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# Before-after analysis of genetic and epigenetic markers in garlic: A 13-year experiment

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ABSTRACT

*Allium sativum* is an important crop species in Argentina, one of the major garlic exporting countries in the world. Because garlic is an agamic propagated species, its breeding is based on the occurrence of plants with desirable traits, which are attributed to genetic mutations. In this work, we took advantage of a long term field experiment in a garlic line destined to genetic improvement to evaluate bulb weight and genetic and epigenetic stability and dynamics over 13 years. Average bulb weight increased from 65.3 g in the first year to 84.8 g the 13th year. Using AFLP (Amplified Fragment Length Polymorphism) and MSAP (Methylation Sensitive Amplified Polymorphism) techniques, we assessed genetic and epigenetic stability in plants of *A. sativum* taken at first, third and 13th year of field culture. We detected that 82.47 and 19.61% of the AFLP and MSAP fragments were present in the tree years of sampling. The application of selection pressure led to a reduction in genetic polymorphism and an increase of bulb weight, but did not influence epigenetic polymorphism, indicating that it is independent of genetic variability. We detected changes in the amount of each methylation pattern (unmethylated, hemimethylated and internally methylated) among the different years analyzed while 82.47 and 19.61% of the genetic and epigenetical loci were stable during the time of culture. Although some epiloci showed stability along the 13 years of culture, others presented gradual variation, while others were polymorphic within samples from the same year of culture. Finally, we discussed the implications of the high epigenetic variability of an agamic propagated species and its possible effect on the phenotype.

## 1. Introduction

Garlic (*Allium sativum* L.) is a widely cultivated crop species used as a spice and with medicinal purposes since ancient times. Argentina is one of the major garlic exporting countries and most of its production is carried out with monoclonal cultivars (García-Lampasona et al. 2012), which are expected to present no genetic variability given that all plants of a cultivar descend from a single plant. However, there is limited genetic variability due to mutations that enables garlic genetic improvement. This is a time consuming process because new monoclonal cultivars are obtained selecting plants/bulbs with distinguishable and better traits than the original population and the occurrence of differential traits is seldom (Burba 1993). Garlic breeding centers on the size of the bulb, as it is one of the fundamental characteristics for its commercialization.

It has been proved in different species that the phenotype is influenced by epigenetic marks (Cubas et al. 1999; Manning et al. 2006; Aversano et al. 2011; Dyachenko et al. 2014; Xia et al. 2016; Seymour and Becker 2017). In populations with low genetic variability, epigenetics can act as an important source of phenotypic variability (Róis et al. 2013). In addition, it is presumed that epigenetic contribution has higher potential in the adaptation of asexual species, since epigenetic differences accumulate easily (Verhoeven and Preite 2013). Knowing that garlic cultivars show wide phenotypic plasticity dependent on environmental factors–such as soil type, meteorological conditions, latitude, altitude, and cultural practices–(Volk et al., 2009; Hoogerheide et al. 2017) it is expected that epigenetic mechanisms exert some influence on garlic phenotype.

Many studies reporting genetic variability in garlic cultivars (Azuara Hernández et al. 2008; Chen et al. 2013; Chen et al. 2014; García Lampasona et al. 2003; Paredes et al. 2008; García-Lampasona et al. 2012; Gimenez et al. 2016; Manzum et al. 2014; Liu et al. 2015; Wang

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et al. 2016; Egea et al. 2017; Hoogerheide et al. 2017) proved that among various molecular markers AFLP is an excellent technique for detecting this variability when information on the genome is limited. It is known that garlic cultivars display a wide genetic, physiological and phenotypic variability; however, genetic variability has not been studied over time. In this sense, having a more comprehensive knowledge of the sources of genetic and epigenetic variation and the stability of markers over generations is crucial for germplasm utilization in garlic breeding. Here we took advantage of a long term field experiment in a garlic line destined for improvement and evaluated bulb weight and genetic and epigenetic status over 13 years of field culture with the goal of understand phenotypic, genetic and epigenetic stability over generations.

## 2. Material and methods

Plant materials used in this work come from a garlic genetic line destined for improvement. Plant cultivation was carried out at the Experimental Station La Consulta, INTA (National Institute of Agricultural Technology) 33° 42.7′ S and 69° 04.4′ W; 950 m.a.s.l. The experimental station is located in the Uco Valley, east of Los Andes mountains, and in an arid zone of Argentina characterized by low rainfall (200 mm per year), rigorous winters (-7 °C min – 25 °C max) and hot summers (7 °C min- 35 °C max).

