, have a great role for successful propagation, preservation, and breeding and for its subsequent production in garlic. Tissue culture has been applied to the establishment of germplasm banks in various parts of the world where valuable garlic collections are maintained for medium and long term (cryopreservation).Somaclonal variation and genetic engineering could play an important role by generating genetic variation for genetic improvement of garlic. The some achievements on herbicide resistance, insect and diseases resistance have been obtained by genetic transformation. However, the somaclonal variants and genetic transformation needs more investigations to identify the optimal explant type with appropriate culture condition. DNA markers such as RAPDs, AFLPs, SSR and DNA fingerprinting have been used for phylogenetic and taxonomic studies, germplasm conservation, detection of fertile genotypes, disease resistant genotypes of this spice, medicinal, and cash crops in the world as whole.*

Keywords: *Allium sativum (garlic), Genetic engineering, Molecular marker Tissue culture*

1. INTRODUCTION

Garlic (*Allium sativum L.* 2n=16), is one of the most important edible bulbous crops of the world, which belongs to the family Alliaceae and genus Allium. It is adiploid species of obligated apomixes, therefore its reproduction system is vegetative through its cloves. Garlic is the second most widely used of the cultivated *Allium* next to onion (Rubatzky and Yamaguchi, 1997).

Garlic is originated in the arid and semi-arid areas of Central Asia and spread to other parts of the world through trade and colonization (Etoh and Simon, 2002).Today, garlic is grown in temperate and tropical regions all over the world, and many varieties have been developed to suit different climates. Worldwide the area covered by garlic exceeds 1,199,929hectares with a production of 17,674,893 tones. In Ethiopia,16,411.19 ha of land was covered under garlic cultivation with a production of about 159,093.58 tones (CSA, 2014).

However; numerous crop management problem, the nature of propagation, diseases and insect pest limited the supply of production as required (Getachew and Asfaw, 2000).Biotechnological tools such as micro propagation, meristems culture (in order to obtain virus-free plants), somaclonal variation, and genetic transformation, have a great role for successful propagation, preservation, and breeding and for its subsequent production in garlic.

Therefore; the objective of this paper is to review recent efforts achieved towards garlic crop improvement through biotechnology.

2. IMPORTANCE OF GARLIC

Garlic is the most popular spices and used for seasoning in many foods as well as for economical, medicinal and religious purposes. It is mentioned in the Bible and Qur’an. It can be processed to

dehydrated chips, flakes, granules, and powder (Rabinowitch and Brewster, 1990). Steak houses invariably offer garlic bread, and garlic butter has many uses in home and restaurant cooking; every cook has a clove or two at the ready as well as dehydrated powder (Nonnecke, 1989).

It has high medicinal properties which a secret armory consists of more than 33 active sulfur-containing substances that do battle with enemies such as bacteria, viruses, and fungi. The major content in the garlic root is sulfur containing compound allicin (thio-2-propene-1-sulfinic acid S-allyl ester) (Tattelman, 2005). Allicin has the wide range of biological and pharmacological activities, such as anticoagulation, antihypertensive, antimicrobial, antibiotic, antiparasitic, antimycotic, antiviral, antitumoral, anti-oxidant, anti-aging, antiplatelet, detoxifies heavy metals, fibrinolusis, hypolipidaemic (lipid-lowering) and immune enhancer and modulator (Munchberg*et al.,* 2007).

In Ethiopia, it has diverse economic and dietary importance which is used as ingredients of local stew wot and has tremendous medicinal value (Metasebia and Shimelis, 1998). The crop is produced as a cash crop to earn foreign currency by exporting to Europe, the Middle East and USA. Being a cash crop in many parts of the country, increasing its productivity per unit area and production will enable farmers get encouraging returns and contribute its role in achieving the food security goals.

Now days it is started to produce by small and commercial growers for both local use and export (Getachew and Asfaw, 2000). Out of the total production, greater than 64% was used for household consumption and 22% was for market.Dehydrated garlic and extracts are also fast replacing fresh bulbs for industrial and home usage in the production of drugs, insecticides and explosives (Pursglove, 1992; kilgori et al., 2007).

