C-BANDING PATTERNS AND ALLOCATION OF ALLICIN GENES ON CHROMOSOMES OF TWO GARLIC CULTIVARS

ANWAR GEHAN M. * AND ATA ABDELTAWAB M.

Department of Genetics, Faculty of Agriculture, Minia University, Egypt

** Correspondence Author***:** *gehan_ma@mu.edu.eg*

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Garlic (*Allium sativum*) is a diploid species belonging to the genus *Allium* of the *Liliaceae* family, order Asparaguses' is well known that Garlic originated from the main center of *Allium* diversity that stretches from the Mediterranean basin to central Asia (Ipek and Simon, 2001). The soft-neck cultivars are stronger and more pungent, and the top-setting cultivars are usually milder, sweeter, and nuttier in flavor (Woodward, 1996). A wide range of morphological diversity has also been observed in Garlic, including flowering ability, bulbils traits, and flowering traits (McCollum 1976; Lallemand *et al*. 1997; Hong *et al*. 2000; Senula and Keller 2000 and Simon and Jenderek 2003; Osman *et al*. 2007 and Ata and Osman 2009).

The diploid number of common Garlic is 2N=16 with different karyotypic formulas. Early published data by Bozzini (1991) indicated that Garlic karyotype has consisted of six metacentric chromosomes, four sub-metacentric chromosomes, and six acrocentric chromosomes. The karyotype of Egyptian garlic clones appears to be different compared to those published during the last two decades (Elmamlouk *et a*l. 2002; Ata 2005; Osman *et al*. 2007; Mahmoud *et al*. 2017; Ata *et al*. 2020; Helmey 2020 and Anwar *et al.* 2021).So far, reasonable indications have been confirmed to prove that the garlic karyotype is not identified because of the apomictic nature of its propagation and the accumulation of particular structural aberrations (Elmamlouk *et al.* 2002; Ata 2005; Osman *et al*. 2007; Ata and Osman 2009; Anwar and Ata 2017; Anwar *et al*. 2017; Mahmoud *et al*. 2017; Helmey and Anwar 2018; Anwar *et al*. 2020; Ata *et al*. 2020; Helmey 2020 and Anwar *et al.* 2021).Using Differential staining of chromosomes (i. e C-banding) and molecular cytogenetic techniques (I. e., FISH) may aid in karyotyping and identifying the garlic genome. C-banding patterns were extensively examined on different garlic genotypes (Cortes *et al*. 1983; Cortes and Escalza 1986; Pich *et al.* 1996 a & b and Yüzbaşioğlu and Ünal 2004 and Ata 2005). Therefore, it could be concluded that the position and distribution of the constitutive heterochromatin on chromosomes might differ depending on genotypical and procedural differences (Ata 2005).

FISH (Fluorescent *In Situ* Hybridization) has been used in many plant species to recognize chromosomes accurately, using species-specific repetitive sequences, ribosomal genes, and even unique sequences (Maluszynska and Heslop-Harrison 1991). FISH also allowed the visualization of telomeres and confirms the relationship between chromosomal aberrations and the genetical and cytological instabilities. In some *Allium* species, chromosomal localization of multi-gene families were detected by FISH (Seo *et al.* 1997 and Lee *et al.* 1998). For instance, FISH of rRNA genes has been described for *Allium* species such as *A. sativum* (Hizume *et al*. 1995), *A. fistulosum* (Irifune *et al*. 1995), *A. wakegi* (Hizume 1994) and *A. victorialis* var platyphllum (Seo *et al*. 1997). With ribosomal probes, active and inert rDNA sites can be localized on chromosomes by FISH (Lavania 1998). By using FISH techniques, Pich *et al.* (1996a) tested the presence of *A. cepa* satellite sequence repeat in 27 species of *Allium*.

