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Genetic transformation of garlic (*Allium sativum* L.) with tobacco chitinase and glucanase genes for tolerance to the fungus *Sclerotium cepivorum*

Erika Lagunes-Fortiz[1], Alejandrina Robledo-Paz[1]*, M. Alejandra Gutiérrez-Espinosa[1], José Oscar Mascorro-Gallardo[2] and Eduardo Espitia-Rangel[3]

[1]Colegio de Postgraduados, Km 36.5 Carretera México-Texcoco, 56230. Montecillo, Edo. de México, México.
[2]Departamento de Fitotecnia, Universidad Autónoma Chapingo, Km 38.5 Carretera México-Texcoco, 56230. Chapingo, Edo.de México, México.
[3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). Km.6.5. Carretera Celaya-San Miguel de Allende s/n, 38110. Celaya, Guanajuato, México.

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Garlic yield and quality have decreased due to white rot disease caused by *Sclerotium cepivorum* Berk. A transformation protocol to introduce tobacco chitinase and glucanase genes into garlic embryogenic calli using *Agrobacterium tumefaciens* has been established. LBA4404 strain having pC2301CHGLU plasmid with *TaCh, glu*, *gus* and *nptII* genes (coding for chitinase, glucanase, β-glucuronidase and neomycin phosphotransferase, respectively) was used. 30 putative transgenic clones were obtained from inoculated calli after six months. Histochemical assay revealed high *gus* activity in 43% of the clones. Molecular analysis of transgenic plants showed 92% of the clones carried *TaCh* gene. Eight culture media for plant regeneration from transgenic calli were evaluated; MTDZ-1 (thidiazuron 1 mg/l) medium induced the highest number of plants (38.4 plants). Transgenic plants were grown in the greenhouse and they developed normally. *S. cepivorum in vitro* bioassays showed 41 to 60% of mycelial invasion in the transgenic plants, and 80% in non-transgenic plants (control). Transformed plants were not completely resistant, but they showed a delay in fungal infection. This is the first report on the introduction of fungal resistance genes in garlic.

Key words: *Allium sativum, Agrobacterium tumefaciens*, *Sclerotium cepivorum*, glucanase, chitinase.

INTRODUCTION

Garlic (*Allium sativum* L.) is a monocotyledonous plant, cultivated for its medicinal and nutritional properties. Worldwide, garlic production has increased significantly during the last decade; thus between 1999 and 2010, the area of garlic cultivation grew by 23%, while garlic production increased by 65%, and reached 16,095,538 t, with 13.4 t average per hectare. China is the largest producer of garlic in the world, with 13,374,400 t per year (77% of the global garlic production), followed by India with 833, 970 t and Korea with 271,560 t (FAOSTAT, 2010). Although, Mexico is not among the major garlic producing countries, this crop represents an important source of jobs and foreign exchange, as more than 30% of its products are exported. White rot disease, caused by the fungus *Sclerotium cepivorum* is found in all the garlic producing areas, affecting considerably its quality and yield. Several strategies such as, use of fungicides, application of synthetic *Allium* oil, crop rotation, soil flooding,

*Corresponding author. E-mail: arobledo@colpos.mx. Tel: +52-595-9520200. Ext: 1581. Fax: +52-595-9520262.

addition of compost, solarization and mulching, and the use of antagonist have been implemented for fungus control (Ulacio-Osorio et al., 2003). The application of these methods has not been successful, due to the long viability (up to 20 years) and the large quantity of reproductive structures of the fungus (sclerotia) found in the soil (Coley-Smith et al., 1990; Delgadillo et al., 2002). Likewise, fungi are able to evolve to infect resistant plants throughout the gene recombination (Parlevliet, 2002).

Garlic is reproduced vegetatively and does not form true seeds, for this reason, there is no genetic recombination that would allow selection of resistant genotypes to the fungus (Rabinowitch and Brewster, 1990); therefore, genetic transformation is a feasible alternative for garlic improvement. Genetic engineering can produce plants that may exhibit long-lasting tolerance to one or multiple pathogens, through the ability to introduce genes from distantly or not related species (Wally and Punja, 2010).

There are a few reports of garlic transformation using biolistic particle delivery or mediated by *Agrobacterium tumefaciens*. Barandiaran et al. (1998) reported the transfer and expression of *uidA* gene into different garlic tissues of 'Morado de Cuenca' cultivar, only after endogenous nuclease activity was inhibited. Later, Ferrer et al. (2000) introduce by bombardment particle method, *uidA* and *bar* genes. They found maximum expression of *uidA* gen in calli and leaves. Kondo et al. (2000) were the first to use *A. tumefaciens* for the genetic transformation of garlic. They were able to develop a stable transformation system of garlic using highly regenerative calli.

Sawahel (2002) showed that biolistic transformation can lead to the expression and stable integration of a DNA fragment into immature cloves of 'Giza 3' cultivar. Park et al. (2002) obtained transgenic plants resistant to herbicide chlorsulfuron after bombarding calli of garlic 'Danyang' cultivar with *ALS* gene coding for acetolactate synthase.

Zheng et al. (2004) developed a reliable transformation system to produce garlic plants containing *Bt* resistance genes which conferred resistance to beet armyworm (*Spodoptera exigua*). Khar et al. (2005) studied the transitory expression of the reporter gene *gusA* in two garlic cultivars after infecting them with *A. tumefaciens.* Eady et al. (2005) recovered garlic transgenic plants from immature embryos using *A. tumefaciens* containing the vector pBIN *mgfp-ER* which includes the modified *gfp* reporter gene and the *nptII* selectable marker gene. Later, Kenel et al. (2010) developed a method for garlic transformation from immature leaves of cv Printanor using *A. tumefaciens* which contained the *mgfp*-ER reporter gene and *hpt* selectable gene. Regenerated transgenic plants survived in the glasshouse and matured into healthy plants.