3. Reproduction System of Garlic

Both asexual and sexual method of reproduction is possible. However; 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). 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 (Kamenetsky, 2007).

4. Constraints to the Production, Use & Improvement

4.1. Challenges of Breeding

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.

Moreover, vegetative propagation has various constraints for the crop:

- A low multiplication rate
- Expensive and short-term storage that requires wide spaces
- Transmission of phytopathogens (fungi, viruses, nematodes) through generations which can cause a yield decrease of up to 70%, and
- Loss of product quality (Kamenetsky, 2007).

4.2. Diseases and Pests

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). Some carlaviruses, like Common Latent Virus (GCLV) and Shallot Latent Virus (SLV) can also infect the garlic plant (Messiaen*et al.,* 2004).

One of the most widely spread diseases in garlic producing countries is white rot, caused by the fungus *Sclerotiumcepivorum,* 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 (DelgadilloSánchez, 2000). 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 that infest plants from early developmental stages and cause severe foliage damages. 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 (Ditylenchusdipsaci) causes the root knot disease, characterized by yellowing and rolling of leaves, as well as rotting of the bulb's base.

5. Overview of Biotechnology Application in Garlic (Allium Sativum) Improvement

Agricultural biotechnology is a collection of scientific techniques used to improve plants, animals and microorganisms. Biotechnology enhances breeders' ability to make improvements in crops that are not possible with traditional crossing of related species alone.

Biotechnological tools such as micropropagation, meristems culture (in order to obtain virus-free plants), somaclonalvariation, and genetic transformation, have contributed high to propagation, preservation and breeding of garlic (Herdt, 2006 and Fiserova*et al*., 2016).

5.1. Tissue Culture

5.1.1. Micro Propagation

Table1. *Overview in vitro propagation techniques used in garlic*

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

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

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

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

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

Although 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. Meristem culture enabled virus-free plants to be produced in various regions in the world. The highest percentage (100%) of virus-free plants was obtained when meristems of 3mm were cultivated on MS medium containing 50mg/L virazole through chemotherapy.

Four to five years would be necessary to obtain virus-free elite seeds that can be established in the field. The economic analysis indicated a net profit increase from 50.3 to 244.5% (depending on the genotype) for garlic seed producers (Xu*et al*., 2000).

Micropropagation can be carried out via two morphogenetic ways in garlic:

- Organogenesis, which results in the formation of organs (shoots or roots), and
- Somatic embryogenesis, which leads to the formation of structures having a similar or equal morphology to that of a zygotic embryo.

Morphogenetic ability solves the problem of garlic that callus grows older and the emergence of abnormal plants increases (Novak, 2009).

- *Organogenesis*

Meristem culture is a technique used for obtaining virus-free plants**,** and also for micro propagation. Regenerating garlic plants from meristems and shoot ,roots or bud formation from callus was achieved using a combination of 6-furfuryl amino purine (kinetin), indol-3-acetic acid (IAA) and 2, 4-diclorophenoxiacetic acid (2,4-D).

Garlic is propagated vegetatively, are known to harbor a number of viruses. Some of these viruses are latent, i.e., cause no visible symptoms, whereas others reduce yield considerably. However, meristem culture enables to eliminate viruses in garlic.

The yield of virus-free line was 76% higher than that of the field-propagated line. This technique also facilitates international exchange of disease-free germplasm. Regenerating garlic plants from meristems and shoot or bud formation from callus was achieved using kinetin, IAA and 2, 4-D (9.28µM, 11.4µM and 4.5µM) respectively (Messiaen*et al.,* 2004).

The application of this protocol enables the production of 256 plants from one shoot-tip in 10 months.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), 380 shoots could be produced starting from a single clove (Haque*et al.,* 1997). Thus, bulbs derived from root meristems were smaller than the ones derived from bud meristems. Approximately 75% of the regenerated plants established successfully when transferred to greenhouse.