Alliinase is a vital enzyme occurring in Allium species that converts precursors of sulfuric compounds, cysteine sulfoxides, into a biologically active substance termed allicin. Alliinase is encoded by members of a multi-gene family that has not yet been sufficiently characterized, namely concerning the copy numbers occurring within the genome and the polymorphisms among the family members (Ovesná *et al.* 2015). Sun *et al.* (2020) reported that the alliinase gene family has extended considerably in the Garlic genome: there are 60 alliinase genes in garlic of them, 24 are duplicated genes mainly scattered in 10 genomic regions. A total of 38 alliinase genes were expressed in several tissues, and 21 showed dynamic expression changes in developing bulbs. Thus, in the present study the description of C-banding patterns, and allocation of allicin genes using FISH signals of a bolter (Egaseed 2) and non-bolter (AZO 3) garlic cultivar have been carried out.

MATERIALS AND METHODS

1. Materials

Two garlic cultivars called AZO 3 (soft-neck, non-bolting and white bulb) and Egaseed 2 (hard-necked, complete bolting and purple bulb) of common garlic (*Allium sativum* L.) were kindly obtained from Vegetative Crops Branch, Horticulture Department, Faculty of Agriculture, Minia University. The materials used in the present work are shown in Figs. (1a-c).

2. Preparation of root tips for cytogenetic analyses

Root tips in 1-2 cm length were taken from germinated cloves and pretreated with 0.05 colchicine for an hour at room temperature and immediately fixed in cooled Farmer's fixative (3 ethanol: 1 acetic acid glacial) at 4ºC for 24 hours and then stored in ethanol 70%. After fixation, the roots were used for both conventional preparations and differential C-banding technique on the mitotic chromosomes.

Fluorescence in situ hybridization (FISH) technique was also carried out in the fixed tissues.

3. Chromosome preparations

3.1. Conventional staining of mitotic chromosomes

Some of the fixed root tips were taken from germinated plants of AZO3 and Egaseed 2 and then hydrolyzed using 1 N HCl at 60ºC for six minutes. These root tips were squashed in Acetocarmine stain and microscopically examined. The cells with well chromosome spreads were counted and photographed using Soft Imaging System (SIS) program (Version 3.0) edited in 1999 by Soft Imaging System GmbH, Germany, with a C-4040 zoom digital camera as a unit of Olympus BX51 microscope.

3.2. Chromosome preparations for C-banding

A number of the fixed root tips taken from AZO3 and Egaseed 2 cultivars were hydrolyzed in a mixture of 1% Cellulase and Pectinase for a few minutes at 37ºC. The hydrolyzed root tips were squashed in a drop of 45% acetic acid and covered with coverslip. The slides were immerged in liquid nitrogen, then the cover slips were removed using straight razor. The slides were directly passed in a series of ethanol 90, 95 and 98% and air dried under room temperature. Traditional C-banding technique of Sumner (1972) was used with some modifications (Ata 2005). Slides were treated with HCl (0.2N) at 10ºC for one hour, then washed in distilled water three times and incubated, in $BaOH_2.8H_2O$ at 50ºC for 2-3 minutes then in 2XSSC at 50ºC for 30 minutes, and finally stained with 4% Giemsa for 2-3 minutes. About 50 C-banded cells were examined each from AZO 3 and Egaseed 2 varieties. Good preparations were scored and photographed using Soft Imaging System (SIS) program (Version 3.0) edited in 1999 by Soft Imaging System GmbH, Germany, with a C-4040 zoom digital camera as a unit of Olympus BX51 microscope.

3. 3. Florescent *In Situ* Hybridization (FISH)

Chromosome preparations for FISH were made from the fixed roots of AZO 3 and Egaseed 2 plants as declared above in C- banding procedure till passed in a series of ethanol 90, 95 and 98% and air dried under room temperature.

3. 3. 1. Probes preparation and Labeling

Probe was formed by the polymerase chain reaction (PCR) using Allicin primer (F- CTC AAC TCA TCC ATG GAC TCG TCA TCT CT, R- GAT CGT ACG TTA GAT CGA TGT GTG C). The probe was labeled using Prime-It® Fluor Fluorescence Labelling Kit by mixing 250 ng of DNA template (Genomic DNA or PCR product) with 26µl of distilled water and 10µl of random 9-mer primers. This mixture was heated at 97ºC for 10 minutes in water bath, and then placed directly on ice for 10 minutes. After that, 1µl of fluor-

12-dUTP, 10µl of 5X nucleotide buffer and 1µl (5U) of exonuclease-free Klenow enzyme were added to the previous mixture and mixed gently.