Plants were grown in an alluvial soil, deep fine sandy loam Typic Torrifluvent (La Consulta series). This soil have values for medium total nitrogen content (709 mg/kg), medium to high for available phosphorus level (6 mg/kg), high exchangeable potash level (360 mg/kg), alkaline pH (7.8) and 1.25% organic matter. Clay content is about 8%; 30% is silt and 62% is sand, and almost 40% of sand was smaller than 250 µm. Water field capacity is 17% by weight, permanent wilting point is 9.4% by weight and bulk density is 1.35 kg/dm$^3$ (Lipinski and Gaviola 2011).

The crop was grown and cultured in the field according to the standard agronomic practices used by the growers at Experimental Station La Consulta INTA. In the mid-summer cloves weighing 4 g approximately were planted at 10 cm clove-clove distance in lines separated by 60 cm on field fertilized with animal manure at 1 kg/m2. The crop was irrigated every 10 days in winter and every five days in autumn and spring. Plants were harvested at the end of spring and were placed in a horizontal dryer. After 45 days, the bulbs were separated from the leaves, cleaned, and its weight was registered. Taking advantage of a long term experiment in which the weight of 100–300 bulbs was measured annually, we analyzed the data from years 1, 3, 5, 7, 10 and 13 with Kruskal Wallis using Infostat/P (Di Rienzo et al., 2014). Average minimum and maximum temperatures at EEA La Consulta were calculated for each month during the culture of garlic.

Fully expanded leaves of garlic plants were collected six months after cloves plantation in three different years named Y1, Y3 and Y13, corresponding to the first, third, and thirteenth year of culture. Three biological replicates were analyzed each year. DNA extraction was performed following Murray and Thompson (1980) protocol. The AFLP (Amplified Fragment Length Polymorphism) technique was performed following the general steps described by Vos et al. (1995) using six primer combinations (Suppl table). Only the reproducible amplification products from the triplicate reactions were scored. The MSAP (Methylation Sensitive Amplified Polymorphism) technique was conducted following the general steps of Xiong et al. (1999) using six primer combinations (Suppl table). Two isoschizomeric methylation-sensitive enzymes, *Hpa*II and *Msp*I, in combination with *EcoR*I were used to assess methylation in CCGG sequences. These enzymes have different sensitivity to certain methylation pattern of cytosines. *Hpa*II will not cut if either of the cytosines is fully (double-strand) methylated, but will cut if external cytosine is hemimethylated (single strand). While *Msp*I will not digest if the external cytosine is fully or hemi (single strand)-methylated, but it will digest if the internal cytosine is fully methylated. Based on the absence or presence of a band, cytosine methylation patterns were classified as unmethylated, hemimethylated, and internally methylated. AFLP and MSAP products were denatured at 90 °C in 4 µl of loading buffer, resolved by polyacrylamide gel (6%) electrophoresis at 85 W for 150 min, and visualized by silver staining.

Fragments from the AFLP and MSAP techniques were scored into a binary character matrix indicating presence (1) or absence (0). Only fragments within the 200–600 bp range were scored. A methylation status matrix was built from the *Hpa*II and *Msp*I datasets, being assigned into four categories according to the methylation pattern as follows: “1” when fragments are present in both *Hpa*II and *Msp*I (unmethylated sites); “2” fragments only present in *Hpa*II lane (hemimethylated CHG sites); “3” fragments only present in *Msp*I lane (full methylated CG sites) and “0” lack of fragment in both lanes (fully methylated 5´-CCGG sites or absence of the site) (Xu et al., 2004). The methylation status matrix was transformed into a methylation binary matrix, generating one line (or locus) for each methylation status and detailing only the presence (1) or absence (0) of the specific status. Based on MSAP data, number of methylated, hemimethylated, and unmethylated loci were determined for each year of culture. ANOVA was performed to compare the methylation patterns between years using Infostat software (Di Rienzo et al., 2014). For Venn diagram analysis we estimated the loci with fragment presence for each year (Y1, Y3 and Y13) and then performed pairwise comparisons to calculate the fragment shared by all three years, the fragments present in two years, and fragments exclusive from one year.