Chitin and glucan are the main components of the cell wall of many plant fungal pathogens. The production of enzymes capable of degrading the cell walls of pathogens fungi is a component of the defense response of the plants (Ceasar and Ignacimuthu, 2012). For this reason, the introduction by genetic engineering, of genes coding for enzymes such as chitinases and glucanases capable of hydrolyzing the cell walls of these microorganisms, into the garlic genome, could confer tolerance or resistance to fungi (Punja, 2001). Several protocols for genetic transformation of garlic have been published (Park et al., 2002; Robledo-Paz et al., 2004; Zheng et al., 2004; Eady et al., 2005; Khar et al., 2005; Kenel et al., 2010), but a few have incorporated genes conferring agronomically important traits.

Therefore, the present paper aimed at developing a transformation protocol to obtain transgenic plants tolerant to *S. cepivorum* fungus. Genes coding for tobacco chitinase and glucanase were transferred into garlic embryogenic calli by *A. tumefaciens*. It was possible to regenerate transgenic plants which were able to express these transgenes and show higher tolerance to fungal infection. To our knowledge, this is the first report on the introduction of fungal resistance genes in garlic.

MATERIALS AND METHODS

A description of the protocol of garlic genetic transformation by *A. tumefaciens* strain to transfer genes coding for tobacco glucanase and chitinase is shown in Figure 1.

Induction of embryogenic calli

Garlic cloves 'ABEN' cultivar (white garlic) from Guanajuato, Mexico, were treated with a fungicide solution of Fungimycin 100 [18.1% (w/v) streptomycin and 2.0% (w/v) oxytetracycline] 1.0 g/l and Daconil [75% (w/v) chlorothalonil tetrachloro isothalonitrile] 1.0 g/l for 16 h. Then cloves were soaked with a chlorinated solution (1.8% active chlorine) for 20 min. Treated cloves were rinsed with sterilized distilled water. Disinfected cloves were placed in flasks with 250 ml of medium prepared with half the concentration of salts and vitamins of Murashige and Skoog (MS) culture medium (1962) supplemented with 10 g/l sucrose and 6 g/l agar (Merck, Darmstadt, Germany), pH 5.7 ± 0.1. Cloves were incubated at 24°C under 16 h of daily illumination (27 mmol/m^2/s). After three days, tips of the emerging roots (explants) were cut and cultured in a callus induction medium (CIM) containing N6 salts (Chu et al.,1975), MS vitamins, 20 g/l sucrose, 7 g/l agar and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.7 ± 0.1. Explants were incubated in complete darkness for 8 weeks.

Strain and plasmid

A. tumefaciens strain LBA4404 was used containing pC2301CHGLU plasmid (11621 base pairs) with tobacco (*Nicotiana tabacum*) *TaCh* and *glu* genes coding for a chitinase and a glucanase, respectively, *gus* reporter gene, *nptII* plant selection gene (conferring resistance to kanamycin) and rifampicin resistance gene. All genes were under control of the Cauliflower Mosaic Virus 35S promoter (CaMV 35S) (Figure 2).

Inoculation of calli with *A. tumefaciens*

A. tumefaciens strain LBA4404 was streaked out on YEP culture

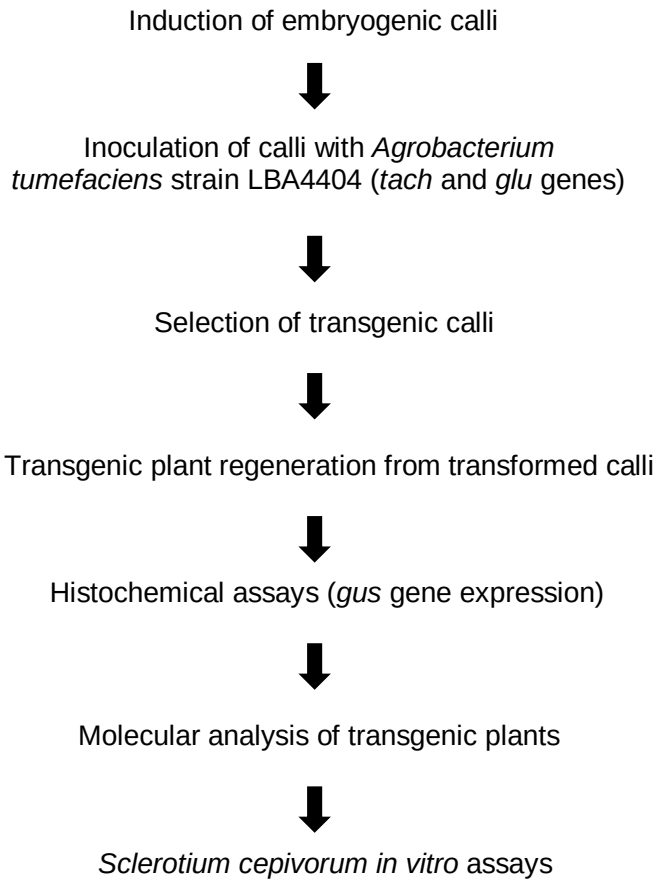

Figure 1. Genetic transformation protocol of garlic 'ABEN' (*Allium sativum* L.) with tobacco chitinase and glucanase genes mediated by *Agrobacterium tumefaciens*.

medium [10 g/l yeast extract, 14 g/l agar, 10 g/l peptone (DIFCO, Detroit, USA), supplemented with 5 g/l sodium chloride, 50 mg/l kanamycin and 50 mg/l rifampicin; pH 7.0 ± 0.1. Culture medium was sterilized for 15 min at 121°C. A single bacterial colony was collected from a plate and suspended into YEP liquid medium and incubated at 28°C for two days under constant shaking at 230 rpm, until it reached an optical density of 1.0 (OD_{600}).