The mixture was incubated at 37ºC for 30 minutes. The labeling reaction was stopped by adding 2µl of stop mix, and then stored overnight at 4ºC in the dark. Unincorporated nucleotides were removed by rainfall by adding 1/10 volume of 3M sodium acetate and 2.5 volumes of 100% (v/v) cold ethanol to the reaction tubes. The tubes were incubated on ice for 20 minutes and centrifuged for 30 minutes at 12000 rpm. The pellet was washed with 70% (v/v) cold ethanol and then dried in air. The pellet was re-suspended in 10 µl of hybridization buffer (50% formamide and 10% dextran sulfate in 4XSSC buffer) and preserved at -20°C for use.

3. 3. 2. Slides pretreatment and the procedure of FISH

Procedure of FISH was carried out according to the method of Abd El-Twab and Kondo (2008) with few modifications. The dried slides were rinsed in 2XSSC for 10 min to refresh chromosomes, then, denaturized in a preheated formamide solution (70% in 2XSSC) at 73ºC for 2 min. The slides dehydrated in an ice cold ethanol series (70, 80 and 100%) for 5 min. the probe mixtures were denatured at 93ºC for 10 min followed by 20 min on ice. 15µl of probe per slide were added and covered with para-film without air foam. The slides were then incubated in a preheated humid chamber at 37ºC overnight for hybridization. The cover slips fall off by floating, then the slides were washed twice in 2XSSC for 10 min at room temperature and washed anther twice in 4XSSC for 10 min and allowed to air-dry. Each slide was stained by one drop of PI (600µl PI in 5ml glycerol). The fluorescence signals were observed with an epifluorescence microscope with Olympus BX 51 WB filter cassette (yellow-red-color), and microphotographs were taken by CCD camera (Olympus C-4040). Images are pseudocolored and merged using Adobe Photoshop 7.0 software.

RESULTS AND DISCUSSION

1. Mitotic chromosomes

Regardless of the differences in the karyotypic formula between the studied cultivars (Egaseed 2 and AZO 3) and the presence of the known structural aberrations, which were indicated elsewhere in detail (Anwar and Ata 2017; Anwar *et al.* 2017; Mahmoud *et al.* 2017; Helmey and Anwar 2018; Anwar *et al.* 2020; Ata *et al.* 2020; Helmey 2020 and Anwar *et al.* 2021), the diploid number was clearly counted as 2n = 16 chromosomes of the almost all examined cells (more than 200 cells of each variety) as shown in Fig. (1 a and b). The absence of numerical abnormalities was also documented.

2. -banding of the two garlic clones (AZO 3 and Egaseed 2)

2.1. C- banding at the interphase and prophase

Data in Table (1) showed considerable differences of C- banding characteristics (including number, size, and intensity) between the interphase and prophase stages and among the plants of the two studied garlic cultivars. For instance, the interphase cells of the Egaseed 2 cultivar showed two C-band blocks (One heavy large and one sharp small block) as shown in Fig. (2 a), while only one heavy large C-band block was seen at prophase (Fig. 2 b). In the same manner, three large C-band blocks (two heavy and one faint) were observed at the interphase of the AZO 3 cultivar (Fig. 2 c), while two large blocks (one heavy and one faint) were observed at the prophase (Fig. 2 d).

