

## Characterization of some garlic clones using morphological, cytological, molecular and chemical techniques

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### Abstract

Four different clones of garlic (Balady, EGA 3, EGA 4 and Growers clone) cultivated in Egypt were differentiated using morphological, cytogenetical, molecular and chemical characterization. Results of the morphological and cytogenetical characters analyses showed that there were considerable variations for all studied traits except for three; leaf width, stem diameter and chromosome numbers. Additionally, SSR (simple sequence repeats) and ISSR (inter simple sequence repeats) markers showed a high level of genetic variation among the four garlic clones. Moreover, Headspace Gas Chromatography and Mass Spectroscopy (HS GC/MS) was used for identifying and comparing the volatile constituents. The resulting peaks showed thirteen major constituents [prop-2-en-1-ol, 5-hexenal, trans-2-trans-7-nonadiene, 1-propene-3,3'-thiobis, 1-propene, 3-(methylthio), disulfide, methyl-2-propenyl, camphene, disulfide, di-2-propenyl, trisulfide, methyl-2-propenyl, diallyltetrasulphide, 2-vinyl-[4H]-1,3-dithiin, trisulfide, 1- trisulfide, di-2-propenyl, 1,3-dithiane and trisulfide, dimethyl ] in the four analyzed clones. It could be concluded from this study, that the percentage of active volatile constituents in each clone may be used as chemical markers to distinguish between these clones. Accordingly, HS GS/MS is a considerably simple and fast method that can be used for initial identification of all four clones under the same conditions using the above compounds. The analyses can be performed on both entire fresh or powdered plant forms.

### Key words

Garlic, Morphological, SSR and ISSR markers, headspace sampling, Gas Chromatography, Mass Spectroscopy

### 1. Introduction

Garlic (*Allium sativum*L., *Liliaceae*) is considered to be one of the most essential vegetables throughout the world. The prominence of garlic is due to its use not only for culinary but also for beneficial and medicinal purposes in both traditional and modern medicine [1]. It is an asexually propagated crop and it displays great morphological diversity in bulb color and shape, leaf size, scape presence and height, flower color, fertility and bulbil (topset) development inflorescence [2-5]. Variation in garlic performance of clonal species may be due to microbial infection, which is passed from generation to generation by asexual propagation [6]. Additionally, such diversity probably results from cross pollination between wild ancestors when this plant species still had the ability of sexual reproduction or due to the accumulation of mutations throughout the history of garlic cultivation [2 and 7].

Garlic represents a sizeable group of useful plants with a large nuclear genome [8]. The genome of diploid garlic ( $2n = 2x = 16$ ) is assessed at 15.9 Gbp, 32 times larger than the rice genome. Although full sequencing of the garlic genome remains a challenging task, transcriptome assembly by next generation sequencing may be used for the effective generation of functional genomic data [9]. Different molecular techniques have been developed to study garlic diversity using various biochemical and molecular markers, such as isozymes,

randomly amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), inter simple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLPs) markers. SSRs are reported to be more adaptable than restriction fragment length polymorphism (RFLP) or RAPD, and they have been commonly utilized in plant genomic studies [10]. Therefore, these markers would be suitable for evaluating genetic variation among asexually propagated garlic clones.

ISSR primers target simple sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome and advance rapidly [11-13]. It is a PCR-based method established by Zietkiewicz *et al.*, [14]. This technique has been used to fingerprint the different plant species and cultivars [15 and 16] and successfully to evaluate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species. It can rapidly differentiate closely related individuals and have been effectively used to measure genetic diversity among closely related cultivars which were difficult to differentiate with other molecular markers [17-20].