Embryogenic calli were inoculated with *Agrobacterium* using three methods: 1): calli were soaked in the bacterial suspension for 5 min, then were placed on a sterilized paper towel to remove excess suspension; 2): calli were air-dried in a laminar flow cabinet for 10 min, soaked in the bacterial suspension for 5 min and placed on a sterile paper towel; 3): calli were pricked with a hypodermic needle and air-dried in the laminar flow cabinet for 10 min; they were immediately inoculated with 50 µl of the bacterial suspension. Ten Petri dishes with 300 mg fresh weight of callus were inoculated (per each method).

Selection of transgenic calli (clones)

Inoculated calli were placed on medium CIM and subcultured every four weeks on CIM fresh medium containing 100 mg/l kanamycin until non-inoculated calli (control) did not grow (20 weeks).

***Gus* assays**

Six month after the inoculation of calli with *A. tumefaciens*, a histochemical assay based on Jefferson et al. (1987) method was performed in order to evaluate *gus* gene expression. Sections of calli growing in kanamycin were placed in 50 µl reaction buffer containing 0.5 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and incubated for 24 h at 37°C. Blue spots were counted under a stereo microscope.

Molecular analysis of transgenic clones

Detection of *TaCh* gene in genomic DNA of regenerated plants was carried out by polymerase chain reaction (PCR). DNA was extracted from 40 mg of fresh transformed plants which displayed a positive reaction in GUS assay, and also from non-transformed plants, according to the protocol of McGarvey and Kaper (1991). Primers used to amplify the 800 bp fragment corresponding to chitinase gene (*TaCh*) were TaCh1 (5´-GGATCCCTAGCCTTGGGCGAAGTTC-3´) and TaCh2 (5´-TCTAGAATGGAGTTTTCTGGATCAC-3´). PCR reaction mix consisted of 5 µl 10X PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 1 U of Taq DNA polymerase, 2 µl of each primer (10 X), 2

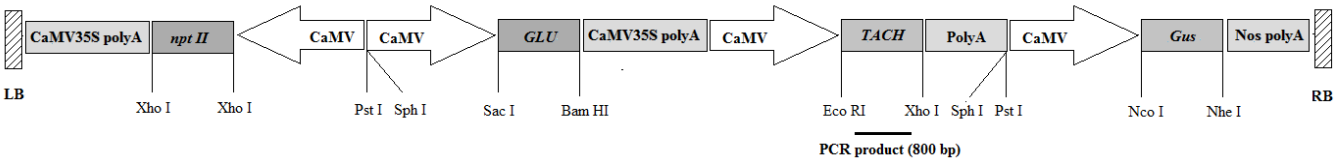

Figure 2. Representation of the T-DNA region of pC2301CHGLU and the product amplified by PCR. RB, right border; LB, left border; CaMV35S, 35s promoter of Cauliflower Mosaic Virus; PoliACaMV, terminator of Cauliflower Mosaic Virus; *glu* and *TaCh*, tobacco chitinase and glucanase, respectively; *nptII* gene, neomycin phosphotransferase; *gus* gene, β-glucuronidase; Nos polyA, terminator of Nopaline Synthase.

Table 1. Media used to induce plant regeneration from calli transformed with tobacco genes *TaCh* and *glu.*

Medium	Growth regulator	Supplement
M47-1	BA, 2 mg L^{-1}	Coconut milk, 100 ml L^{-1}
M47-2	GA_3, 1 mg L^{-1}	Coconut milk, 100 ml L^{-1}
M48-1	BA, 2 mg L^{-1}	Coconut milk, 150 ml L^{-1}
M48-2	GA_3, 1 mg L^{-1}	Coconut milk, 150 ml L^{-1}
MTDZ-1	TDZ, 1 mg L^{-1}	
MTDZ-2	TDZ, 2 mg L^{-1}	
MZEA-1	ZEA, 1 mg L^{-1}	
MZEA-2	ZEA, 2 mg L^{-1}	
MEB	IBA, 0.2 mg L^{-1}	

BA: N_6-benziladenine; GA_3: gibberellic acid; TDZ: thidiazuron; ZEA: zeatin; IBA, indolebutiric acid.

µl of ADN (0.1 µg/µl) and 38 µl of sterile distilled water. The amplification was performed on a MJ Research™ thermocycler with the following program: a cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s; a cycle at 72°C for 10 min, and finally 4°C. PCR products were analyzed on a 1% agarose gel, after a 60 min run at 100 V and staining with ethidium bromide (5 µg/ml) for 10 min.

Plant regeneration from transformed calli

Culture media tested for somatic embryos regeneration consisted of mineral salts and vitamins of MS medium, 30 g/l sucrose and 7 g/l agar, and different growth regulators and supplements (Table 1). Media was adjusted to pH 5.7 ± 0.1 and then sterilized at 121°C for 20 min.

Resistant kanamycin calli (1.5 g fresh weight) were placed into 250 ml flasks containing 30 ml of embryos regeneration media. After somatic embryos differentiation, they were transferred to MEB medium to induce shoot and root elongation (Table 1). The experiment was conducted in a complete randomized block design with three repetitions. The experimental unit consisted of a flask with 1.5 g of transgenic calli. Two months after, normal plants (NP, plants having color and shape of typical garlic plants), and vitrified plants (VP, embryos and microplants with a crystalline, hyperhydra-ted and translucent appearance) were evaluated.

Data were analyzed using GLM program of S. A. S. (Statistical Analysis System, 1999) Version 8. 0. by analysis of variance (ANOVA). Means comparison was done with Tukey test (α= 0.05).