2.2 C-banding at metaphase cells

Data in Table (2) clearly showed that there are no considerable differences in C-banding patterns on the metaphase chromosomes between the two studied garlic cultivars. It should be noted that C-bands were not detected around the centromeric regions on any chromosome. Most of the examined cells showed five C-bands scattered on three chromosomes. Of them, two neighbored thin sharp bands were localized in the middle of the short arms of the two homologous chromosomes (pair no.6) of both cultivars (Fig. 3 a and b). Sometimes, these two bands were absent. However, a large, heavy C-band block was always found on the short arm of only one copy of the 7^{th} chromosome at all examined cells of both garlic cultivars (Fig. 3 c and d). This single band was dominant and has never disappeared.

2. 3. Fluorescent *In Situ* Hybridization (FISH) of Allicin probe

As shown in Table (3), Fluorescence *In Situ* Hybridization (FISH) technique was applied to detect the number and location of the Allicin gene scattered along with chromatin of interphase and metaphase cells of two studied garlic cultivars (AZO 3 and Egaseed 2). Four FISH signals of the Allicin probe were clearly observed at the interphase stage of the Egaseed 2 cultivar. Furthermore, these signals were localized in four different compartments of the interphase nuclei (Fig. 4a). Likewise, four FISH signals were localized on four metaphase chromosomes of the Egaseed2 cultivar (Fig. 4b). On the other hand, three FISH signals of the Allicin probe were detected in three different compartments at interphase cells of the AZO3 cultivar (Fig. 4c). In the same manner, two signals were scattered on three metaphase chromosomes (Fig. 4d).

In the current study, the conventional staining of the metaphase chromosomes has confirmed that the diploid number of two studied Garlic cultivars (Egaseed 2 and AAZO 3) is 2n=16 without numerical aberrations, regardless of the appearance of the well-known structural aberrations. Data recorded herein is in accordance with several reports

(Konvicka and Levan 1972; Verma and Mittal 1978; Elmamlouk *et al.* 2002; Ata 2005 and Osman *et al.* 2007). The karyotype exhibited a significantly high variation between and within studied cultivars. The differences were especially pronounced in the two satellite-carrying chromosome pairs. (Anwar *et al.* 2017; Mahmoud *et al.* 2017; Anwar *et al.* 2020; Ata *et al.* 2020; Helmey 2020 and Anwar *et al.* 2021). An inter and intra-karyotypic variation has also been identified (Ata *et al.* 2020). It was not easy to define which chromosome of the eight pairs could be called SAT chromosomes because the secondary constrictions (SC) and the satellites (SAT) were shown on all 8 chromosome pairs but with variable percentages. Otherwise, chromosome pairs nos.5, 6, and 7 were more frequent SAT chromosomes. A preponderance of the "basic" karyotype (2 sets of chromosomes) was demonstrated for bolting Central Asian clones by Hong *et al.* (2000). In contrast, incomplete-bolting cultivars from the Iberian Peninsula usually exhibited "non-basic" karyotypes (e.g., heteromorphic pairs) and variations in centromere location and/or satellites.

Karyotypic variation was also examined using the C-banding technique. In plants, C-bands are often non-centromeric and correspond to blocks of constitutive heterochromatin. However, most of these bands contain late-replicating DNA. Similarly, they are sometimes rich in A-T base pairs, closely adjacent to each other (Schubert 1984). In the present work, metaphase chromosomes of either bolting (Egaseed 2) or on-bolting (AZO 3) garlic cultivars showed two different C-banding patterns, first with five bands scattered as two neighbored thin sharp bands on the short chromosome arms of each copy of the homologous pair no.6. In addition to these four C-bands, a large stable heavy c-band block localized on the short arm of only one copy of chromosome no.7. The second type showed the only one stable thick heavy C-band, which localized on the short arm of only one copy of chromosome no.7. Data reported herein are contradicted with those of Cortes *et al.* (1983); Cortes and Escalza (1986); Yüzbaşioğlu and Ünal (2004) and Ata (2005) in which they recognized C-bands at the secondary constrictions on chromosome no. 4, 5, 6, 7 and/or eight as well as an additional telomeric band on the long arm with the interstitial band on the short arm of the chromosome 7. Additionally, a telomeric C-band was determined on the short arm of chromosome 4. Likewise, Cortes and Escalza (1986) used two modified C-banding methods on Garlic. In one of them, C-bands were present mainly on secondary constrictions and in some telomeres. The other method allowed preferential staining of centromeres and NORs. In Egyptian Baladi accession, Ata (2005) recognized C-bands on the short arms of chromosome pairs 4, 5, and 8.