Garlic has many medicinal uses and comprises a complex mixture of different phytochemical constituents, that differ considerably among different strains and probably clones. These constituents also vary according to geographic origins and drying methods [21-23]. Garlic odour and flavour components are produced enzymatically from precursors as allin which is converted to allicin and other sulphur compounds when the

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plant is cut or crushed. Many of these components are volatile and thus are well-suited for headspace analyses [22 and 24]. Diallyltrisulphide and diallyldisulphide were described as the main constituents of garlic oil from (*Allium sativum*L.) [25]. Therefore, this study aimed the characterization of the phenotypic and genotypic dispersion and chemical composition of four garlic (*Allium sativum*L.) clones cultivated in Egypt using some morphological, cytological and molecular features. The study was extended to include the estimation of volatile compounds using the Headspace Gas chromatography and Mass spectroscopy (HS GC-MS).

## 2. Experimental

### 2.1. Plant material

Bulbs of four cultivated garlic clones, Balady (white bulb color), EGA 4 (white color), EGA 3 (Purple color) and Growers (Purple color) were used in the present study. The former three clones were kindly provided by Egyptian Agricultural Company for Seed Production (EGAS) while Growers clone was provided by Horticulture Department (vegetable branch), Faculty of Agriculture, Minia University.

### 2.2. Morphological Studies

#### *Germination and Storability percentage*

Germination percentage of the four garlic clones was recorded in September (the suitable cultivation date of garlic in Egypt) in a laboratory experiment. Fifteen cloves of each clone were used and the germination percentages were recorded after 30 days of sowing. To evaluate the storability percentage of the present material, the number of healthy and unhealthy cloves of ten bulbs/clone was recorded after five months of harvesting.

### 2.3. Field experiment

Twenty cloves/clone (from used 4 clones) were separated and planted in the experimental farm of Faculty of Agriculture, Minia University. After five months of cultivation, some morphological traits such as plant height (the height of the true stem to the terminal point of the tallest leaf in centimeters), number of leaves (the number of all leaves above the soil), width of leaves (the third right leaf in centimeters) and the number of flowering plants were recorded. At harvesting time another four different characters; the distance between bulbs and bulbils, stem diameter (5 plants/ clone), bulb diameter (5 bulbs /clone) and the average number of cloves /bulb (using 5 bulbs /clone) were calculated.

### 2.4. Cytological study

#### *Mitotic preparations of root tips*

Growing roots (1-2 cm) of cloves of the four garlic clones were collected and pretreated using 0.05% colchicine at room temperature for three hours and immediately fixed with Farmer's fixative solution for 24 h [3]. Acetocarmin-squashed preparations were made from the root tips and cells with well-spread chromosomes were chosen. Number of chromosomes and number of secondary constrictions were recorded at ten

good metaphase cells. Good metaphase spreads were photographed microscopically using CCD camera (Olympus C-4040).

## 2.5. Molecular analyses

#### *DNA isolation*

DNA of the four garlic clones was extracted in 600 µl of Cornel extraction buffer (500 mMNaCl; 100 mMTris-HCl, pH 8.0; 50 mMEDTA and 0.84% SDS, equilibrated to 65 °C, mixed with 0.38 g Sodium Bisulfite/100 ml buffer, and then the warm buffers pH was adjusted to 7.8-8.0 with NaOH). For quantifying the amount of DNA, 10 µl of the re-suspended nucleic acid was mixed with 990 µl of double distilled water and the absorption at 260 and 280 nm was measured using spectrophotometer. DNA purity and concentration were estimated according to Sambrook *et al.*, [26].

#### *PCR conditions for SSR analyses*

SSR analyses was performed using two microsatellite primers (Asa08: F: 5'-TGATTGAAACGAATCCCAC-3'; R: 5'-GGGGGTTACCTGAACCTGTT-3' and Asa18: F: 5'-TCAAGCTCCTCCAAGTGTCC -3'; R: 5'-TCGGGATATGACAGCATTTG- 3') developed in *Allium sativum* [27]. The amplification program used was described in [28].

#### *PCR conditions for ISSR analyses*

ISSR analyses was performed using D24 [(CA) 6 CG] and HB13 [(CTC) 3 GC] primers [29]. Amplification was performed in a thermal cycler (Thermo Hybaid) programmed for 1 cycle of pre-denaturation at 94 °C for 2 min; and 35 cycles at 94 °C for 30 s, 44 °C for 45 s, and 72 °C for 1.5 min; followed by 20 min of post extension at 72 °C.