S. cepivorum in vitro assays

Sclerotia collected from soils infested with *S. cepivorum* in Guanajuato, Mexico were soaked in a chlorinated solution (1.0% of active chloride) and rinsed three times in sterilized distilled water. Subsequently, they were dried on sterile paper towels and placed in Petri dishes containing ODA culture medium (200 g/l onion, 14 g/l dextrose, 18 g/l agar). Plates were incubated in dim light (15 µmol/m^2/s) 30 days after sclerotia were collected. Prior to the challenging bioassays of transgenic plantlets with the fungus *S. cepivorum*, trials were carried out in which transformed calli were challenged by fungal sclerotia. These trials consisted of placing five segments of transgenic callus to form a circle and in central part, the fungal sclerotia on a Petri dish containing PDA medium (Potato-Dextrose-Agar) (DIFCO, Detroit, MI, USA). Likewise, 5 cm long transgenic plantlets from independent lines (clones) were used in the bioassays with the fungus *S. cepivorum* according to the methodology proposed by He et al. (2008). A mature sclerotium was placed at the shoot base of a plantlet in a Petri dish with moistened filter paper and sealed in order to maintain 100% humidity. The Petri dishes were incubated at room temperature with a 10 h photoperiod (15 µmol/m^2/s). Six plants of five different clones and non-transformed plantlets were inoculated. Percentage of mycelial invasion was measured 10 days after inoculation. The data were analyzed by the GLM procedure of S. A. S. (Statistical Analysis System, 1999) software Version 8. 0., using the least significant difference test (LSD, 0.01) for means comparison.

RESULTS

Selection of transgenic calli (clones)

Only calli inoculated by method 2, which consisted of drying them for 10 min before immersing them into the

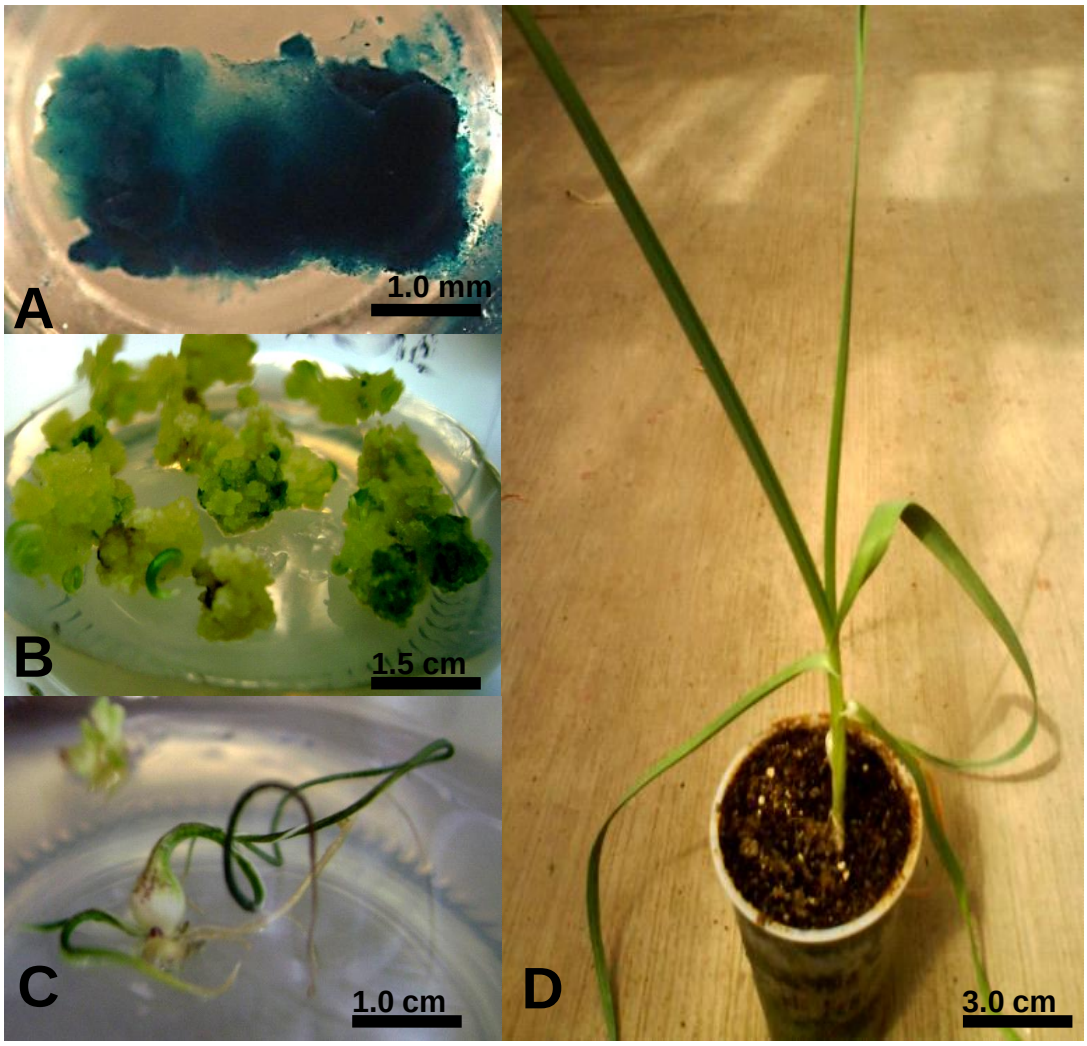

Figure 3. A) Expression of *gus* gene in six months calli after inoculation with *A. tumefaciens* containing pC2301CHGLU plasmid. B) Transgenic garlic *embryogenic calli* regenerating seedlings. C) Garlic microbulbs. D) Garlic transgenic plant conditioned in the greenhouse.

bacterial suspension for 5 min and eliminating the excess of it with a sterile paper towel, led to recovery of 30 kanamycin resistant cell colonies (clones) after six months of culture, due to the death of calli inoculated by methods 1 and 3 in selective medium with antibiotic (kanamycin). Resistant cells were yellow and had high rate of growth. On the other hand, non-transformated calli and those that were inoculated by methods 1 and 3 turned brown and soft and after four or five months of culture on selective medium, they died.