Indeed, the number and intensity of C-heterochromatin blocks have shown considerable differences between the interphases and the prophases within the same cultivar and between bolter and non-bolter Garlic. Alterations in C-banding

between individuals and even within the different tissues may be attributed either to certain structural chromosomal variations such as reciprocal translocations, chromosome breaks and/or inversions or to the transformation of euchromatin into heterochromatin and vice versa. Consequently, heterochromatin duplication has occurred for silencing neighboring genes (Avramova 2002).The karyotypes of some Brazilian garlic clones were obtained using conventional staining and with chromomycin A3 (CMA) and 4, 6-diamidino-2-phenylindole (DAPI) fluorochromes (Bacelar *et al.* 2021)..

In the present work, three allicin signals were localized on the chromatin of both interphase and metaphase of the non-bolter cultivar (AZO 3), whereas four signs were scattered on chromosomes of the bolter cultivar (Egaseed 2).A relationship may be found between the number of allicin gene signals and bolt differentiation in Garlic. Ovesná *et al.* (2015) used Intron length polymorphism (ILP) markers to differentiate between the bolting and non-bolting genotypes, and detected that the intron polymorphisms of allicin genes were newly developed. So far, some hindrances have come across during mapping these genes. For instance, of 60 alliinase genes assembled on garlic chromosomes, Sun *et al.* (2020) could only map four tandem duplicated allicin genes on one chromosome (no.5), despite using the bioinformatic tools to achieve the chromosome assembly and genome mapping. Results reported herein may support future studies of genome mapping in Garlic.

CONCLUSION

The present work confirmed that no considerable differences between a bolter garlic cultivar (Egaseed 2) and other non-bolter (AZO 3) on the level of diploid chromosome number and the distribution of C-banding heterochromatin patterns, while number and allocations of allicin FISH signals showed clear differences between the bolter and non-bolter garlic cultivar. Data reported herein may play an important role in future study with respect to garlic genome mapping of allicin genes.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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SUMMARY

In order to study c-banding patterns and the allocation of the allicin gene on Garlic chromosomes, some cytological examinations on two Garlic cultivars (Egaseed 2 and AZO 3) were done. The diploid number of plants of the two studied cultivars was 2n = 16 without numerical aberrations. Considerable differences in heterochromatic C- banding patterns (including number, size, and intensity) between the interphase and prophase stages and among the plants of the two studied garlic cultivars were also observed. The interphase cells of a bolter cultivar (Egaseed 2) showed two C-band blocks, while only one heavy large C-band block was seen at the prophase. Likewise, three

large C-bands were found at the interphase of a non-bolter cultivar (AZO 3), while two large blocks were observed at the prophase. No valuable variations of C-banding patterns were observed in the metaphase chromosomes between the two studied cultivars. C-bands were mainly scattered in the interstitial region on the short arm of both the 6th and 7th chromosomes. On the other hand, four FISH signals of Allicin genes localized in four different compartments were observed at the interphase stage of the Egaseed 2 cultivar. Likewise, four FISH signals were localized on four metaphase chromosomes of the Egased 2 cultivar. Three FISH signals of Allicin genes were detected in three different compartments at interphase cells of the AZO 3 cultivar. In the same manner, three signals were scattered on three metaphase chromosomes. It could be concluded that the considerable differences in the number and allocation of allicin genes along with bolter and non-bolter garlic genomes are crucial and may play an essential role in the future study of gene mapping.