Products of SSR and ISSR amplifications were confirmed by electrophoresis in 2% agarose gels stained with ethidium bromide, visualized on UV light and photo-documentation was performed using gel documentation system (G: Box, syngenc UK Gel Doc.). Sizes of the amplified fragments were estimated according to the standard ladder of 100 bp.

## 2.6. Headspace Gas chromatography and Mass spectroscopy (HS GC/MS) analyses

Metabolites of the peeled crushed cloves samples were analyzed using GC/MS, headspace sampling technique (Automated headspace GC/MS analyses.).Cloves of the four garlic clones were peeled, crushed and left in the measuring vial for 20 min at 120 °C before injection in GC/MS. Shimadzu GC/MS Model (QP-2010 Ultra) was equipped with a Head Space AOC-5000 auto injector and a Rtx-5 MS fused-silica column of 30 meters in length, 0.25 mm ID; 0.25 µm film thickness. Column oven temperature was 40 °C, with a hold time of 2 min, with a rate of 5 min/ml till 210 °C then with a hold time of 5 min. The total running time was 41 min. The carrier gas used was helium, total flow was 13.9 ml/min and column flow was 0.50 ml/min with linear velocity of 14.4 cm/sec. The injector was operated at 250 °C and 45.8 KPa pressure with an injection speed of 500 ul/sec.

GC runtime was 47 min, connected to a flame ionization detector (FID), with a temperature of 230 °C, makeup gas was helium, makeup flow of 40 ml/min, H<sub>2</sub> flow of 40 ml/min and air flow of 400 ml/min. The mass spectrometer used had a scan range of 35-350 amu. The constituents were identified after comparison with those available in the computer library (Willey 9.lib.) attached to the GC/MS instrument.

The GC analysis has been performed at the MEPACO factory (Arab for Pharmaceuticals and Medicinal Plants company) Enshas Factory, Enshas El Raml, Belbis, Sharkeiya, Egypt.

## 2. 7. Statistical analyses

Morphological experiments were carried out in a complete randomized design and analyses of variance (ANOVA) between the means was performed according to [30] using MSTATC program (version 4.0) edited in 1985 by the MSTAT development team, Michigan University and Agricultural University of Norway.

## 3. Results and discussion

The aim of the presented study is to identify a simple applicable method to distinguish between garlic clones that are used in pharmaceutical or food industry.

### 3. 1. Morphological characters

Data of Agronomical traits such as germination percentages, plant height (cm), number of leaves, width of leaves (cm), percentages of flowering plants, bulb diameter (cm), stem diameter (cm), number of cloves, distance between bulbs and bulbils (cm) and storability percentage, of plants of the four garlic clones are shown in (Table 1). The percentages of germination (rooting and shooting) and storability of Balady were the highest, while those of EGA 4 and Growers clones showed lower rates. The high mean value of bulb diameter was observed in EGA 4 (5.38) while the lowest value (4.96) was observed in EGA 3. Two morphological traits (percentages of flowering plants and the distance between bulbs and bulbils) were varied among the studied clones. These two morphological traits were considerably differentiated among complete bolting clones (EGA 3), incomplete bolting clones (EGA 4) and non-bolting clones (Balady and Growers clone).

To our knowledge, according to the newly expected parameter (variation in distance values between main bulbs and bulbils which contain the region of inflorescence organization), the probability of the positive relationship between this distance and differentiation of flowering buds in garlic clones is possible. Florogenesis comprises a number of processes, each controlled by specific genes of the given genotype [31 and 32]. Non-significant differences were found in additional two morphological traits (width of leaves and stem diameter) among the four garlic clones. Bulbs of Balady clones (Balady and EGA 4) had a significantly high number of cloves (39 and 47 respectively) when compared to those of EGA 3 and Growers clones (15 and 10 respectively). The average plant height ranged from 49 cm in Growers clone to 78 cm in EGA 3. Mean number of leaves varied from 7 in Balady to 11 in Growers

clone. It has repeatedly been shown that a wide range of morphological and botanical diversity in garlic (*Allium sativum*), including traits like those of the present work depend upon the genotypic- environmental interaction [33 and 34].