## 3. Results

Experimental Station INTA La Consulta has a garlic breeding program and an active germplasm bank that maintains cloves/seeds of different garlic cultivars obtained from several countries of the world. Over 13 years of evaluation, garlic plants traits showed progressive changes in size, clove and leaf number, and, particularly, in bulb weight (Fig. 1). In the 1st year, bulbs weighted 65 g in average and display a large dispersion with bulbs weighing between 40 and 82 g. The third year showed less dispersion and bulb weight sharply diminished to 47 g. In the fifth and seventh year the dispersion was reduced by a progressive increase in the average bulb weight to values of 82.2 g. By the 13th year the average weight increased to 84.8 g, and showed less dispersion (bulb size ranged between 76 and 102 g). The time in which bulbification begins depends, first, on plant exposure to low temperatures during the first months of culture (March-August) and then, on the

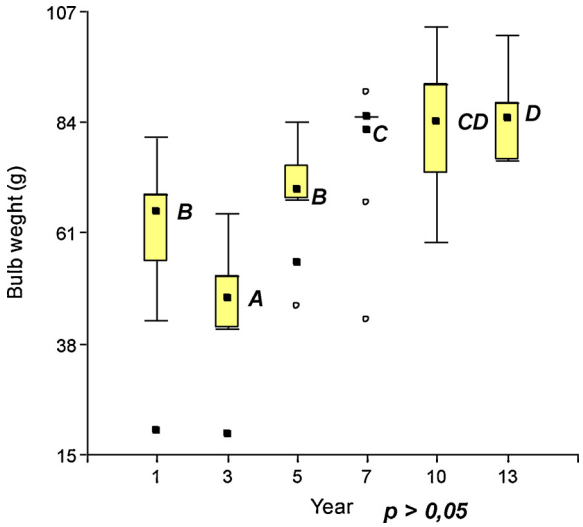


**Fig. 1.** Weight of bulbs harvested in year 1, 3, 5, 7, 10 and 13. Bars with the same letter are not significantly different.

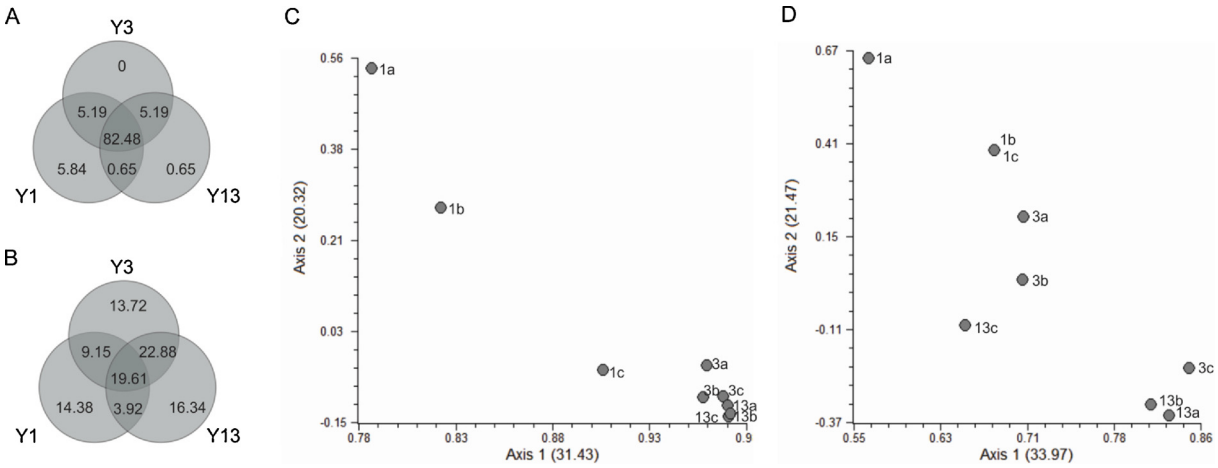


**Fig. 2.** Venn diagrams showing the distribution of AFLP (A) and MSAP (B) fragments in garlic samples taken at first, third and 13th year (Y1, Y3 and Y13) of field culture. Principal coordinate analysis of Dice genetic (C) and epigenetic (D) distance showing distribution of garlic samples taken in first year (1a, 1b and 1c) third year (3a, 3b and 3c) and 13th year (13a 13b and 13c).

hours of light. Hence we analyzed average minimum and maximum temperatures during the months of garlic culture (Suppl. Figure). In August of the 7th year, the maximum temperature was the highest registered reaching 30 °C, 8.7 °C higher than in other years. In July of the 10th year and June of the 13th year minimum temperatures were 4 °C lower than other years reaching −11 °C.