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Table (1): the number, size and intensity of C-bands at the interphase and prophase of two garlic cultivars.

cultivars	Number of examined cells	Cell stage	Band number	Band size and intensity
Egaseed 2	50	Interphase	2	One heavy large and one sharp small block
		Prophase	1	Heavy large block
AZO3	50	Interphase	3	Two heavy large blocks and One large faint
		Prophase	2	One heavy large block and One large faint

Table (2): Number, size and position of C-bands at metaphase chromosomes of the two studied garlic cultivars.

cultivars	Number of examined cells	Cell stage	Chromosome number	Band number	Band size and intensity		Band position
Egaseed 2	50	Metaphase	6 (two homologous)	2	small	Thin sharp	middle of the short arm one near the centromere
			7(only one of the two homologous)	1	Large block	Heavy dark	middle of the short arm
AZO3	50	Metaphase	6 (two homologous)	2	small	Thin sharp	middle of the short arm one near the centromere
			7(only one of the two homologous)	1	large blocks	Heavy dark	middle of the short arm

Table(3): Number and localization of detected Allicin FISH signals in the nuclei at interphase and metaphase cells of Egaseed2 and AZO3 garlic cultivars.

cultivar	Cell stage	No. Signal	Signal localization
Egaseed2	Interphase	4	Distributed at four different compartments
	Metaphase	4	Scattered on four chromosomes
AZO3	Interphase	3	Distributed at three different compartments
	Metaphase	2	Scattered on three chromosomes

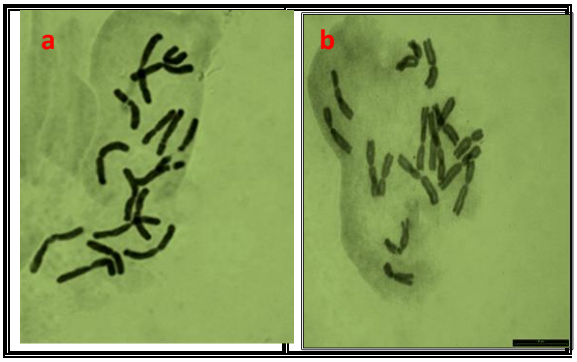

Fig. (1): Metaphase plates showing 16 chromosomes of garlic varieties a: from Egaseed 2 and b: from AZO 3 plants, the scale bar= 20 microns

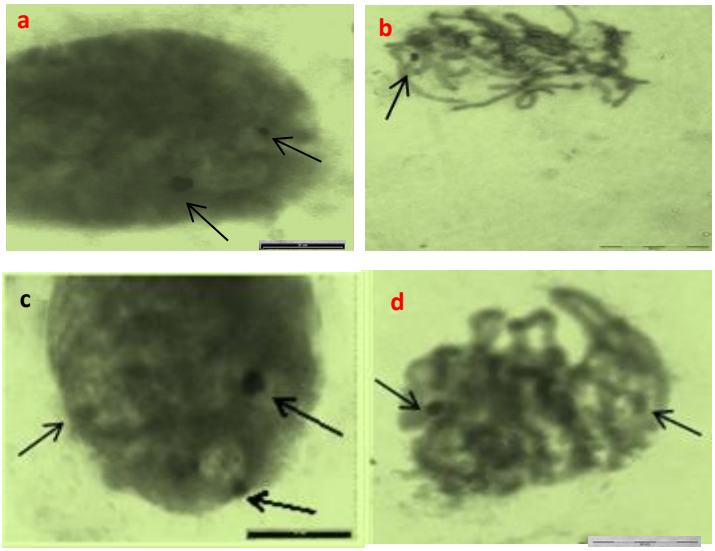

Fig. (2): C-banding patterns in Egaseed 2 and AZO3 garlic (*A. sativum*). a: two bands at the interphase and b: one band at the prophase of Egaseed 2 c: three bands at the interphase and d: two bands at the prophase of AZO3. All C-band blocks are indicated by arrows. Scale bar= 20 microns.