### 3.2. Cytological study

Almost all examined cells in plants of the studied garlic clones showed a diploid number of  $2n=16$  (Table 1). Chromosomes were mitotically regular, in spite of visual variation in individual chromosome morphology. For instance, metaphase plates in plants of Balady and EGA 3 clones Figure 1 (a & b) often showed high frequencies of cells bearing four satellited chromosomes (Four secondary constrictions on pairs no.6 and no.7), while those of Growers and EGA 4 clones Figure 1 (c & d) usually had one Sat-chromosomes (one secondary constriction either pairs no.6 or no.7). Variation in number of secondary constrictions could be due to unequal exchanges among ribosomal genes on homologous and non-homologous chromosomes. Ribosomal DNA sequence homogeneity detected in *Allium sphaerocephalon* supported this conclusion [35].

### 3. 3. Molecular analyses

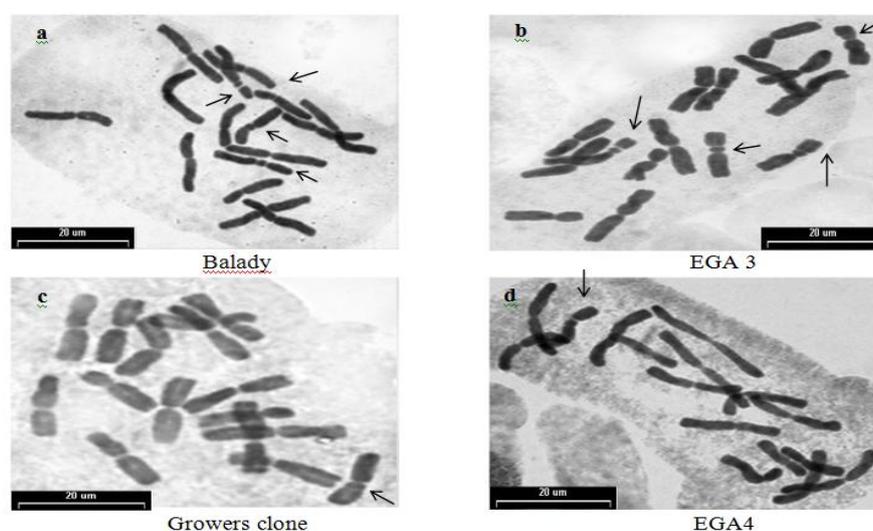
Total number of amplified fragments, number of monomorphic, polymorphic, unique bands, the percentage of polymorphism and mean frequency of bands obtained of the used two SSR and two ISSR primers are shown in (Table 2). As shown in (Figure 2) and (Table 2), the number of amplified bands varied between Asa08 (six bands) and Asa18 marker (three bands). Percentage of polymorphism which identified by SSR primers between Asa08 (100) and Asa18 (66.7) are shown in (Table 2). Asa08 SSR primer produced only one unique band in EGA3 clone (Figure 2a). The two ISSR Primers were polymorphic conferring a 100% of polymorphism (Table 2). The availability of a relatively high number of polymorphic ISSR markers reflects the heterozygosity of the genome. These results reveal the ability of ISSR technique to detect genetic polymorphism in both vegetative and sexually propagated species. Three unique bands with molecular weights of 279, 833 and 1066 bp were detected from Growers clone by using D24 ISSR primer (Figure 2 d). The high level of genetic variation observed in this study is consistent with results obtained from previous studies of garlic carried out using different molecular markers [36-38], thereby confirming the great diversity among garlic clones [39-40].