Taking advantage of DNA samples of garlic leaves obtained in the first, third, and 13th year of evaluation, we performed a genetic and epigenetic characterization. Employing six primers combinations for both AFLP and MSAP assays, we detected 154 and 212 loci, respectively. We found 82.47% fragments shared among Y1, Y3 and Y13 samples. The genetic similarity was lower than expected for a clonally propagated species because there was high genetic polymorphism (69.48% for the whole data set). This high polymorphism was mainly attributed to the high genetic polymorphism found in Y1 samples (64.82%), which was also manifested in the Dice genetic distance (Fig. 2C). Y3 and Y13 samples showed lower genetic polymorphism (20.27 and 7.29% respectively), in agreement to the Dice genetic distance results (Fig. 2C). AFLP Venn diagram analysis (Fig. 2A) showed that restriction sites were highly dynamic over time: 5.85% fragments were only present in Y1 samples and were lost before the third year of culture; 5.19% fragments were shared among Y1 and Y3 samples, but were lost before the 13th year; 5.19% were gained after the first year of culture and shared among Y3 and Y13 samples, and only 0.65% fragments were new in Y13 samples. The genetic polymorphism detected throughout the AFLP assays showed certain degree of instability which could be classified in four classes: 1) stable loci (82.48%, Fig. 2A), 2) gradually variable loci (5.19 + 5.19% fragments), 3) annually variable monomorphic loci (0.65% for Y1 and 0% for Y3 and Y13), and 4) annually variable polymorphic loci (5.19% and 0.65% for Y1 and Y13). Beyond all genetic changes there is 83.13% of AFLP fragment shared (82.48 + 0.65) among Y1 and Y13 samples.

Regarding the MSAP assay, we detected 19.61% fragments shared among Y1, Y3 and Y13 samples (Fig. 2B) and high epigenetic polymorphism (69.81%). In contrast to the genetic polymorphism, epigenetic polymorphism was high for samples taken in the three different years with values of 72.22, 82 and 76.04% for Y1, Y3 and Y13, respectively. This high epigenetic polymorphism is manifested in the Dice epigenetic distance as samples arranged randomly with no apparent pattern (Fig. 2D). MSAP Venn diagram analysis (Fig. 2B) showed that 14.38% fragments present in Y1 samples were lost before the third year of culture; 9.15% fragments were shared among Y1 and Y3 samples but were lost before the 13th year of culture; 13.72% fragments were unique of Y3 samples, being gained after the first year of culture and lost before the 13th; 22.88% fragments were shared among Y3 and Y13 samples, being acquired after the first year and remained until the 13th. As in the AFLP assay, MSAP fragments found some variability, which could be classified in four classes: 1) stable loci along culture (19.61%, Fig. 2B), 2) gradually variable loci (9.15 + 22.88% fragments), 3) annually variable monomorphic loci (1.31, 0.65 and 0.65% fragments for Y1, Y3 and Y13 samples) and 4) annually variable polymorphic loci (13.07, 13.07 and 15.69% fragments for Y1, Y3 and Y13 samples respectively). Finally, there was a large epigenetic variation, with only 23.53% (19.61 + 3.92%) fragments shared among Y1 and Y13 samples.

This epigenetic variation is the result of changes in methylation patterns. From the first to the third year, we detected an increase of internally methylated and hemimethylated sites and a decrease of unmethylated sites (Fig. 3). From the third to 13th year, we found a decrease in internally methylated and hemimethylated sites and an increase in unmethylated sites. Regarding the three methylation patterns distinguishable by MSAP assays, internal CG methylation was the most abundant in all years, hemimethylated was the second, and unmethylated was the least in abundance, except for Y13 samples. However, no significant statistic differences were found.