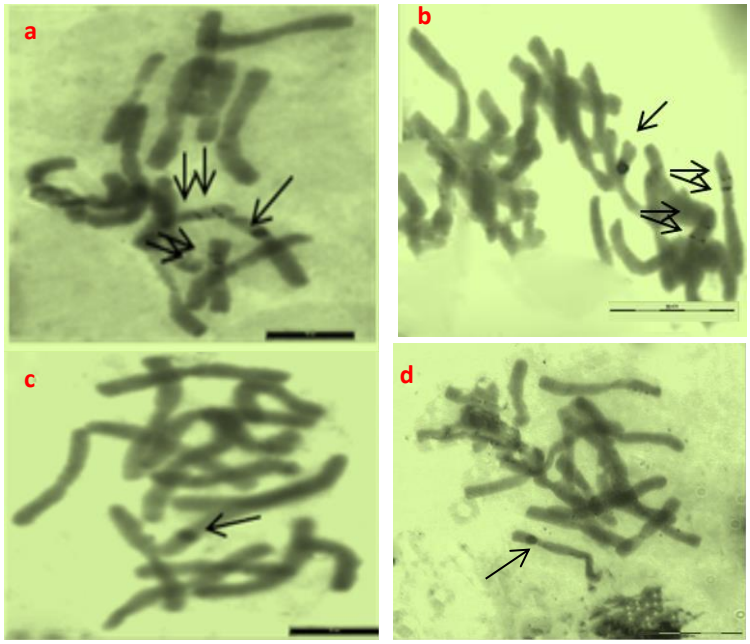

Fig. (3): C-banding patterns at metaphase of Egaseed 2 and AZO3 garlic cultivars. a: metaphase plate with one large c-band block and two adjacent c-bands on the two homologous copies of chromosome no.6 in Egaseed 2 cultivar, b: metaphase plate with one large c-band block and two adjacent c-bands on the two homologous copies of chromosome no.6 in AZO3 cultivar, c: metaphase with only one large dark c-banding block on one homologous copy of chromosome no.7 of Egaseed 2 and d: metaphase with only one large dark c-banding block on one homologous copy of chromosome no.7 in AZO3, Scale bar= 20 microns.

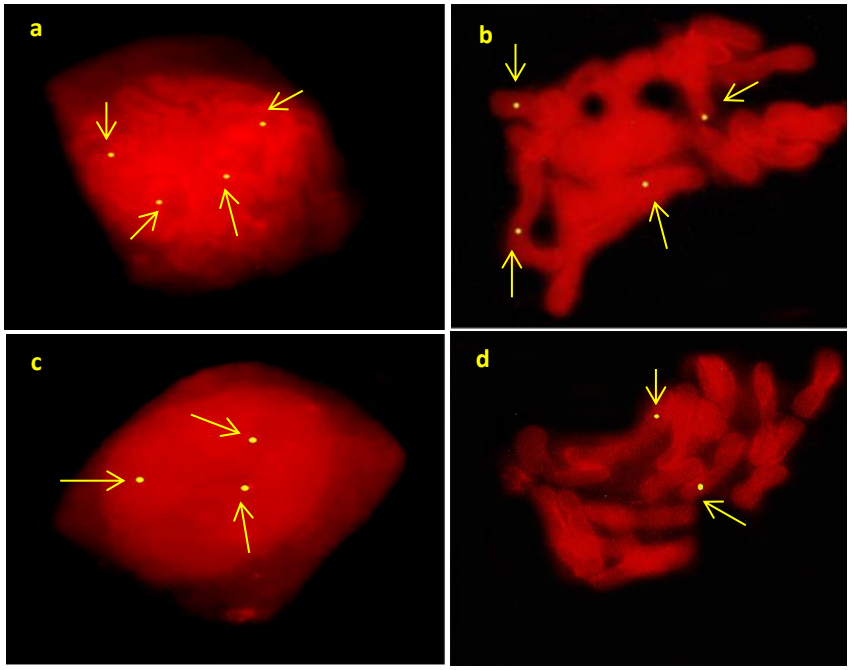

Fig. (4): Yellow-colored FISH signals of allicin probe. (a): at the interphase of Egaseed2, (b): at the metaphase plate of Egaseed 2, (c): at the interphase of AZO 3 and (d): the metaphase plate of AZO 3. Arrows indicate fish signals.