Gus assays

Histochemical assay was carried out six month after calli inoculation. Thirty clones expressed the *gus* gene at different magnitudes: 43% (13 clones) showed a uniform dark blue color (Figure 3A), while the rest of the clones had a more subdued color.

Plant regeneration from transformed calli

Statistical analysis showed significant differences among clones for the normal plants variable (NP), but not for culture media and the interaction between both. Vitrified plants were not different among clones, media and their interaction. Although 17 transgenic clones were exposed to different regeneration media, only five of them (30%) showed response to the different culture media (Figure 4). Clones 12, 9 and 15 produced the highest number of normal plants (60, 50 and 40 plants for each 1.5 g of callus, respectively). There was no statistical difference for the number of normal plants with regard to the culture media (Figure 5). When regenerated plants were cultivated on a medium with a high concentration of sucrose

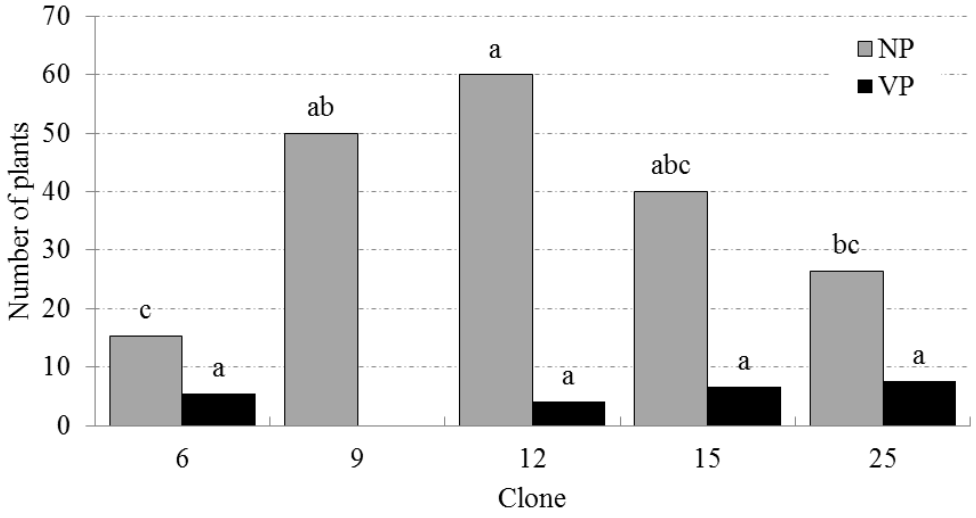

Figure 4. Average number of normal plants (NP) and vitrified plants (VP) formed on transgenic calli (clones) grown on eight different culture media. Bars with the same letters are not statistically different ($p \leq 0.05$).

(60 g/l) at 4°C for four weeks, followed by incubation at 26 ± 2°C (Figure 3B), these developed microbulbs (Figure 3C). Once microbulbs were transferred to soil (peat moss) and grown in the greenhouse, they resulted in plants with leaves similar in shape and color to those of non-transformed garlic plants (Figure 3D).

Molecular characterization of putatively transformed clones

12 out of the 13 putative transgenic clones (*gus*-positive) analyzed by PCR amplified a 800 bp fragment corresponding to *TaCh* gene (chitinase), which mean 92% of them carried this transgene (Figure 6).

S. cepivorum in vitro bioassays

20 days after testing with sclerotia, it was possible to observe that the entire surface of the culture medium was invaded by the mycelia and fungal sclerotia except in areas near the calli (Figure 7A and B). Furthermore, *in vitro* bioassays in which transgenic plantlets were challenged by fungal sclerotia showed significant differences in the extent of invasion of the mycelium. The percentage of mycelial invasion (PMI) of *S. cepivorum* varied from 41 to 60% in transgenic plantlets, whereas in non-transgenic plantlets (control), it was 80%. Plantlets of clones 6, 9 and 25 showed the lowest percentage of invasion by *S. cepivorum* (41.2, 43.6 and 49.3%, respectively) (Figure 8).

DISCUSSION

Fungal diseases cause substantial losses in many crop plants. The production of enzymes capable of degrading the wall cells of pathogen fungi is an important mechanism of the defense in plants. For this reason, the incurporation of genes coding for enzymes such as chitinases and glucanases which may degrade the wall of the fungi has been used to produce fungal resistant or tolerant transgenic plants (Mei et al., 2004; Moravcikova et al., 2007; Zhu et al., 2007; Sridevi et al., 2008).

Agrobacterium system is considered as the first option for plant transformation since it has several advantages over other transformation systems, such as: the stable integration of foreign DNA into the genomic DNA with little rearrangement of foreign DNA sequence, low incidence of silencing, a simple segregation pattern due to low number of copies, the ability to generate lines that lack the marker selection genes, and a low cost as it does not require sophisticated equipment for transgene transfer (Jones et al., 2005; Luo et al., 2004).