### 3. 4. Headspace Gas chromatography and Mass spectroscopy (HS GC/MS) analyses

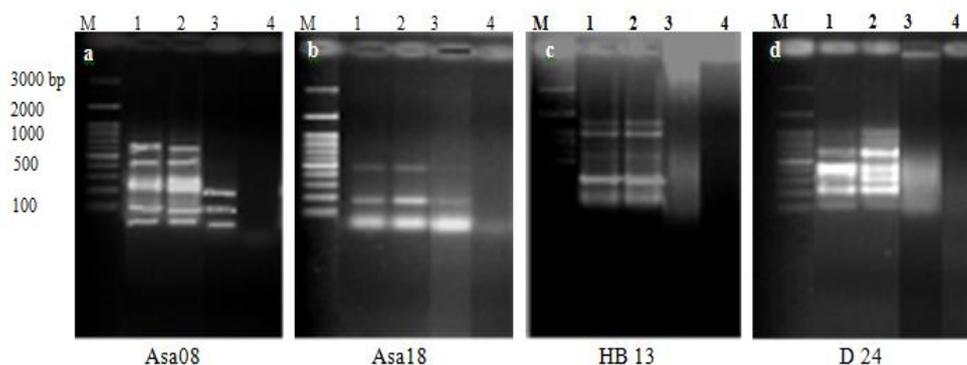
In order to identify the volatile constituents of the 4 different garlic clones, garlic cloves were analyzed by GC-FID, GC/MS using headspace technique. Analyses evidently indicated the presence of various sulfur containing compounds. It is quite clear that the major constituents are aliphatic sulphides, thiosulfonates (S<sub>2</sub>O<sub>3</sub>), thiols (RSH) (strong garlic odour), mostly the transformation products of allicin (Table 3). Thirteen major constituents were found in all four different clones; diallyl sulfide, methyl allyl sulfide, methyl allyl disulfide, diallyl

**Table 1:** Morphological and cytogenetical traits measurements of the four garlic clones (Balady, EGA 4, EGA 3 and Growers clone)

Characters	Clones				L. S. D. at alpha 0.05	
	Balady	EGA 4	EGA 3	Growers clone		
Morphological characters	Percentages of Germination (%)	100	40	60	15	
	Middling Plant height (cm)	73	77	78	49	5.061
	Middling Number of leaves	7	10	10	11	0.7289
	Middling Width of leaves (cm)	1.76	1.92	1.82	1.87	Non-significant
	Percentages of flowering plants (%)	0	6	60	0	
	Middling Bulb diameter (cm)	5.06	5.38	4.96	5.10	0.3540
	Middling Stem diameter (cm)	1.18	1.10	1.02	0.78	Non-significant
	Middling Number of cloves	39	47	15	10	4.5790
	distance between bulbs and bulbils (cm)	12.5	45	75	8.5	
Percentages of Storability (%)	96	86	55	50		
Cytogenetical characters	Chromosome numbers	16	16	16	16	
	Number of secondary constrictions	4	1	4	1	

**Figure 1:** Normal metaphase stage cells of the four garlic clones showing Sat-chromosome (arrows), Scale bar=20 Microns**Table 2:** Total number of monomorphic, polymorphic and unique PCR fragments, percentage of polymorphism and mean of band frequency obtained by using two SSR and two ISSR primers for studied garlic clones

Primers	Monomorphic bands	Unique bands	Polymorphic bands		Total number of bands	Polymorphism (%)	Mean of band frequency
			without Unique	with Unique			
Asa08	0	1	5	6	6	100	0.542
Asa18	1	0	2	2	3	66.7	0.750
D 24	0	3	4	7	7	100	0.393
HB 13	0	0	5	5	5	100	0.500

**Figure 2:** Electrophoretic patterns of two SSR and two ISSR primers used in the present work. M: 100 bp ladder marker and lanes 1 through 4 refer to: Balady, Growers clone, EGA 3 and EGA 4 respectively.