- *Somatic Embryogenesis*

It is a formation of structures referred to asembryoids that 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) (Al-Zahim*et al.,* 1999).

By this method, 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 (Fereol*et al.,* 2005a).

5.1.2. Somaclonal Variation

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. (Peschke& Phillips, 1991).

Various experiments have been undertaken in garlic in order to generate somaclonal variants that could be used in its improvement. For example, Novak (1983) treated meristems 0.5-0.7mm with a solution of colchicine (3g/ L) 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. The cultivar Frankon is resistant to populations of the nematode Ditylenchusdispsaci found in Israel, however; 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.

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. It also helps to identify cultivar which possesses agronomicaldesirable traits there by grow commercially (Ex. variety Rosado in Argentina).

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*., 2012).

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). As Authors noticed that the technique is very crucial for improving alliicin content in garlic.

5.1.3. Conservation (Preservation)

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, as listed above, germplasm grown in field is exposed to environmental changes, pests and diseases that reduce its quality (Panis & Lambardi, 2006). Hence; 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).

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 (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). 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.

Xu*et al*. (2005) studied the behavior of six genotypes during their in vitro storage. Out of six only two genotypes could be stored for 25 months and had a survival percentage of 100%. They 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. In Germany, one of the biggest gene banks in Europe possesses a collection of 3039 accessions of Allium species including garlic. 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 in 1:1 ratio (Keller et al., 2006).

In the United States, investigations have been carried out the way that 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.

Cultures were incubated in darkness at 4°C for their conservation. Bulblets did 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 to12 and 18 months, while sucrose (0.1 or 0.2M) on bulblets of cultivar Seds 40 with survival rate of 100% after 18 months of maintaining cultures under these conditions. In general it solves problem of garlic sprouting (shown Fig below).

Fig2. *View of Stored garlic at 90 d. Sprouted heads are from short-shelf life cultivars. Heads with low number of sprouting bulbs are from 'Perla' (Bottom left corner)*

5.2. Genetic Engineering (Genetic Transformation)

Genetic engineering is the technique of removing, modifying or adding genes to a DNA molecule in order to change the information it contains.

A great variety of genetic transformation protocols have been developed, but this technology has not been applied with the same efficiency in every species. Even if, genetic engineering is highly required for the species of the genus Allium, particularly garlic, for which only a small number of achievement and publications are available. However; the main achievements on herbicide resistance, insect and disease resistance by gene transformation are discussed below.

5.2.1. Direct Method (Viabiolistic)

It was not until 1998 that a protocol for garlic transformation was reported for the first time.

- *Herbicide Resistance*

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 -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.

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 embryogeniccalli 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. Calli were bombarded with the plasmid pBI22.23 containing the hpt gene 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 embryogeniccalli derived from root tips. 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.

5.2.2. Indirect Method (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.

- *Insect Resistance*

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 Spodopteraexigua. The highest transformation frequency (1.47%) was achieved with the cultivar Printanor and the plasmid pPB34.

Southern hybridization showed that the cry Casquence was stably integrated into the garlic genome. 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. But none of the transgenic in vitro H04 garlic plants survived when transfered to GH. These plants were totally resistant to garlic beet army worm (*Spodopteraexigua)* in bioassays carried out in vitro.Thus providing goodpersepectives for the development of resistant garlic varieties.