In this study, we developed a protocol to introduce tobacco chitinase and glucanase genes to garlic cultivar 'ABEN' by *A. tumefaciens*. Barandiaran et al. (1998) and Sawahel (2002) considered that the genome size (>1010 bp), the monocotyledonous nature of garlic (and other species of *Allium* genus) and the high nuclease activity in their tissues can interfere with the expression of the foreign DNA. However, in this study, easy and effective selection of putatively transgenic cells (clones) was possible when 100 mg/l of kanamycin were included in culture medium. The high proportion of transgenic clones obtained (10 per gram of fresh weight callus) in this work indicated that none of these factors prevented the incurporation of the transgenes into embryogenic cells of the garlic cultivar 'ABEN' and their stable expression. This transformation efficiency contrasts with that reported by Robledo-Paz et al. (2004), who obtained 2.2 transgenic

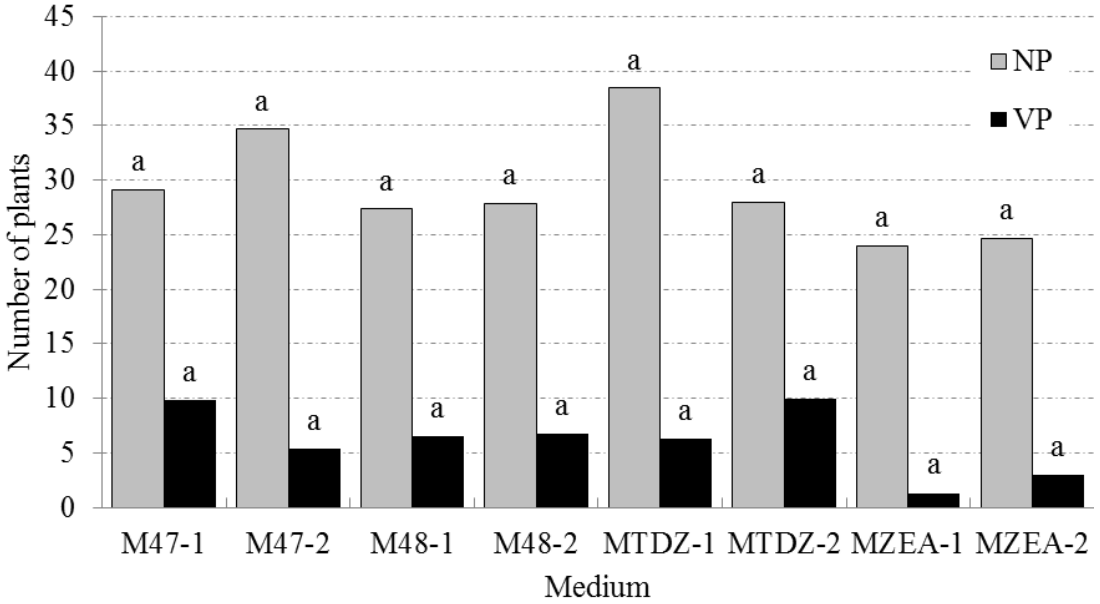

Figure 5. Average number of normal plants (NP) and vitrified plants (VP) formed by the transgenic clones of garlic cultivar "ABEN" grown on different culture media. Bars with the same letters are statistically equal ($p \leq 0.05$).

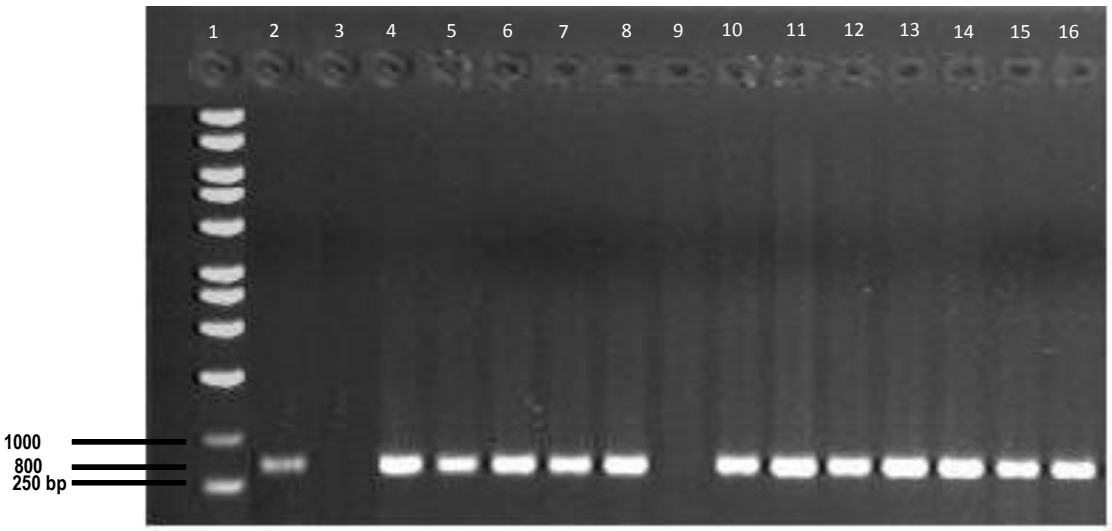

Figure 6. PCR analysis for the amplification of the *TaCh* gene (chitinase) corresponding to the 800 bp fragment. Lane 1: 1kb ladder; lane 2: DNA of the plasmid pC2301CHGLU (positive control); lane 3: negative control (non-transformed plant); lanes 4 to 16; clones 1, 2, 4, 6, 11, 14, 15, 19, 22, 24, 27, 28 and 30, respectively.

clones per gram of fresh weight callus after incorporating the genes *hph* and *gus* into the garlic cultivar "GT96-1" by the biolistic method.

Other garlic transformation protocols have been reported previously. Kondo et al. (2000) developed genetic transformation of garlic using *A. tumefaciens*. They obtained fifteen transgenic shoots per one thousand co-cultured calli. Likewise, Zheng et al. (2004) developed a reliable transformation system to produce garlic resistant plants to beet armyworm (*Spodoptera exigua*). The highest transformation frequency obtained in this paper was 1.47% (one callus line regenerated *in vitro* plants from 68 calli lines on selective medium). On the other hand, Eady et al. (2005) recovered garlic transgenic plants from imma-

Figure 7. Halos of growth inhibition of *S. cepivorum* in the presence of garlic transgenic calli cultivar 'ABEN' grown on PDA medium for 20 and 30 days (A and B, respectively).

mature embryos; a transformation frequency of approximately 0.06% was obtained (two events from 3200 embryos). Later, Kenel et al. (2010) developed a method for garlic transformation from immature leaves of cv Printanor which were inoculated with LBA4404 strain of *A. tumefaciens*; after 12 weeks, they recovered 79.7 transgenic lines from 2,376 garlic leaf pieces (2.3%). Although, these papers also reported stable transforma-tion, it is not possible to compare these transformation efficiencies with results of the present report, because the parameters of evaluation were different.