**Table 3:** Headspace Gas chromatography and Mass spectroscopy (HS GC-MS) analyses

Metabolites	Balady	EGA 4	EGA 3	Growers clone
	%			
PROP-2-EN-1-OL ( styrene)	7.03	19.79	13.91	11.12
5-hexenal	0.58	0.81	0.37	0.21
trans-2-trans-7-nonadiene	1.46	0.65	0.30	37.63
Diallylsulphide (1-propene,3,3`-thiobis)	4.25	3.15	2.42	0.58
Methyl allyl sulfide (1-propene, 3-(methylthio)-	0.45	2.38	1.97	0.26
Methyl allyl disulfide (disulfide, methyl 2-propenyl)	6.01	6.81	1.63	1.95
Camphene	0.11	0.07	0.18	0.11
Cymene	Absent	0.08	0.10	0.12
diallyl disulfide (disulfide, di-2-propenyl)	20.51	25.11	13.59	16.22
methyl allyltrisulfide (trisulfide, methyl-2-propenyl)	12.06	4.55	Absent	4.63
Diallyltetrasulphide	7.91	4.94	3.10	6.39
3-vinyl-[4H]-1,2 dithiin (transformation products of allacin)	0.15	0.15	0.13	Absent
2-vinyl-[4H]-1,3-dithiin (transformation products of allacin)	0.32	0.31	0.22	0.80
Ethanethiol, 2-(diethylboryloxy)-	0.57	0.08	0.12	Absent
Diallyltrisulfide (trisulfide,di-2-propenyl)	17.08	6.93	6.14	15.83
Isobutyl isothiocyanate	0.13	Absent	0.12	0.11
1,3dithiane	0.74	0.53	0.16	0.24
Trisulfide,dimethyl	2.43	0.43	0.05	0.09
Trans $\beta$ ionon-5,6 epoxi (food additive)	Absent	4.74	55.03	37.63
Dimethyl, disulphide	1.89	0.99	Absent	0.18

disulfide, diallyltetrasulphide, 2-vinyl-[4H]-1,2-dithiin, diallyltrisulfide, 1,3-dithiane (Some synonyms were used for some compounds) along with styrene, 5-hexenal, trans-2-trans-7-nonadiene, camphene and ethanethiol. While some compounds were distinctly absent from one clone and present in the other threeclones. For example, cymene and trans  $\beta$  ionon-5,6-epoxi were absent in Balady clone, where isobutyl isothiocyanate was absent from EGA 4, methyl allyltrisulfide and dimethyl disulfide were absent in EGA 3, while ethanethiol, (diethylboryloxy) and 3-vinyl-[4H]-1,2-dithiin were absent in Growers clone (**Table 3**).

The Balady clone contained the highest relative proportions of diallylsulphide.

(1-propene-3, 3`-thiobis), methyl allyltrisulfide (trisulfide, methyl-2-propenyl), diallyltetrasulphide, diallyltrisulfide (trisulfide, di-2-propenyl), dimethyl trisulphide (trisulfide, dimethyl ) and dimethyl, disulphide (4.25, 12.06, 7.91, 17.08, 2.43 and 1.89 %, respectively) while EGA 4, contained the highest content ofprop-2-en-1-ol, methyl allyl sulfide (1-propene- 3-(methylthio), methyl allyl disulfide (disulfide, methyl-2-propenyl) and diallyl disulfide (disulfide, di-2-propenyl) (19.79, 2.38, 6.81 and 25.11 % , respectively). Moreover, EGA 3 had the highest % of trans  $\beta$  ionon-5,6-epoxi, of 55.03 followed by Growers clone with a % of 37.63, only as 4.74% in EGA 4 and completely absent from the Balady clone.

#### 4. Conclusion

Morphological, cytological and molecular characters could be used as applicable tools for differentiating garlic clones. It could be demonstrated that applying HS GC/MS technique as a primary technique for quick identification and discovery of metabolites in minute quantities that may be overlooked using conventional techniques, also this study illustrated that the variation among the four clones was found enough to identify each one separately and differentiate between them.

The experimental set-up is ideal for both screening analyses and low-level trace analyses. This proposed method is rapid, simple, eco-friendly and can successfully differentiate between the four clones.

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