## 4. Discussion

Clonally propagated organisms repeatedly produce genetically identical individuals; however, in *A. sativum* there is some extent of stochastic genetic variation that eventually gives rise to a garlic plant

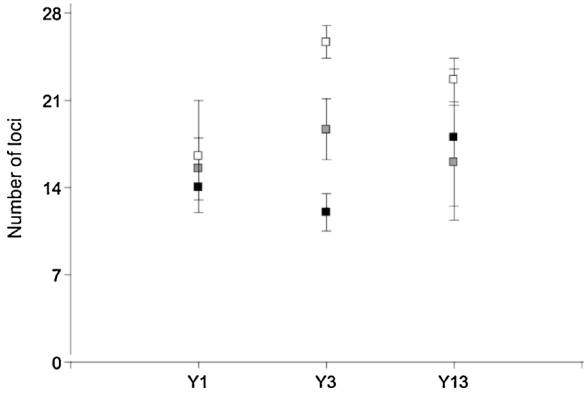


**Fig. 3.** Average number of loci with unmethylated (black), hemimethylated (grey) and internal methylated (white) CCGG in garlic plants at year 1, 3 and 13 (Y1, Y3 and Y13) of field culture.

with outstanding traits and it is selected for crop improvement. In addition, garlic displays high phenotypic plasticity in response to the environment that might arise from epigenetic variation. Therefore, we considered that a crucial step in using genetic and epigenetic information for crop improvement is to understand the stability of genetic and epigenetic patterns over generations. In this work, we evaluated a garlic line along 13 years of controlled culture using AFLP and MSAP markers.

For an epigenetic characterization it is important to assess the stability of the environmental conditions given that drought and heat are stress factors that can alter DNA methylation in plants (Saraswat et al. 2017). The plants evaluated in this study were cultured in the same field with controlled irrigation that is suspended in case of rain. The main variation in culture conditions could be due to changes in temperature. However, average minimum and maximum temperatures were regular and the overall culture conditions were homogeneous throughout the 13 years of analysis (Suppl. Figure). Therefore, there were no great environmental changes that could give rise to major genetic or epigenetic variation.

The garlic line assessed showed a significant increase in bulb weight over 13 years of culture (Fig. 1) and a decrease in genetic variability (Fig. 2C). Particularly striking is the high genetic polymorphism present in Y1 samples (64.48%) and the decrease in polymorphism by the 3rd and 13th years (20.27 and 7.29%). This indicates that the selection of larger bulbs to cultivate the following year led to a reduction of genetic polymorphism obtaining plants with an overcoming trait, which is strongly supported by the large dispersion in bulb weight in the first year and a reduction in dispersion by the 13th year,- outstanding positive correlation between bulb weight variance and genetic variance. The genetic polymorphism detected in the first and third year are consistent with the values of populations composed of many genetic lines (Buso et al. 2008; García-Lampasona et al. 2012; Wang et al. 2016), while in the 13th year the polymorphism was reduced to 7.29%. If plants descended from a single bulb, the expected genetic polymorphism would be 0; however, 7.29% may indicate that all Y13 plants analyzed descend from a single bulb since it is similar to the value registered in a garlic monoclonal cultivar cultured in field (Gimenez et al., 2016). This variation could be caused by mutations due to the inaccuracy of the DNA replication machinery and by transposable elements. Transposition is often neutral or deleterious, yet it is the main source of genetic variation in asexually reproducing species, and it is regulated by DNA methylation (de Carvalho et al. 2016). These mechanisms constitute the solely sources of genetic variability in garlic. Despite the high genetic polymorphism, a large number of fragments were shared by samples from all years. Also, when comparing the samples of each year, there were few overall changes occurring from year to year.

DNA methylation is an epigenetic mechanism that influences the regulation of gene expression (Satyaki and Gehring 2017). New sequencing technologies allow the analysis of methylation profiles on a large scale. By identifying differentially methylated regions (DMRs), a considerable amount of DMRs were found within or near protein-coding genes and are correlated with differentially expressed genes (Garg et al. 2015; Chen et al. 2017; Al-harrasi et al. 2018; Ding et al. 2018). In different plant species epiallele-phenotype relations have been truly established (Cubas et al. 1999; Manning et al. 2006; Martin et al. 2009, Wilschut et al. 2016). These epialleles showed transgenerational heritability and produced phenotypes of agronomic and physiological interest, showing potential for epigenetic improvement. Garlic is a species that depends on environmental changes (temperature and photoperiod) for its normal physiological development to form a bulb and leave offspring -albeit asexual. In this respect we can say that: 1) it is possible that DNA methylation is exerting a certain level of regulation of these mechanisms, and 2) this species has sensitivity to environmental changes and that later influence their physiology and ultimately their phenotype. Supporting these statements, Wang et al (2016) reported that, when performed clustering based on morphological traits, the sizes and individuals present in each group differed somewhat from those clusters based on genotype. They hypothesized that morphological characteristics may differ under different environmental conditions. Hence we consider that is necessary to explore epigenetic variability as a possible source of phenotypic variation in this species.