The transfer of T-DNA from *Agrobacterium* to the plant cells and its integration into the plant genome are influenced by factors such as genotype, explant, vectors, bacteria strain, addition of *vir*-gene inducing compounds (acetosyringone), desiccation of explants, elimination of *A. tumefaciens* after co-culture, etc. (Opabode, 2006).

In this study, embryogenic calli was infected with *Agrobacterium tumefaciens*, this was critical to achieve high frequency of genetic transformation in garlic. These cells had a high division rate that allowed them to duplicate or triplicate their biomass in a relatively short time (four weeks), so the cells that incorporated the transgenes were more likely to form new colonies (clones) in the presence of the selective agent. The role of the quality of the cell or tissue used as starting material for transformation has also been recognized by other authors (Birch, 1997; Myers and Simon, 1998; Robledo-Paz et al., 2004).

Likewise, embryogenic calli were desiccated before inoculation with *A. tumefaciens* and this might have had a positive effect on garlic 'ABEN' cultivar transformation, as it was observed in soya, rice and maize in which the transformation frequency was increased when explants were desiccated prior to or after inoculation (Chen and Fry, 2000; Urushibara et al., 2001; Cheng et al., 2003, 2004). Although, the molecular mechanism of desiccation remains unclear, Simon (1978) postulated that low humidity content causes discontinuity in the membrane, thus forming hydrophilic channels which facilitated the passage of water soluble substances to the dehydrated cells in the first moments of imbibition. Arencibia et al. (1998) suggested that the contact of the bacterial suspension with the dried plant cell during co-culture leads to the rehydration of the plant cells which facilitates the adherence of the bacteria to plant cells and their entrance into the intercellular spaces. In addition, desiccation suppresses the growth of *A. tumefaciens* (Opabode, 2006).

Some authors have included acetosyringone in co-culture medium for garlic transformation (Kondo et al., 2000; Zheng et al., 2004; Eady et al., 2005; Kenel et al., 2010), whereas in the present protocol, acetosyringone was not added to this medium. It was observed that the absence of acetosyringone in the co-culture medium did not prevent the infection of garlic cells by *Agrobacterium.* This indicates that although the addition of acetosyringone can enhance the bacterial ability to infect the plant cells, it is not indispensable for the transgene transfer.

Furthermore, Kondo et al. (2000), Zheng et al. (2004) and Eady et al. (2005) found that co-culture periods of 3, 4 and 6 days were optimal to allow genetic transformation of cells of garlic cv 'Fukuchi-howaito', 'Messidrome', 'Morasol' and 'Printanor'. However, in the present report, only two days were required to obtain this response without overgrowth of *Agrobacterium* which could have a negative effect on the survival of the inoculated cells.

Differences in the regeneration ability of transformed calli (clones) were observed in the present work. These differences can be explained by the fact that each clone arose from a different transformation event. In this regard, Luciany et al. (2006) considered that the most important factors that affect plant regeneration are: the