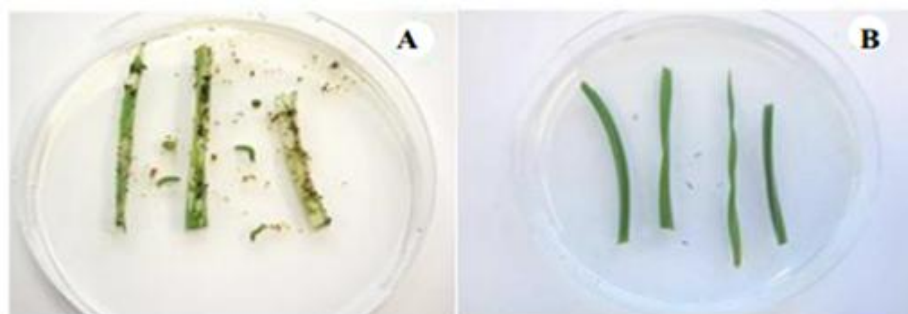

Fig3. *Transgenic Bt garlic. Transgenic cv. 'Printanor' transformed with pPB36 resistant to beet armyworm (right image) and non-transgenic cv. 'Printanor' (left image). From Zheng et al. (2004a), with kind permission of Springer Science and Business Media.*

*(***Source:** *Zhenget al., 2004)*

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.

- *For Diseases Resistance*

Khar*et al.* (2005) studied the transitory expression of the reporter gene gusA in two garlic cultivars after infecting them with aA. 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. Though regenerated transgenic plants through incorporated chitinase and glucanase genes for resistance to the fungus white root rote(Sclerotiumcepivorum)were not resisting as required, it delays the infection speed of the fungus (Eady et al., 2005).

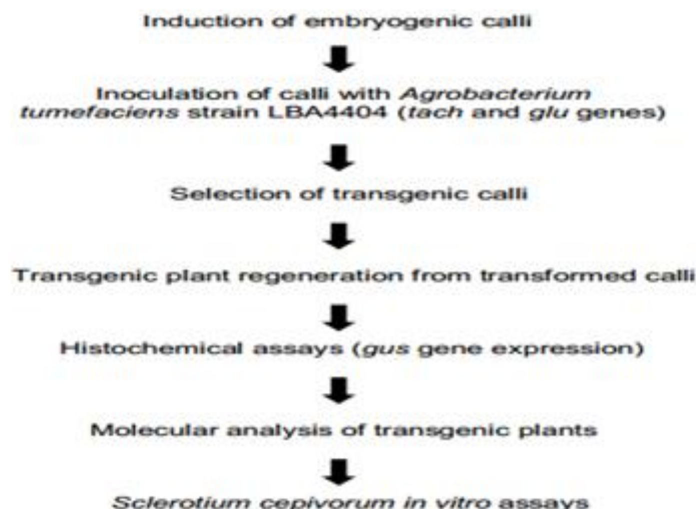

Fig1. *Genetic transformation protocol of garlic 'ABEN' (Allium sativum L.) with tobacco chitinase amd glucanase genes medicated by Agrobacterium tumefaciens.*

(**Source:** *Zhenget al., 2009*)

5.3. Molecular Marker Systems for Garlic

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, especiallyin 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.

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 (Abdoli*et al.,* 2009).

Germplasmexchange 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 for determination of fertile clones; disease resistance and clones producing S-amino acids (Ovesná et al., 2007 and Sánchez-Chiang & Jiménez, 2009).

6. Conclusion

Biotechnology has a great role for garlic crop improvement that help to overcome problems associated with vegetative propagation, the low multiplication rate and disease dispersion through tissue culture.

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). In addition; production of virus-free plants via meristem culture combined with thermotherapy and chemotherapy can reduce losses caused by phytopathogens. 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 also play an important role in the genetic improvement of garlic because they generate genetic variability. The some achievements on herbicide resistance, insect and diseases resistance have been obtained by genetic transformation. However, the somaclonal variants and genetic transformation needs more investigations to identify the optimal explant type with appropriate culture condition that enable formation of somaclones andto establish reproducible, efficient protocols by selecting of suitable target cells for inoculation with Agrobacterium or biolistics respectively.

Moreover, DNA markers such as RAPDs, AFLPs, SSR and DNA fingerprinting have been used forphylogenetic and taxonomic studies, germplasm conservation, detection of fertile genotypes, disease resistant genotypesof this spice, medicinal, and cash crops in the world as whole.

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