In our study the amount of the three epigenetic patterns detected by the MSAP assay showed significant changes along time, and the most abundant of them was internal methylation of CCGG sites. The detection of many internally methylated CCGG sites is typical of species with large genomes in which chromosomes are mostly heterochromatic (Suzuki et al. 2010, Vidalis et al. 2016) and it is involved in the silencing of transposable elements (Rigal and Mathieu 2011; Zakrzewski et al. 2017), which are known to be abundant in onion and garlic genomes (Jakše et al. 2008). These observations establish a link between genetic and epigenetic variation, since DNA methylation is controlling the activity of transposable elements, which in turn are a source of genetic variability under asexuality (de Carvalho et al. 2016).

We detected that epigenetic variability was higher than genetic variability. The values of epigenetic polymorphism in Y1, Y3 and Y13 samples (72–82%) were higher than the 30% previously reported for a field cultured garlic cultivar (Gimenez et al. 2016). Herrera and Bazaga (2016) also reported more epigenetic than genetic changes with 53 and 43% polymorphism, respectively, for *Lavandula latifolia* in a 20 year lapse of time. These results could be due to a higher epimutation rate of DNA methylation than the rate of genetic mutations (Xia et al. 2016). In fact, it has been established in *Arabidopsis thaliana* that epimutation is fivefold greater than nucleotide mutation (Schmitz et al. 2011; van der Graaf et al. 2015). Moreover, garlic epigenetic variability did not decrease correspondingly with genetic variability with the application of selection pressure, implying that there is a high level of independence among each other as it was detected in other plants (Honnay et al. 2008; Paun et al. 2010; Herrera et al. 2016; Herrera and Bazaga 2016).

Regarding the permanence of AFLP patterns over time, the majority of the genetic loci were stable, whereas the vast majority of the MSAP data varied annually and a large amount was gradually variable i.e. the same methylation state was found in two consecutive years. Comparing Y1 and Y13 samples we found that 83.13 and 23.53% of genetic and epigenetic identity remained the same after 13 years, indicating a genetic stability and an epigenetic instability over time. According to Springer (2013) epigenetic marks could be highly stable and heritable –then it may be easily captured in breeding schemes– or could be relatively unstable –then it may lead to an unstable behavior for some traits–. According to our results, both phenomena are occurring simultaneously over different epiloci in garlic, some of them are stable and others are dynamic. These two components explain the behavior showed by garlic epialleles along the years of study. There was a substantial percentage of stable and gradually variable epialleles, which is important for the design of epigenetic improvement. Furthermore, considering that epimutations contribute to heritable phenotypes, the epigenetic variants could be the target of selection (Xia et al. 2016) for an epigenetic improvement scheme. It may be that garlic traits that are currently under selection could be determined by epigenetic marks.

Even though there are several reports studying genetic diversity in garlic cultivars (García Lampasona et al. 2003; Buso et al. 2008; García-Lampasona et al. 2012; Chen et al. 2013; Cunha da et al., 2014; Shaaf et al. 2014), genetic and epigenetic stability has not been assessed in garlic over time. The autonomy of epigenetic variation reported here implies that this is an additional system of inheritance and it takes relevance as an alternative for generating new phenotypes in breeding programs. Since the garlic line analyzed in this study displayed larger epigenetic than genetic variability, we consider that detecting epialleles that lead to an agronomically important phenotype would be advantageous to conduct epigenetic improvement. Moreover, epigenetic variability could be introduced to garlic plants through in vitro culture

as well as through azacitidine treatment resulting in a faster process than genetic improvement.

## Declarations of interest

None.

## Compliance with ethical standards

Authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2018.04.044.

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