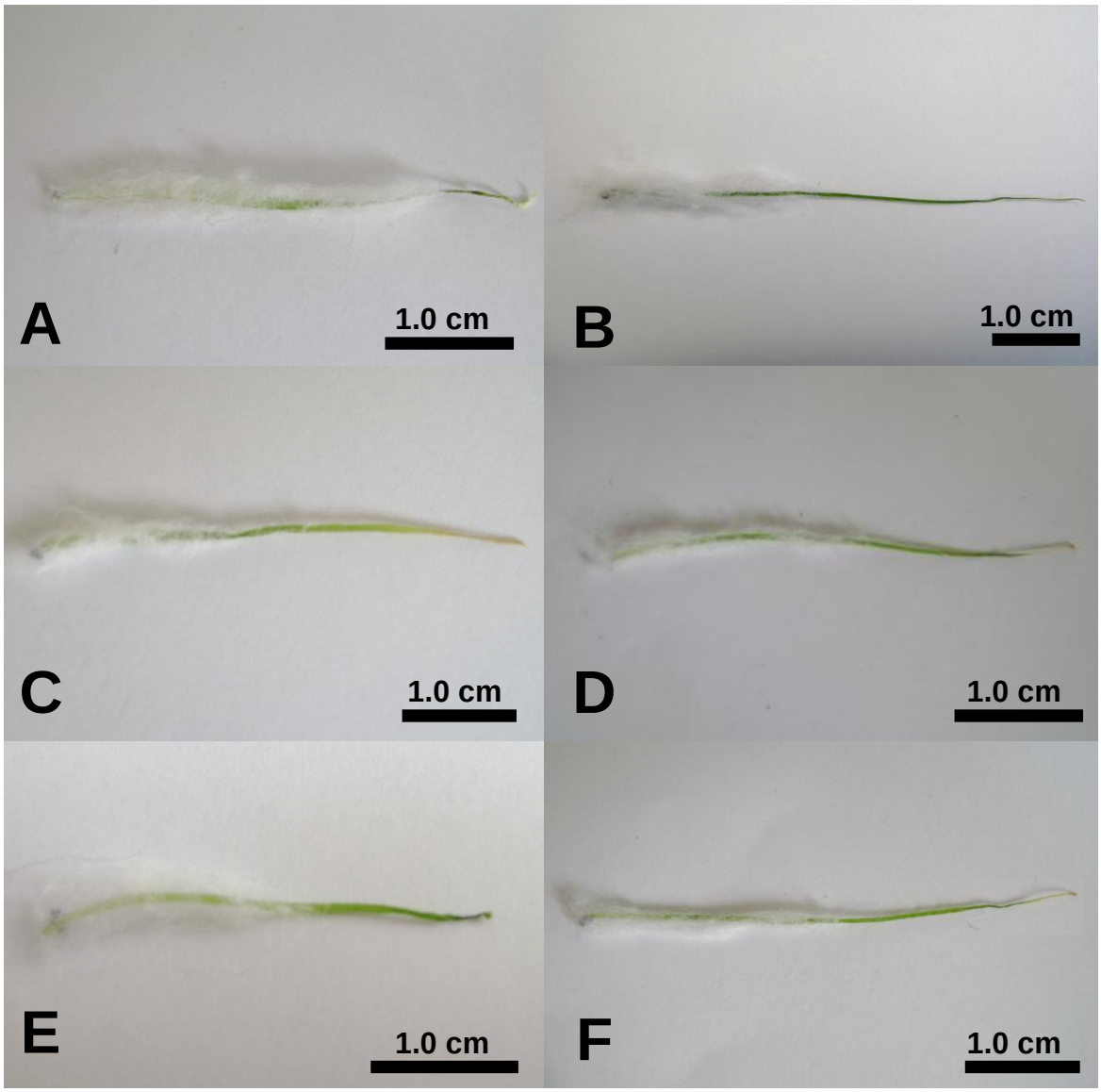

Figure 8. Response of transgenic plantlets tested by the fungal *S. cepivorum,* 10 days after inoculation. A) Control (80% PMI), B) clone 6 (41% PMI), C) clone 9 (44% PMI), D) clone 12 (53% PMI), E) clone 15 (60% PMI), F) clone 25 (50% PMI).

physical condition of the explant, the genotype and the combination of growth regulators in the culture medium. Buitveld et al. (1994) evaluated the plant regeneration of eight leek lines of friable callus and although they observed somatic embryogenesis in all of them, only six regenerated plants.

Although there was no statistical difference for the normal plants with regard to the culture media, MTDZ-1 medium which contained TDZ (1 mg/l) induced the higher number of plants. These results confirm the findings of Murthy et al. (1998) who found that TDZ has a high level of activity at relatively low concentrations (1 and 2 mg/l), and that the exposure of tissues to this compound for relatively short period is enough to stimulate regeneration, which distinguish it from other natural or synthetic regulators. In contrast, other authors reported that TDZ in concentrations of 0.002 to 0.01 mg/l did not promote shoot regeneration in other garlic genotypes (MIV, PI383819, Piacenza, Creole Red and RAL27) (Myers and Simon, 1999).

The use of plasmids which include two or more genes coding for different hydrolytic enzymes has shown higher level of resistance than expression of either gene alone. Beside, this combination reduces the probability of the emergence of resistance-breaking pathogen strains of phythopathogenic fungi, becoming resistant against a

broad range of pathogens (Sela-Buurlage et al., 1993; Jach et al., 1995; Ceasar and Ignacimuthu, 2012).

Moravcikova et al. (2007) co-expressed class I glucanase and class III chitinase in potato plants using plasmid pJL06. Crude protein extracts isolated from transgenic tubers showed growth inhibition of *Rhizoctonia solani*. Wally et al. (2009) introduced acidic wheat class IV chitinase and acidic wheat β-1,3-glucanase genes along with rice cationic peroxidase (*POCI*) gene into carrot genome. Transgenic plants harboring *POCI* alone or in combination with chitinase showed higher resistance to fungal disease. Likewise, transgenic rice plants resistant to rice blast disease were obtained after transfer of two chitinase genes (*RCH10* and *RAC22*) from rice, a glucanase gene (β-*Glu*) from alfalfa and ribosome inactivating protein gene (*B-RIP*) from barley. Also, Sridevi et al. (2008) introduced a rice chitinase (*chi 11*) and tobacco β-1,3-glucanase (*glu*) genes and developed sheath blight resistant transgenic plants. The fungal growth inhibition zones observed when transformed calli of garlic 'ABEN' cultivar were tested by the fungal sclerotia suggests the production and secretion of hydrolytic enzymes (glucanases and chitinases) from the calli, which are the result of the expression of the transgenes introduced into the garlic cells.

Moreover, although garlic cultivar 'ABEN' transformed plantlets were not totally resistant to fungal invasion (since after 20 days, fungal mycelium completely invaded the plantlets), they exhibited a delayed invasion by *S. cepivorum* under the conditions of this bioassay. This delay in fungal infection could increase the tolerance of garlic transgenic plants to this pathogen in advanced stages and reduce crop losses; however, more studies are needed to confirm this hypothesis.

The present protocol is more efficient than other transformation systems as it allows the recovery of a higher number of transgenic clones (Robledo-Paz et al., 2004). Also, it is simple because it does not need inducers (acetosyringone) in co-culture medium, like in other protocols (Kondo et al., 2000; Zheng et al., 2004; Eady et al., 2005; Kenel et al., 2010). Besides, the cells did not need to be pretreated with osmotic agents to increase the transformation efficiency (Uze et al., 1997; Varalaxmi et al., 2013).

Conclusions

In summary, in this research, *A. tumefaciens*-mediated transformation system was developed which allowed the incorporation of tobacco chitinase and glucanase genes into garlic cultivar 'ABEN'. This is the first report on the introduction of fungal resistance genes in garlic. Transformed plants were not completely resistant, but they showed a delay in fungal infection, therefore, *Agrobacterium*-mediated transformation protocol reported here could be a promising method to increase white root disease tolerance and to reduce crop losses.

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